

AMYRIS BIOLOGY HANDBOOK

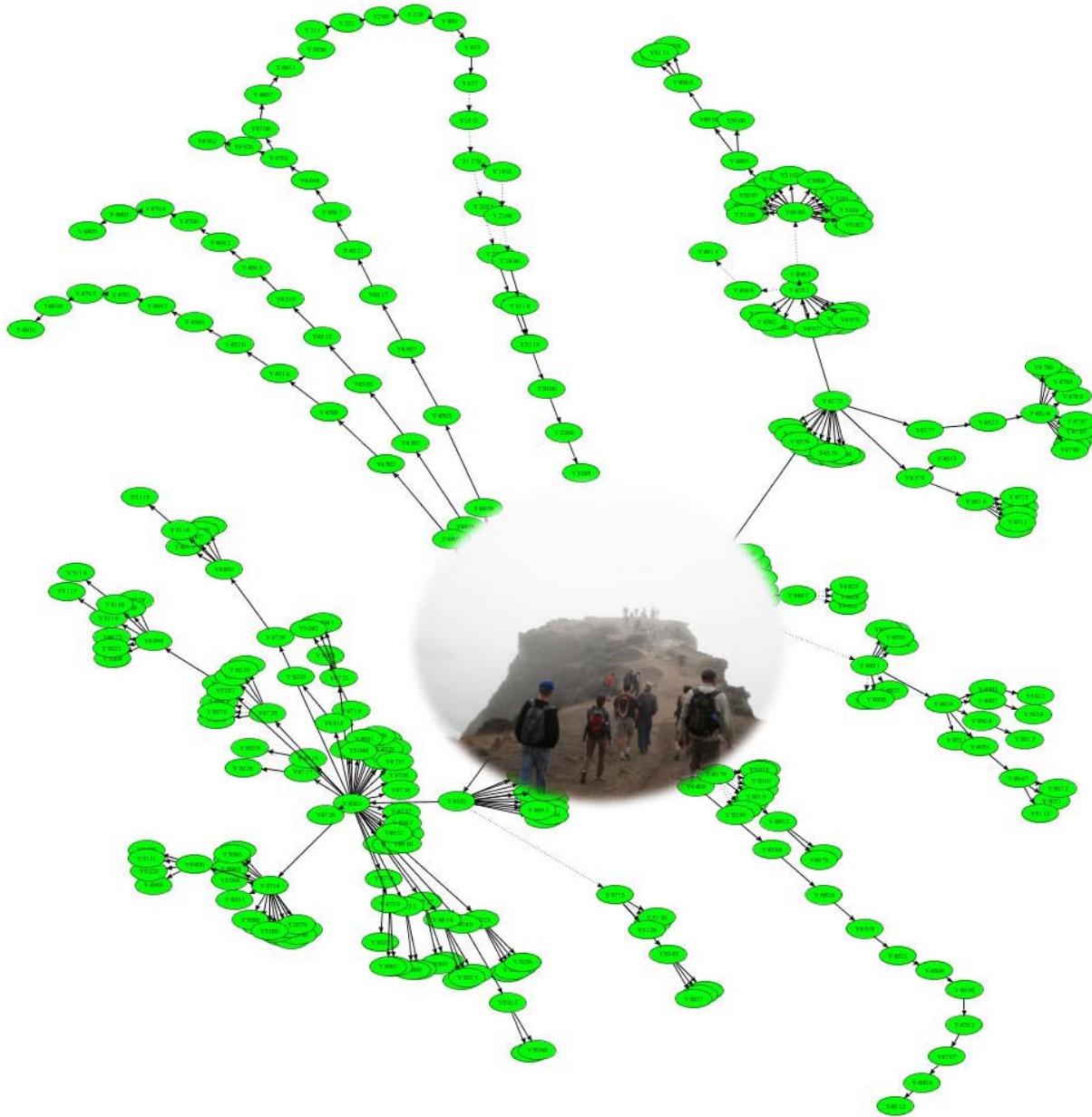


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Our Core Values

We are a learning organization:

- ❖ We learn from everything we do and share everything we learn.
- ❖ We engage and challenge each other constructively.
- ❖ We work collaboratively and across disciplines.
- ❖ We provide opportunities for growth.

We embrace intelligent risk and are not afraid to fail:

- ❖ We encourage creativity and unconventional thinking.
- ❖ We are flexible and adaptive to change.
- ❖ We have high expectations of ourselves and each other.

We seek practical solutions:

- ❖ We address the needs of our target markets and customers.
- ❖ Excellent performance is the norm.
- ❖ We balance perfection and progress.

We are an inclusive organization; resistance is futile:

- ❖ We embrace transparency and open communication.
- ❖ We are open to and respectful of other's ideas.
- ❖ Your voice matters!

Integrity is everything

- ❖ We are socially and environmentally responsible.
- ❖ We participate in our communities.
- ❖ We seek truth and insist upon honesty.

We are a company of owners:

- ❖ Your actions and decisions matter.
- ❖ You represent Amyris!

We are Amorous:

- ❖ We are driven, passionate and committed.
- ❖ We are fun and irreverent.
- ❖ We are humble with our successes and never take them for granted.
- ❖ We are an eclectic bunch and better for it.

1. SAFETY

Our Goal: We are absolutely committed to the philosophy that when the work day is done, people should leave Amyris feeling at least as well as they did when they started their day.

SAFETY TIPS FOR NEW BIOLOGISTS AT AMYRIS (Wendy Goldsby)

The organisms that we work with at Amyris are classified as Biosafety Level-1 or non-hazardous to the humans and the environment. However, because they are technically classified as Genetically Modified, we must contain our strains and not allow them into public areas. It is important to follow all rules with respect to strain handling and disposal. All contamination events should be reported immediately to the contamination control committee (email Chi-Li Liu or CCC).

Basic lab practices

- Wear personal protective equipment (minimum standard: a clean lab coat, safety glasses with side shields, gloves when working).
- Practice good hygiene
- Keep lab areas clean
- Disinfect lab benches with 70% ethanol in the morning and the evening
- Regularly discard old plates and samples
- Label all samples with initials, date, and sample ID-(for sterile solutions date containers with the date opened).
- Keep plates wrapped in storage and seal plate sleeves with clips (instead of tape)
- Call for solid waste pick up if containers become more than ¾ full or 2 days old.
- Regularly dispose of liquid biowaste after deactivation with Bacdown. Properly label all waste containers.
- Use the biosafety cabinet for sterile transfers involving seed stock, antibiotic transfers or other sensitive sterile operations. (NOTE: there are two Class II biosafety cabinets upstairs and laminar flow hoods downstairs)
- Transport all strains in secondary containment
- Take short micro-breaks every 20-30 minutes while pipetting or doing repetitive work. Keep arms lowered while pipetting and supplies within easy reach. Contact EH&S if you are experiencing any discomfort while working. Repetitive stress injury, our primary cause of work place injury, can be prevented if attention is paid to work habits.

If you work with hazardous materials (anything flammable, combustible, toxic, or corrosive) please review the Chemical Hygiene Plan on Safety Superdog. These substances must be stored in secondary containment in approved cabinets and handled in chemical fume hoods. There is a separate waste stream for disposing of hazardous substances. Please review waste handling procedures with the Biology Safety Representative prior to starting your work.

Radioactive materials and mutagens are used in Biology. These materials are stored and used in Lab 200 which is restricted access. You must receive training prior to using these materials. Please contact the Amyris Radiation Safety Officer. Likewise if you wish to prepare and use gels, please contact Arthur Salmon for training. Make sure that you wear the protective face mask (with your safety glasses on) while viewing gels on the UV box and properly dispose of all razor blades in the sharps containers.

Before you begin your experiments, review the primary work tasks with your supervisor and agree ahead of time what safe work practices to adopt. If hazardous substances are used, you may access the material data safety sheets (MSDS) for review through MSDS-online on Safety Superdog. If the procedure involves physical or chemical hazards a more formal safety review may be required. Examples of hazards requiring a more extensive safety review are found in the Chemical Hygiene Plan (Section IIIC). Working alone in the labs on the weekend or late at night is not permitted unless you have approval from your supervisor and the work does not involve hazardous or new and untested procedures.

In case of Emergency

If you become injured or ill from any experiment or procedure at Amyris, please report this immediately to your supervisor and/or EH&S. You will be referred to the Amyris occupational health clinic in Berkeley for treatment of any non-emergency occupational injuries or illnesses. An incident report and insurance forms must be completed with any injury or illness within 24 hours. An incident report is also required for an unplanned event which could have resulted in an injury (near miss). Please review the Amyris Safety Program or Injury Illness Prevention Program on Safety Superdog for additional information on Injury and Illness prevention, reporting, and investigation.

For medical emergencies, please call the receptionist for assistance in contacting emergency medical teams (EMT) or 911 directly if the receptionist is not reachable. Please instruct the EMT on where you are and give them as much information as possible regarding the symptoms. Have someone wait out front to help escort the EMT into the building.

In case of evacuation for fire, chemical spill or gas leak, exit the building by the nearest exit avoiding the central stairwell and the lobby. Proceed to the assembly area, $\frac{1}{2}$ block east on 59th street in the parking area next to the pilot plant. Make sure you check in with your area safety representative (Arthur Salmon). Bring your belongings (purse, wallet or keys) with you if it is safe to do so. Do not try to re-enter the building until an all clear has been given by emergency coordinators.

For lab based emergencies, such as equipment failure or power outages, contact the Biology Safety Representative or the Facilities Director.

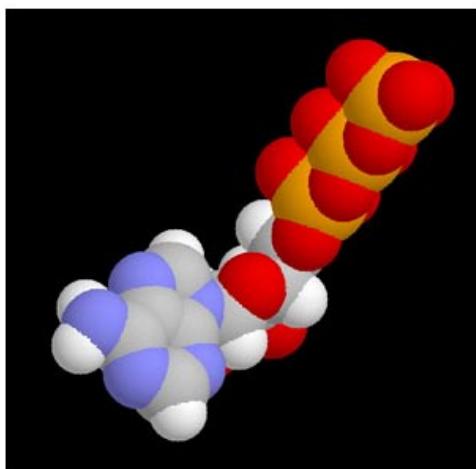
Additional information is contained in Amyris policies on fire prevention and emergency response on Safety Superdog.

Key Biology Safety Contacts:

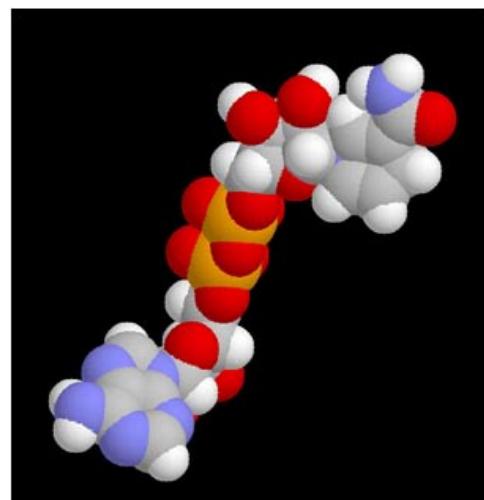
Arthur Salmon	Biology lab manager and area safety representative	X 758
Chris Reeves	Radiation Safety Officer	X 770
Jim Kealey	Director Biology	X 704
Shayin Gottlieb	Biology back-up safety representative	X 750
Wendy Goldsby	Director EHS	X 539
Brandon Friedrikson	Hazardous waste removal	X 595

2. METABOLIC ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR ISOPRENOID OVERPRODUCTION: BACKGROUND

2.1 Introduction to yeast central metabolism, energy production and redox (Gale and Youngnyun)

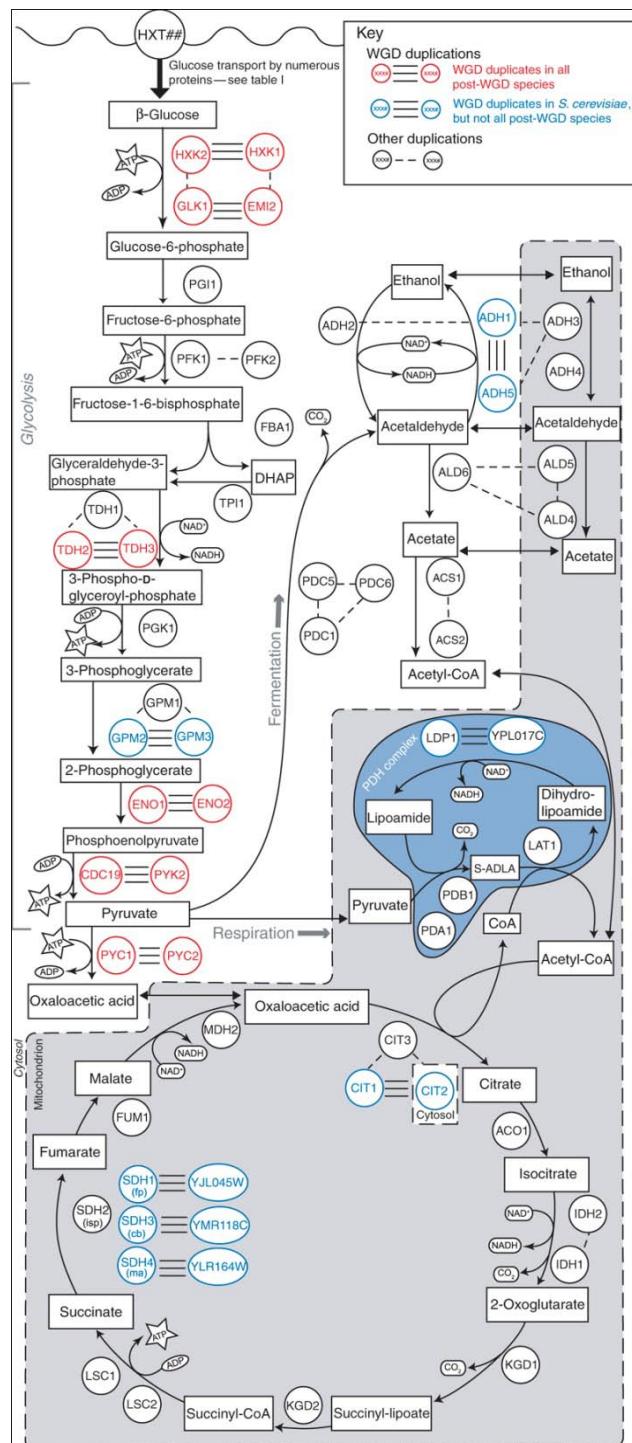


ATP

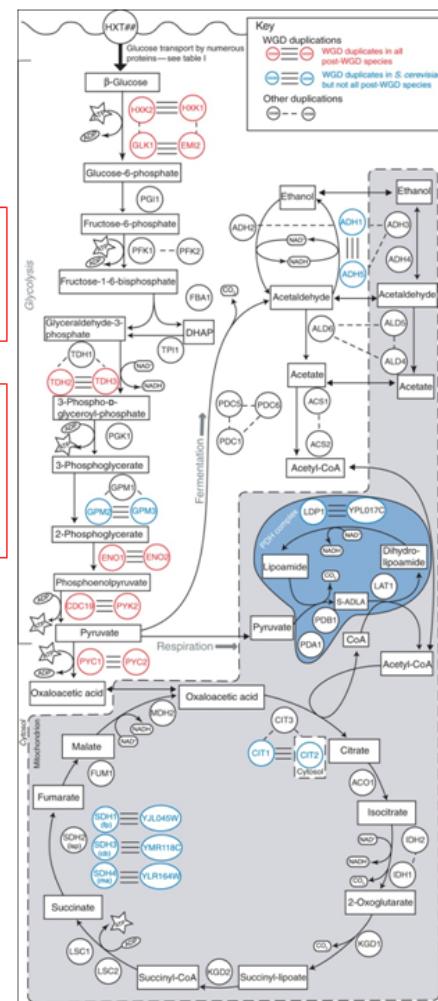
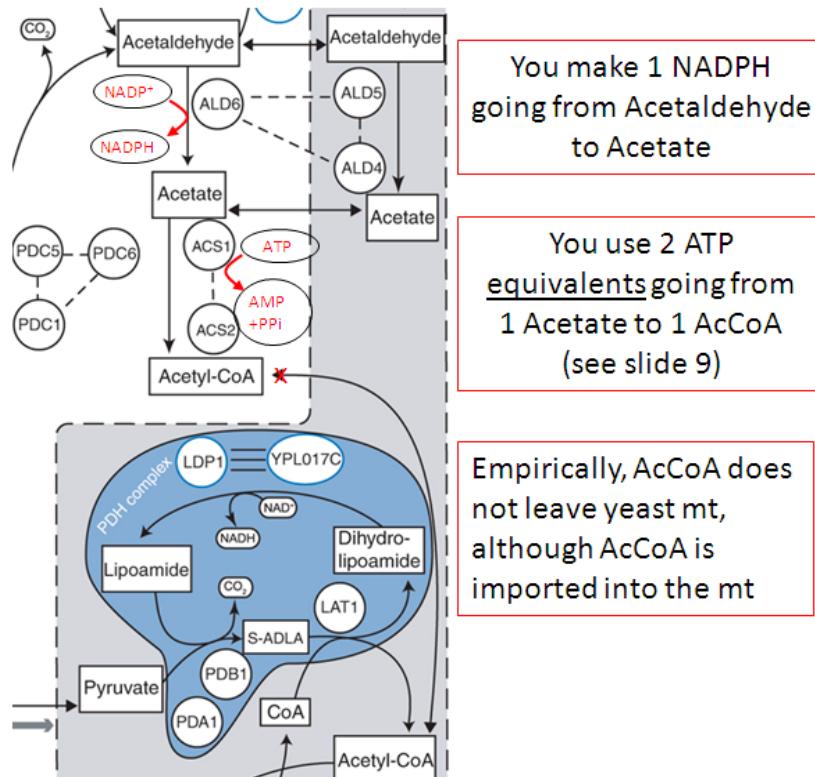


NADH

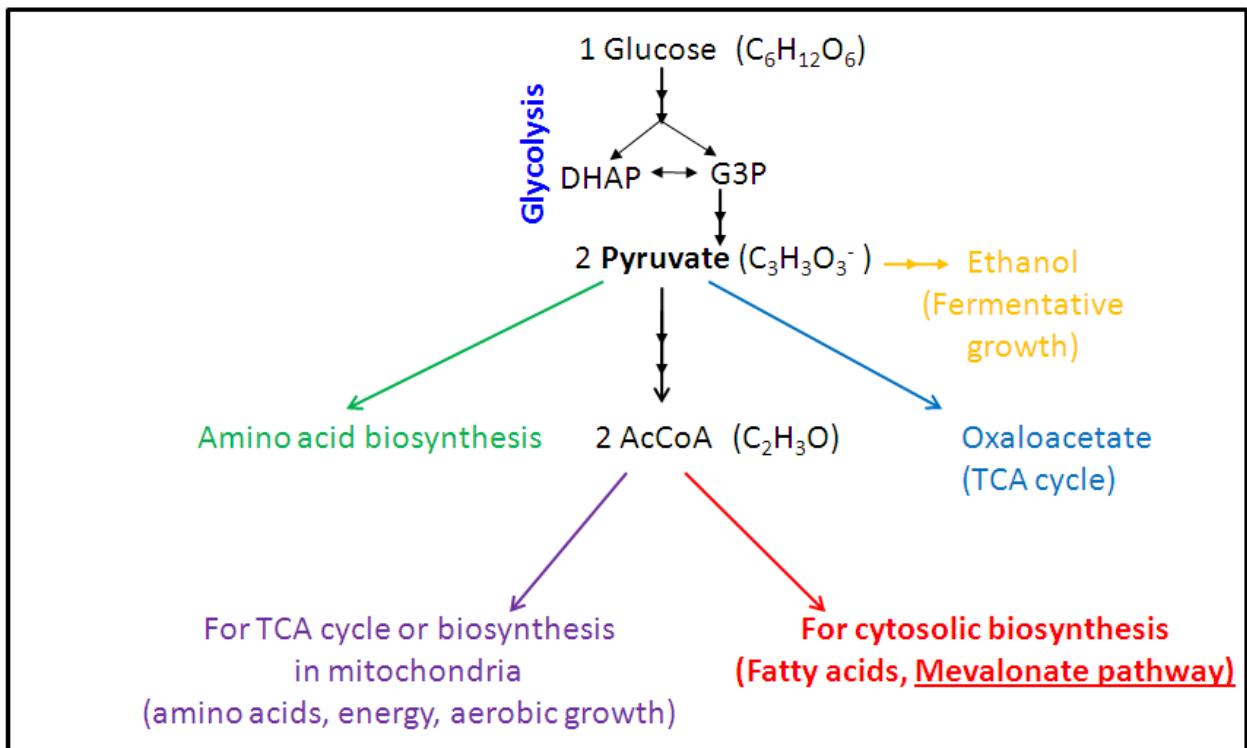
Print out this image for a [helpful overview of yeast metabolism](#) to keep at your desk)



A nice overview of yeast central metabolism
Additional detail relevant to isoprenoid production



Pyruvate is an important (and highly regulated) metabolic branching point in the cell

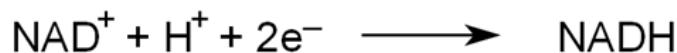
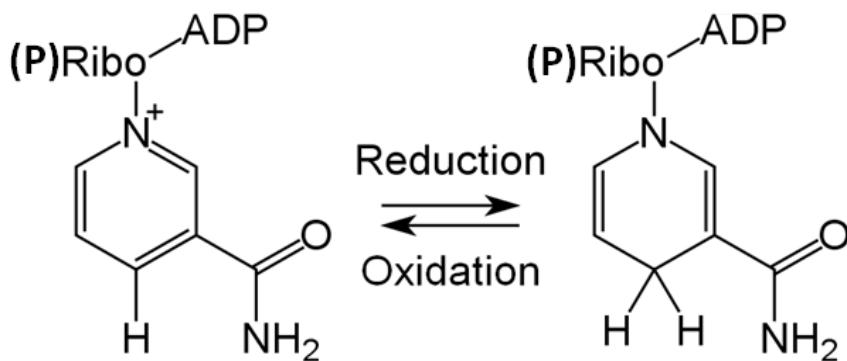


Sucrose = 1 glucose and 1 fructose

Nicotinamide adenine dinucleotide (phosphate)

NAD⁺/NADH and NADP⁺/NADPH are molecules involved in redox reactions; they carry electrons from one reaction to another

NAD(P)^+ oxidized state (less electrons)	NAD(P)H reduced state (more electrons)
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Generally, NADPH is used in biosynthetic reactions, NADH is used in energy production

Stoichiometry of yeast (EtOH) fermentation



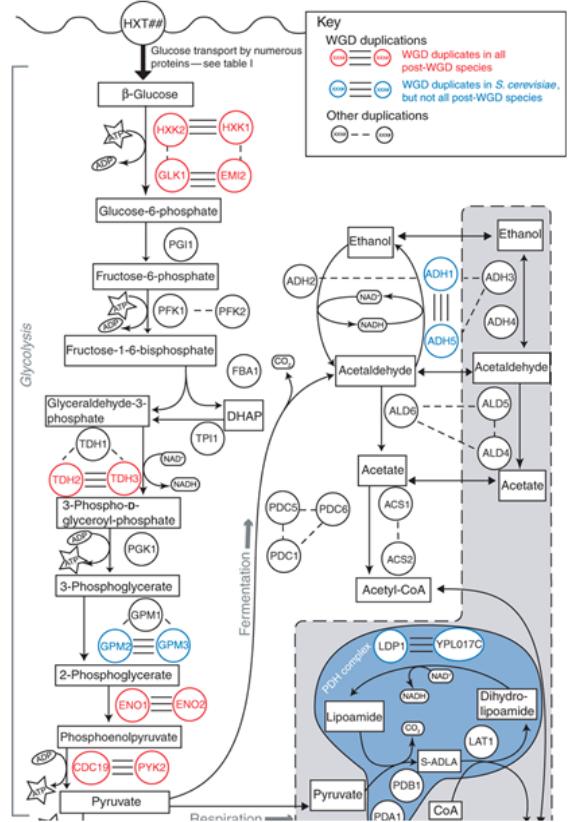
In glycolysis (1 glucose → 2 pyruvate):

- 2 NADH are generated
- 2 ATP are used, 4 ATP are made = 2 ATP are gained

In ethanol fermentation (2 pyruvate → 2 EtOH):

- 2 NADH are consumed
- 2 CO₂ are formed

Therefore, EtOH fermentation is a REDOX balanced process that gains the cell 2 ATP



Stoichiometry of yeast respiration

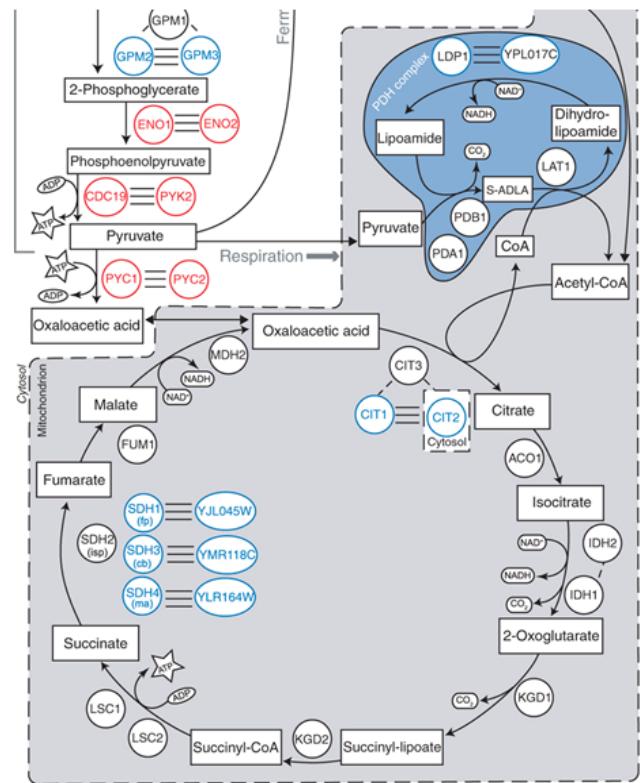


In yeast, P/O = 1

This means only 1 ATP (P) is formed per atomic oxygen (O) converted into water

In the mt, pyruvate is converted to AcCoA via the Pyruvate Dehydrogenase complex (PDH complex)

- No ATP is consumed
- CO_2 and NADH is formed per AcCoA



Respiration, Oxidative Phosphorylation, "Ox-Phos"

In bacteria, this occurs in the plasma membrane

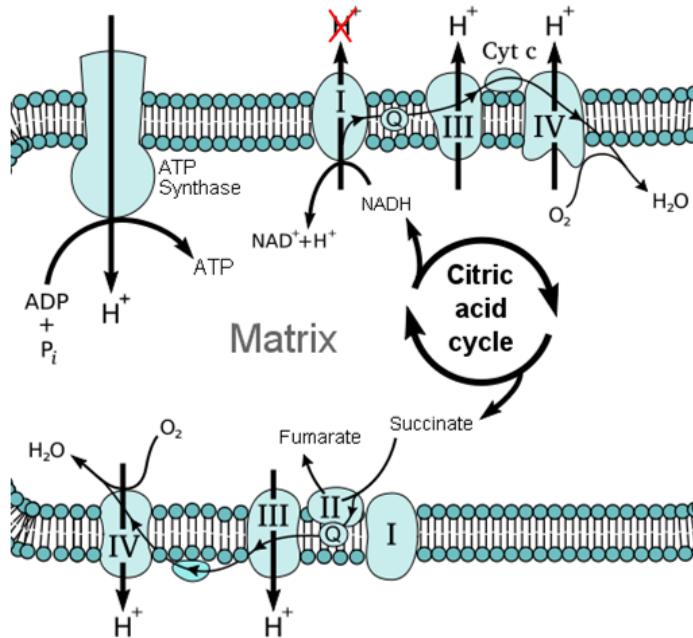
In eukaryotes (yeast) this occurs in the inner membrane of the mitochondria

(Yeast do not have Complex 1, instead they use Nde1p,2p and Ndi1p,2p)

Nde = NADH dehydrogenase, external, takes NADH from the cytosol into complex III

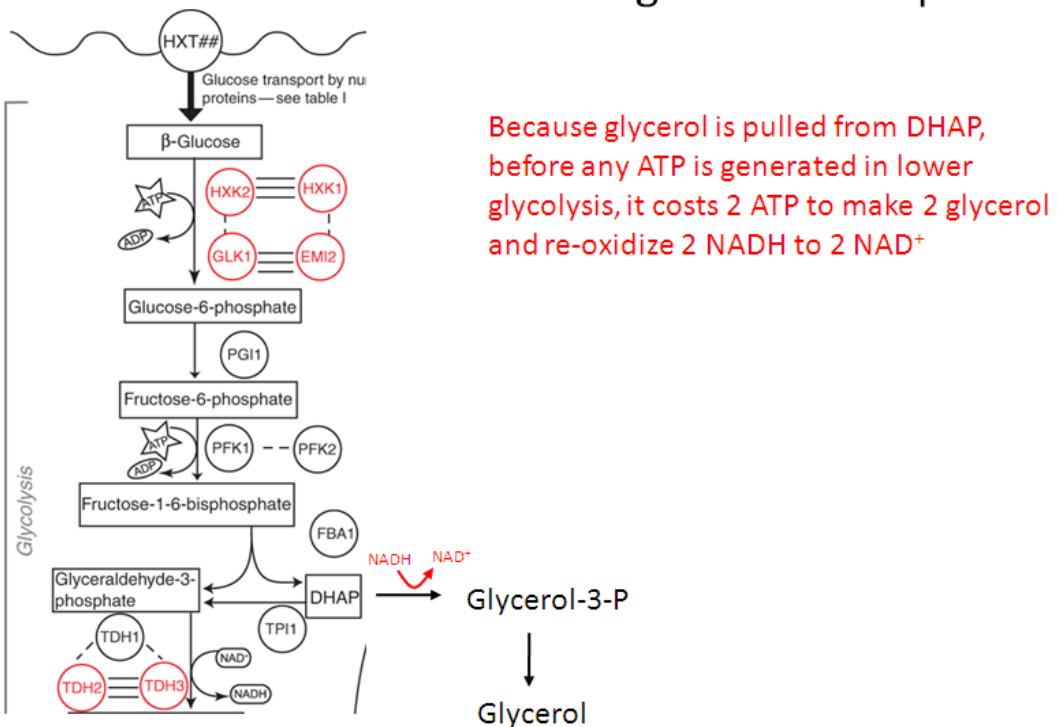
Ndi = NADH dehydrogenase, internal, takes NADH from the mt into complex III

Nde and Ndi do NOT pump H⁺ across the mt membrane

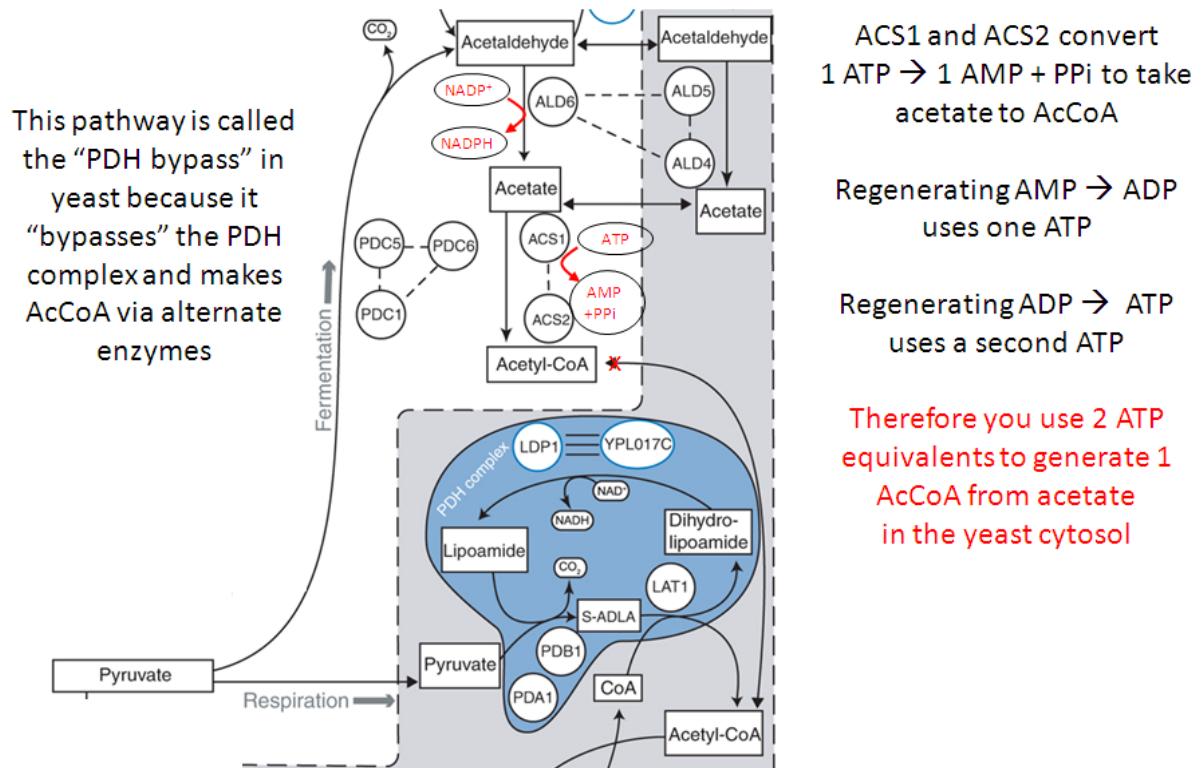


"UQH₂" = also leads to Proton pump and therefore more ATP

Glycerol Production is a way for yeast to re-oxidize extra NADH that is not being used for respiration



Making AcCoA in the yeast cytosol produces one NADPH and is very energetically expensive, costing 2 ATP equivalents per AcCoA

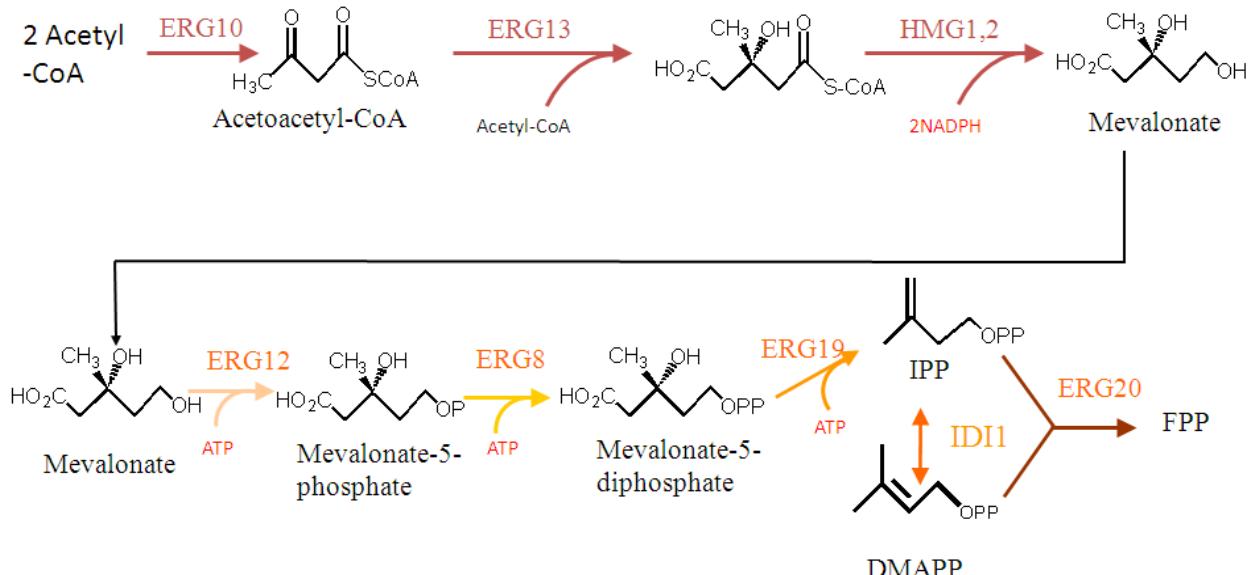


2.2 Yeast Mevalonate Pathway for Isoprenoid Production; Stoichiometry of Farnesene production (Gale and Youngnyun)

- Mevalonate pathway is native to yeast
 - Used to make sterols (ergosterol) and isoprenoids
- Mevalonate pathway is not present in most bacteria
 - Bacteria (and plant plastids) use an alternate pathway to generate IPP and DMAP
 - Known as MEP, DXP, or non-mevalonate pathway
 - *Staphylococcus aureus* is an exception – it contains the mevalonate pathway

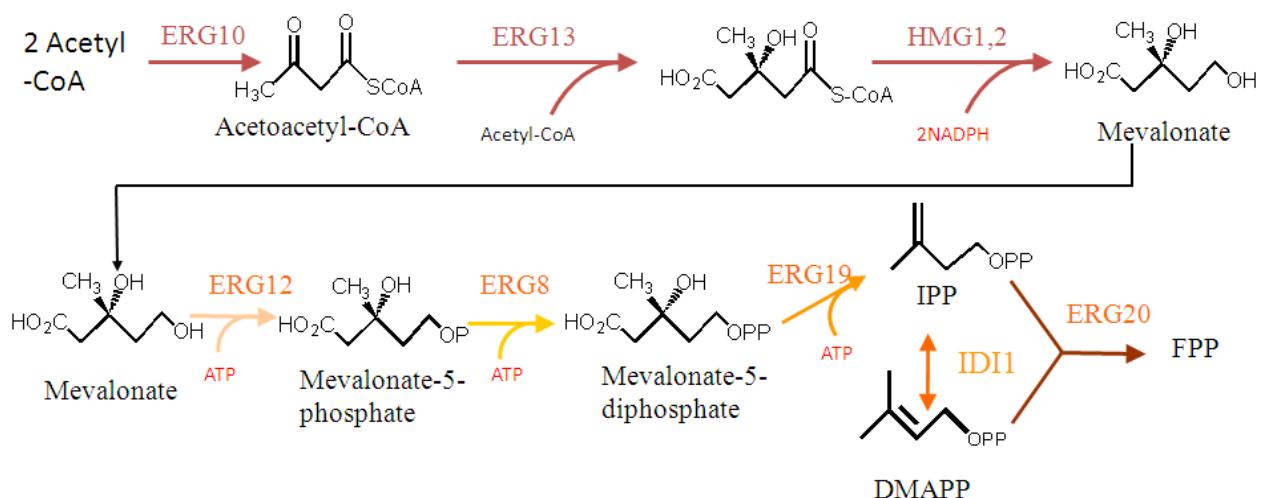
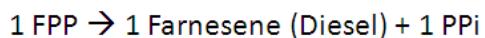
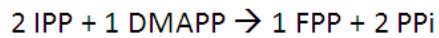
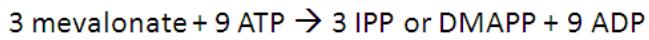
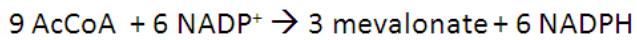
Yeast Mevalonate Pathway for Isoprenoid Production

Mevalonate "TOP" pathway = first three enzymes (produces mevalonate)

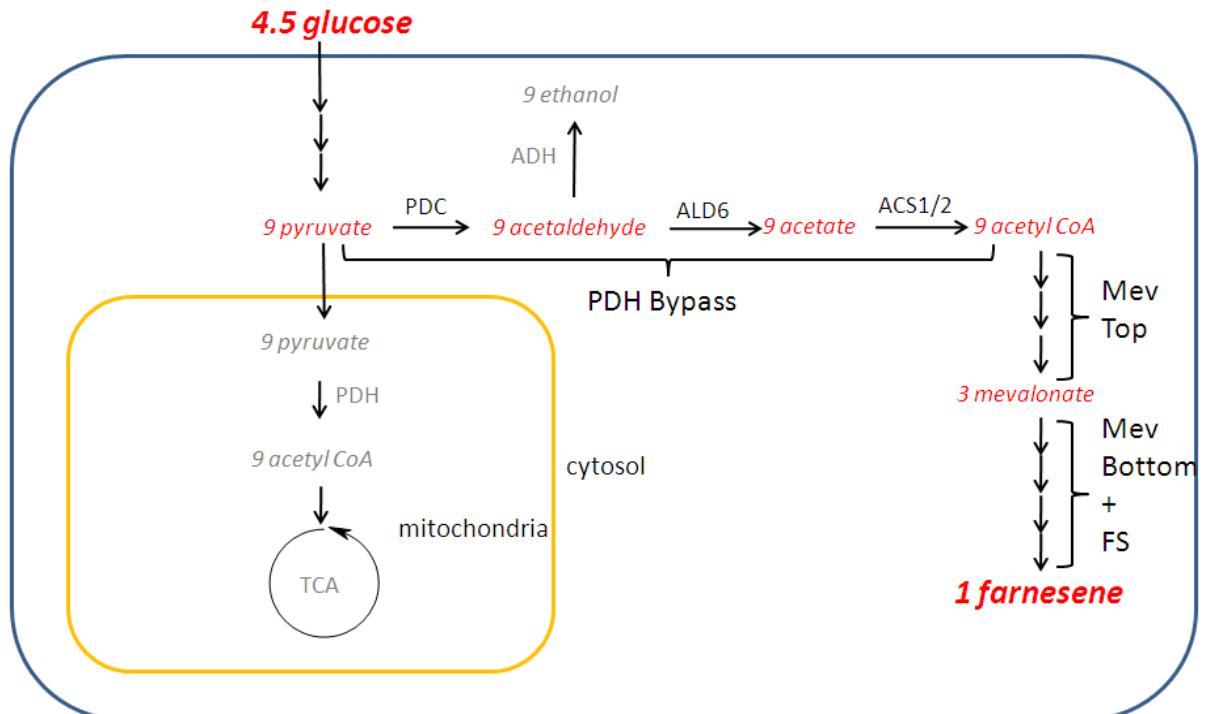


Mevalonate "BOTTOM" pathway = next five enzymes (produces FPP)

Stoichiometry of Farnesene production from AcCoA

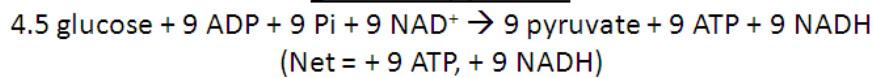


Putting it all together:
Farnesene from Glucose via Yeast Endogenous Pathways

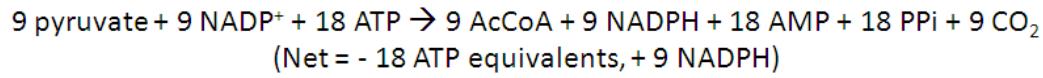


Overall Stoichiometry of Farnesene production from Glucose

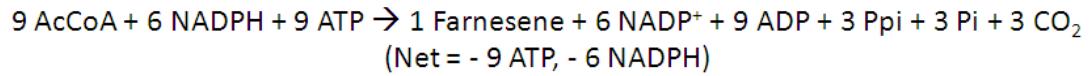
glucose to pyruvate:



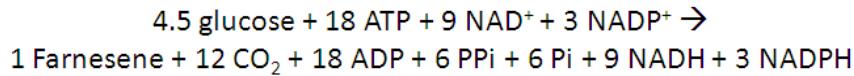
pyruvate to AcCoA:



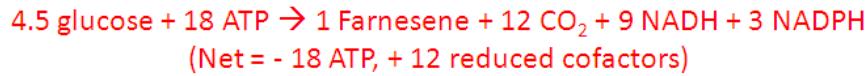
AcCoA to farnesene:



Glucose to farnesene:



Simplified overall:



- Using the PDH bypass in yeast to make AcCoA is very ATP intensive
- Combined with the mevalonate pathway to make FPP, it takes 18 ATP to make 1 Farnesene
- You also lose 45% of your carbon to CO₂
- The cell also is NOT balanced for redox co-factors and ends up OVER-reduced
 - 9 NADH
 - Cytosolic NADH can be used in respiration (via NDE1,2) to produce ATP
 - Otherwise it will be converted to glycerol at the expense of ATP
 - 3 NADPH
 - MUST be converted to NADP⁺ in biosynthetic (catabolic) processes

Yield of Farnesene from Endogenous pathway

$$\text{Yield of product (Y}_{\text{P/S}}\text{)} = \frac{\text{amount of farnesene produced}}{\text{amount of substrate consumed}}$$

Simplified overall:



- Theoretical maximum yield *without redox and ATP balance*
 - $1 \text{ farnesene (mol)} / 4.5 \text{ glucose (mol)} = 204 \text{ g farnesene}/810 \text{ g glucose} = 25.2\% \text{ (g/g)}$

Corrected overall:



- Theoretical maximum yield *with redox and ATP balance*
 - $1 \text{ farnesene (mol)} / 4.77 \text{ glucose (mol)} = 204 \text{ g farnesene}/858 \text{ g glucose} = 23.8\% \text{ (g/g)}$
 - Calculated by computational modeling
 - Assumed no growth in fene production

2.3 Mevalonate Pathway Enzymes in Amyris Yeast

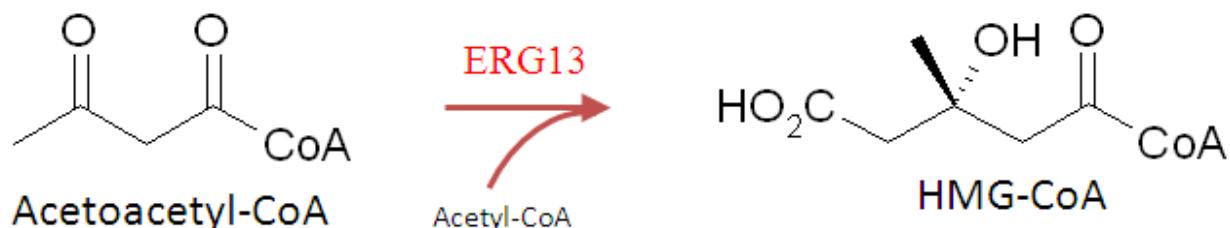
(see also <http://superdog/display/BIO/Fene+Pathway>)

Acetoacetyl-CoA thiolase (ERG10)



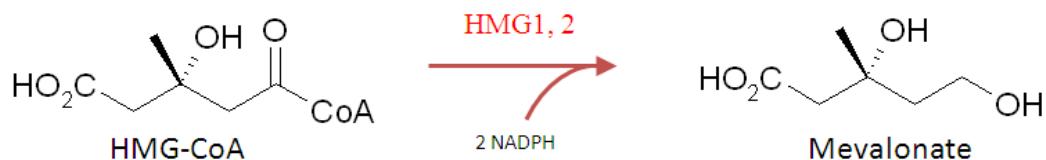
- 2 acetyl-CoA molecules used

HMG-CoA synthase (ERG13 = HMGS)



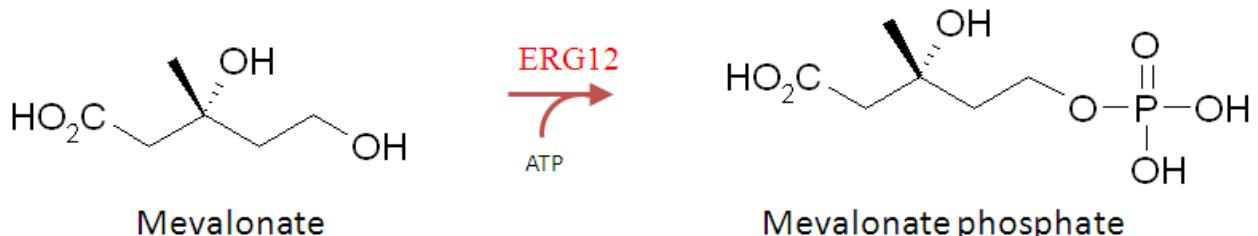
- Another acetyl-CoA molecule used

HMG-CoA reductase (HMG1,2 = HMGR)



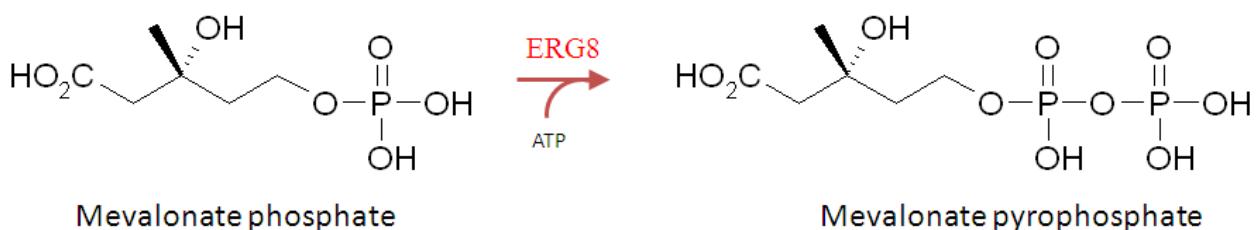
- Native yeast HMGR is membrane-bound. At Amyris a soluble, truncated HMG1 version (tHMGR) is used
- Yeast tHMGR uses only NADPH (2 per reaction)
 - However, there are other HMGR enzymes that can use NADH instead
- This is a known rate-limiting step

Mevalonate kinase (ERG12 = MK)



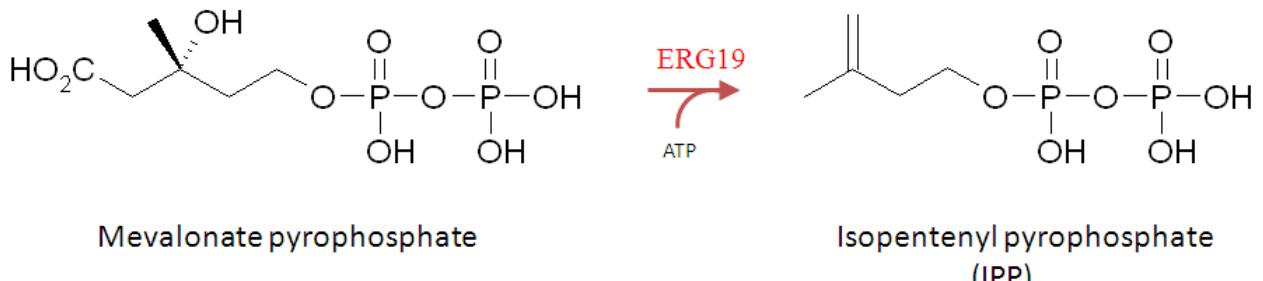
- Uses one ATP

Phosphomevalonate kinase (ERG8 = PMK)



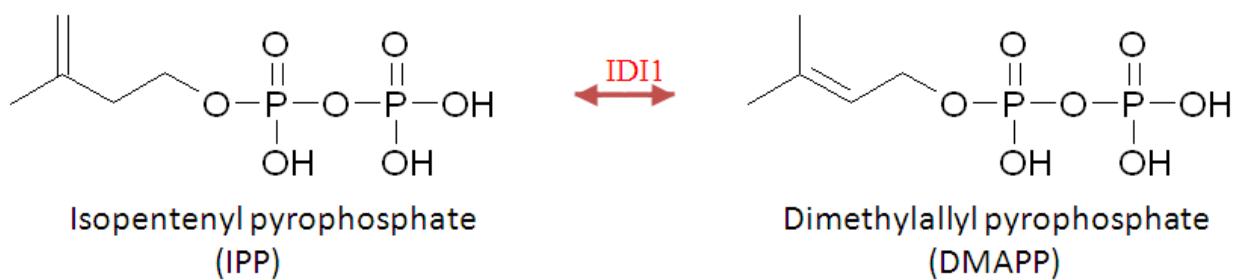
- Uses one ATP

Phosphomevalonate decarboxylase (ERG19 = PMD)



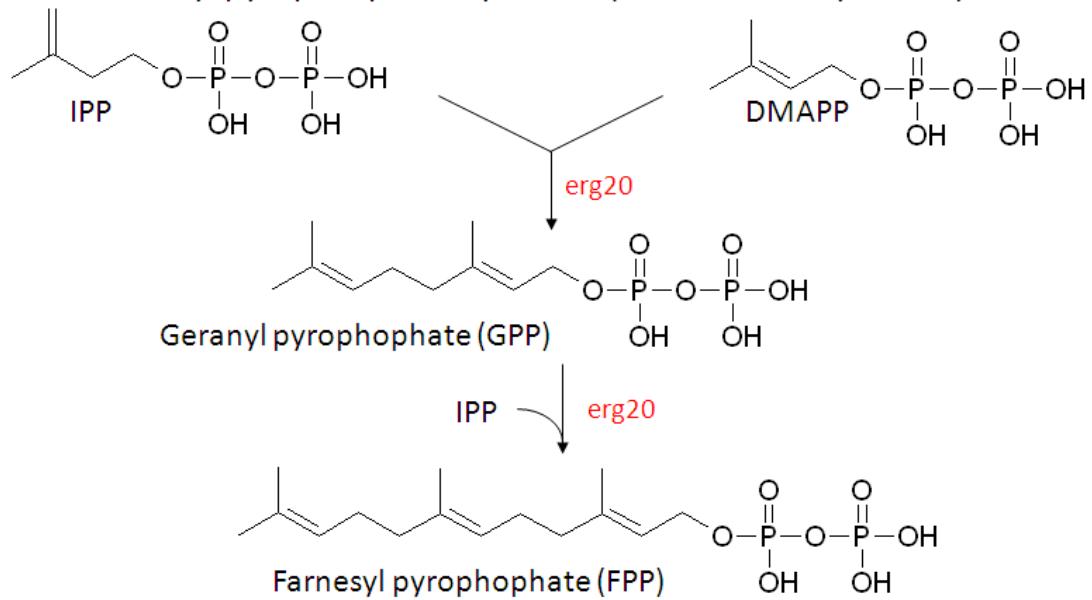
- Uses up one ATP

Isopentenyl pyrophosphate isomerase (IDI1)



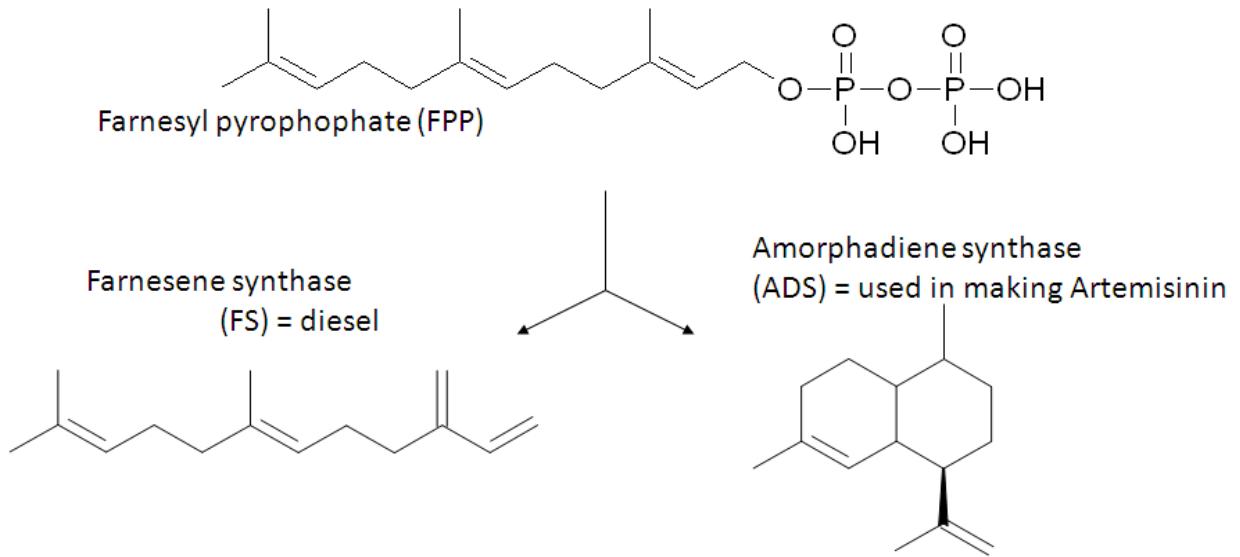
- Isomerase interconverts IPP and DMAPP
- Note that 2 IPP and 1 DMAPP make one FPP

Farnesyl pyrophosphate synthase (ERG20 = FPP synthase)



- It takes 2 IPP and 1 DMAPP to make one FPP
- Results in toxic product! FPP toxicity is a strong stress on our production cells

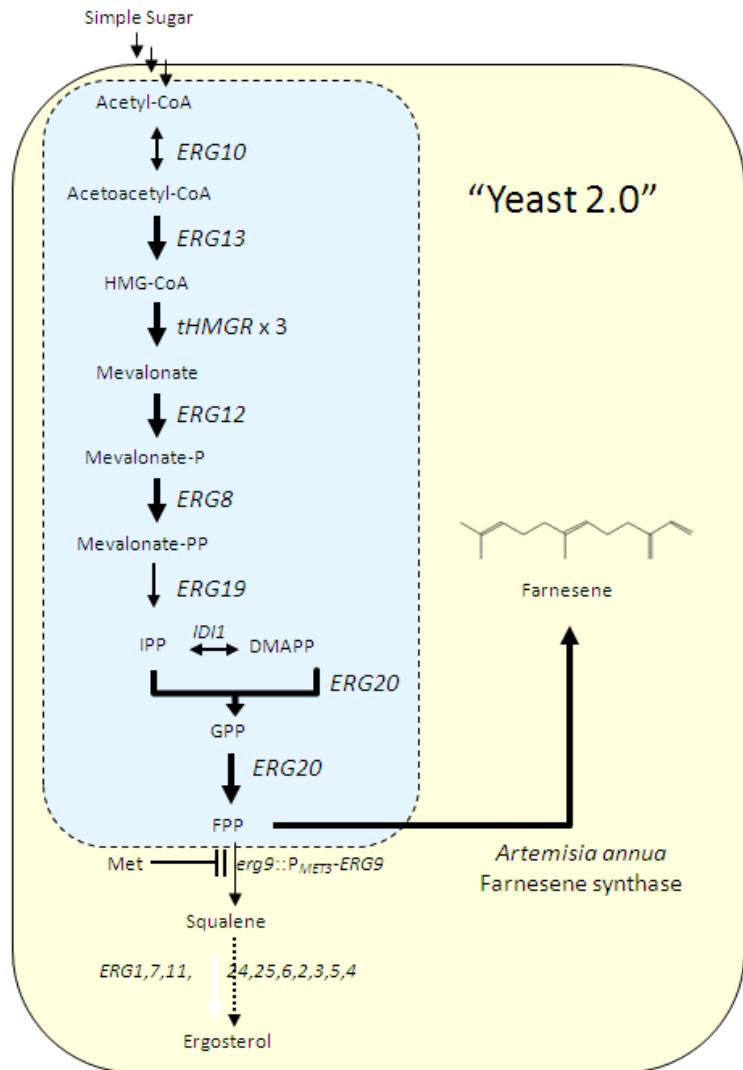
Different terpene synthases are used to take FPP to different isoprenoid products



Mevalonate Pathway in yeast

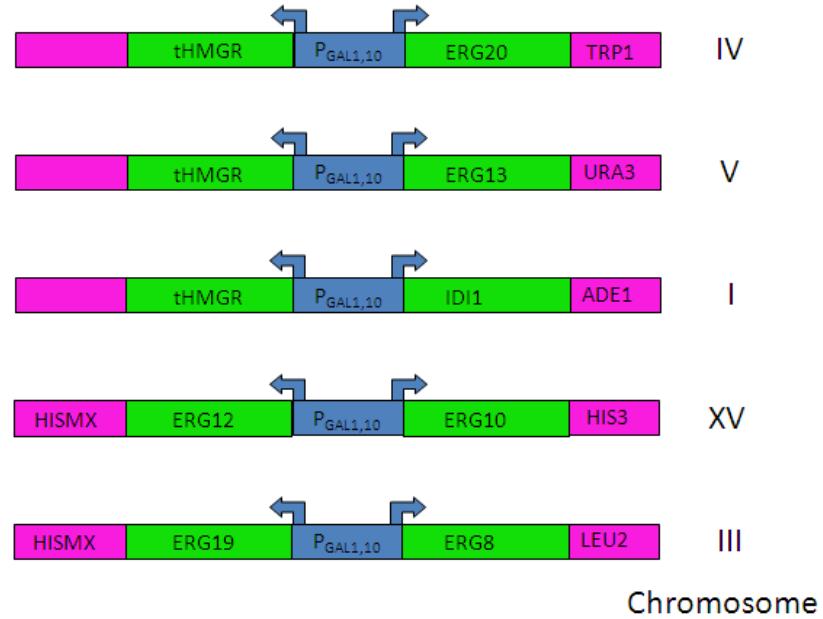
ERG9 = Squalene synthase

ERG9 is down regulated in our production strains to limit the loss of FPP to ergosterol



Extra copies of the mevalonate pathway have been introduced into our yeast strains for increased carbon flux through the pathway

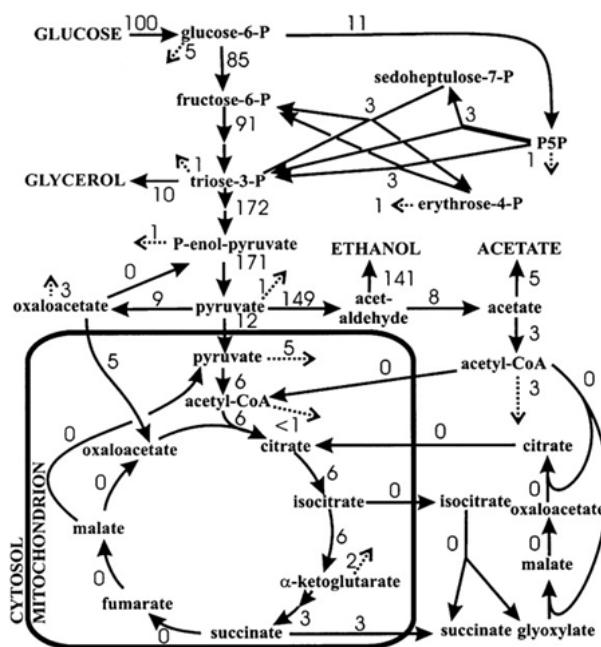
Transcription driven by strong *GAL1/10* promoters



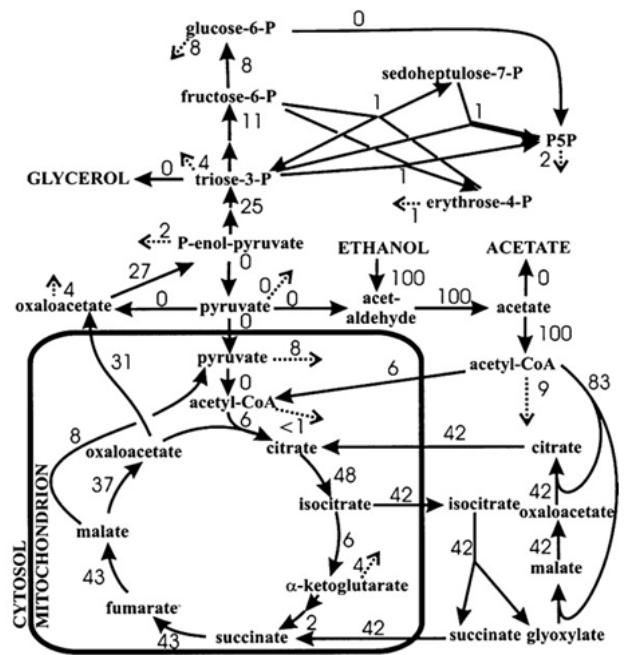
2.4 Metabolic Modeling (Adam Meadows)

2.4.1 Carbon flow in wild-type yeast

Utilizing glucose as a carbon source



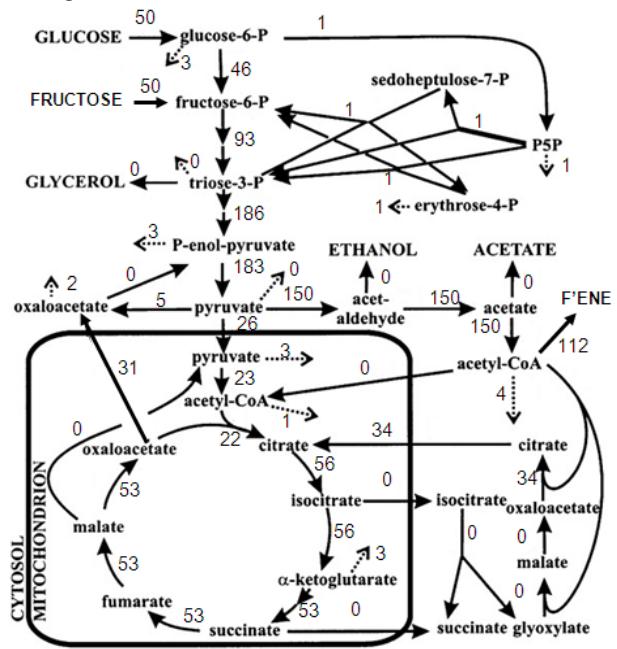
Utilizing ethanol as a carbon source



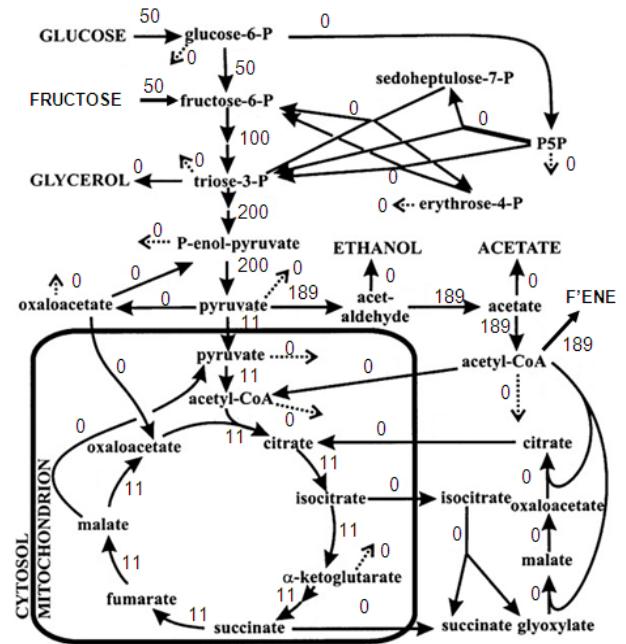
Note: dotted lines denote fluxes to biomass
Fluxes are molar and normalized to substrate uptake rate

2.4.2 Carbon flow in Amyris yeast: Today and tomorrow

Today?: We don't know. Shown is a *hypothetical* flux distribution in an MAPF fermentation for a 13.7% yield strain. We need isotopomer verification to completely understand how carbon is flowing.



Tomorrow: Where we want to end up in a 1st generation strain. Note that this represents an ideal state (max fene yield of 23.8%) which will not be achievable in practice

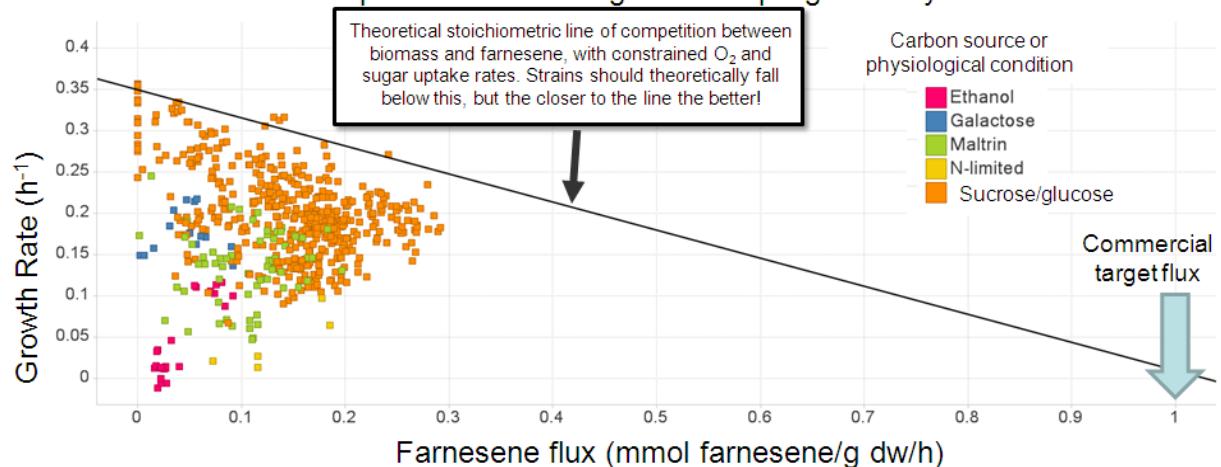


2.4.3 Growth rate-Flux correlations

Maximum farnesene flux and growth rate are generally anti-correlated, but nutrient restriction positively correlates them

Farnesene and biomass compete for shared cellular resources, so strains with higher farnesene fluxes will generally grow more slowly. Farnesene flux and growth rate will be **anti-correlated** in the long run.

However, under nutrient conditions that restrict the availability of shared cellular resources, growth rate and flux become **correlated**. Note the positive correlation in the ethanol data for example. We term this “growth-coupling” at Amyris.



2.5 The awesome power of yeast genetics (Annie and Kirsten)

Haploid/diploid yeast life cycle and Introduction to Yeast Biology



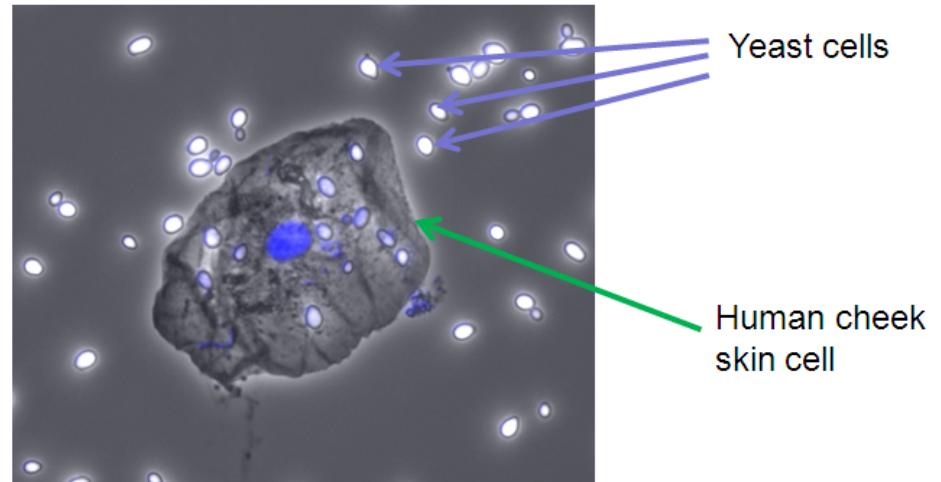
Saccharomyces cerevisiae means “sugar eating fungus for making beer” (think cerveza in Spanish) and it looks like this in a microscope



How big is Yeast?

Saccharomyces cerevisiae have a

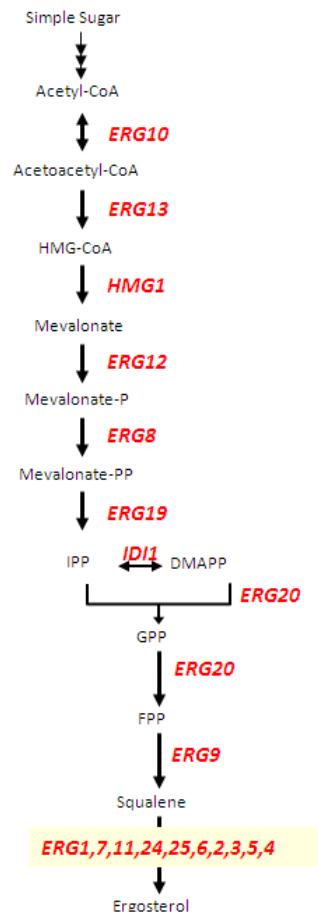
- length of about 5 microns (millionths of a meter)
- volume of about 50 femtoliters (millionths of a billionth of a liter)



Micrograph comparing yeast cells to human cell
(image from Jeff Ubersax)

Yeast genome and nomenclature

- 12 Mbases, 6000 genes on 16 chromosomes (70% of the DNA codes for genes, 10% of genes are essential)
- 3 letters and a number, e.g., *ERG10*
- Named according to how gene was first identified, e.g., genetic screen for mutants unable to make ergosterol (*ERG*) and order of discovery (1, 2, 3, etc.)
- Normal gene is all capitals (*ERG10*), mutant gene is all lower case (*erg10*), and protein is mixed (Erg10 or Erg10p)
- If gene is removed from chromosome (deleted), then chromosomal DNA is denoted *erg10Δ* and behavior is Erg-
- We use “marker” genes as a convenient handle to follow engineered genetic modifications, e.g., *erg10Δ::URA3* is able to grow in the absence of uracil whereas the parental *ERG10* strain cannot



A Broader View of the Metabolic Network in a Cell

The primary focus of our directed metabolic engineering is here.

We can target precise, surgical alterations to DNA that we hope will alter cellular metabolism as desired.

However, other regions of this interconnected metabolic map might be critical for successful re-routing of cellular metabolism, for reasons not yet understood.

Therefore, we also employ **random mutagenesis** of cellular DNA followed by high throughput screening of thousands of mutants.

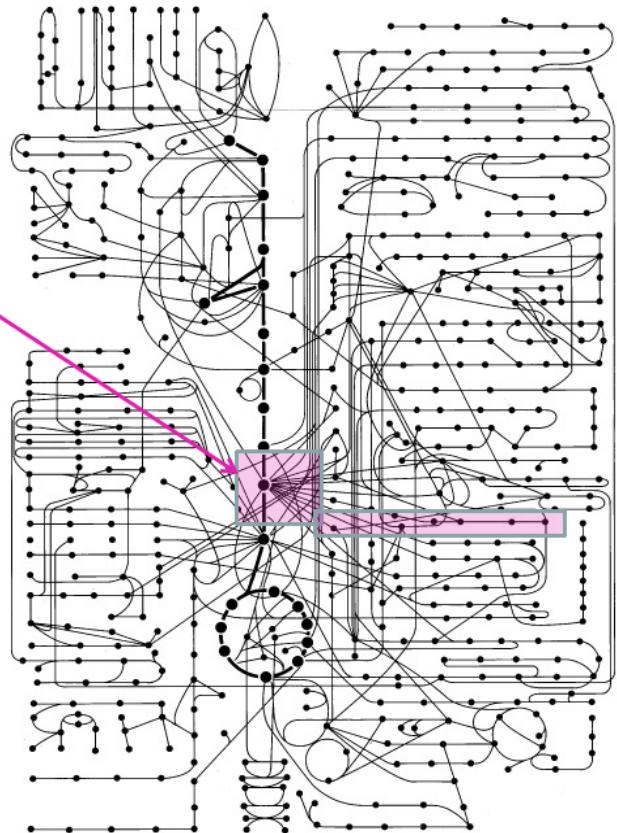


Figure 2–35. Molecular Biology of the Cell, 4th Edition.

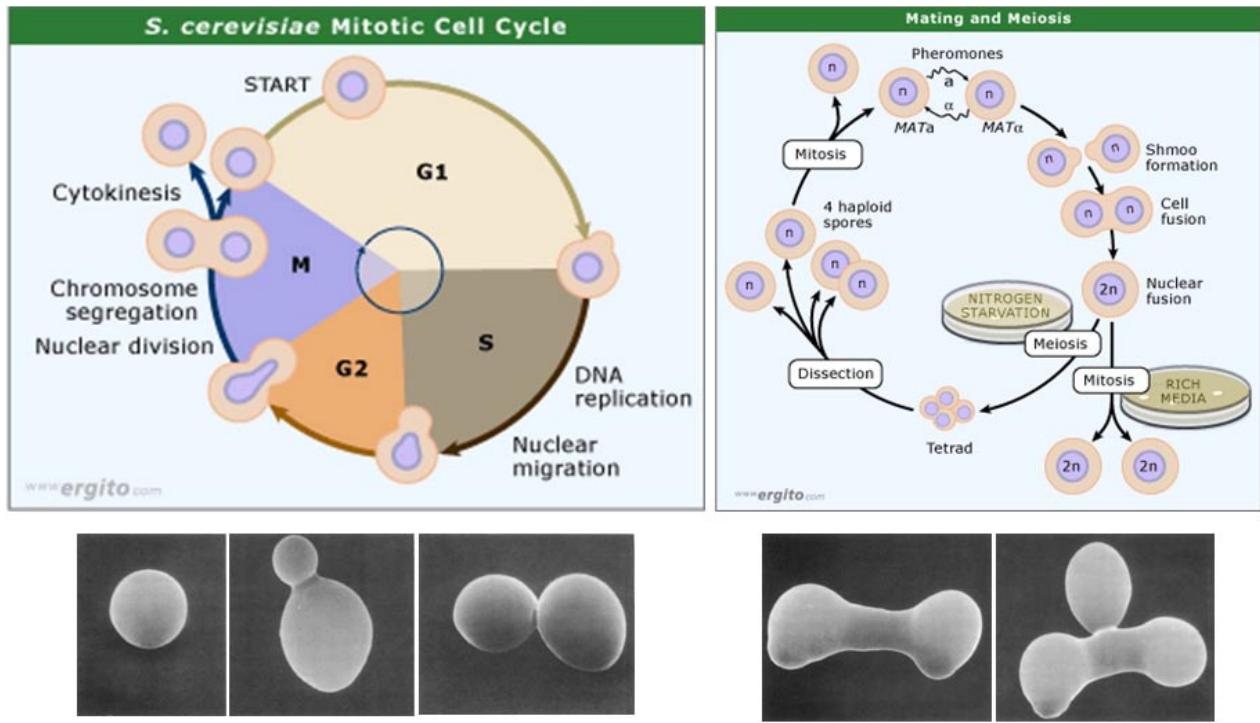
Simple eukaryote for metabolic engineering

Saccharomyces cerevisiae

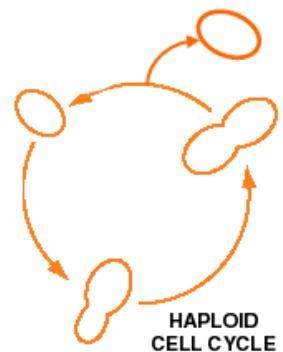
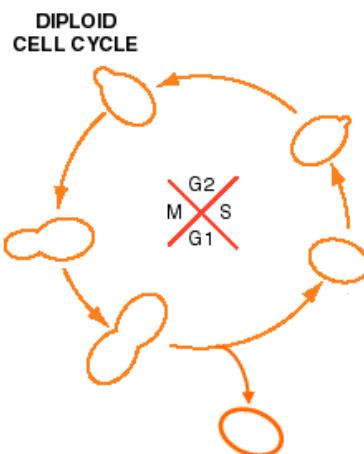


- Rapid growth (2h doubling times)
- Haploid/diploid/sex
(Awesome power of yeast genetics)
- Sequenced
- Easy to transform
- Great at homologous recombination
- 16 chromosomes + mitochondrial DNA, 12 MB

Yeast can be haploid or diploid

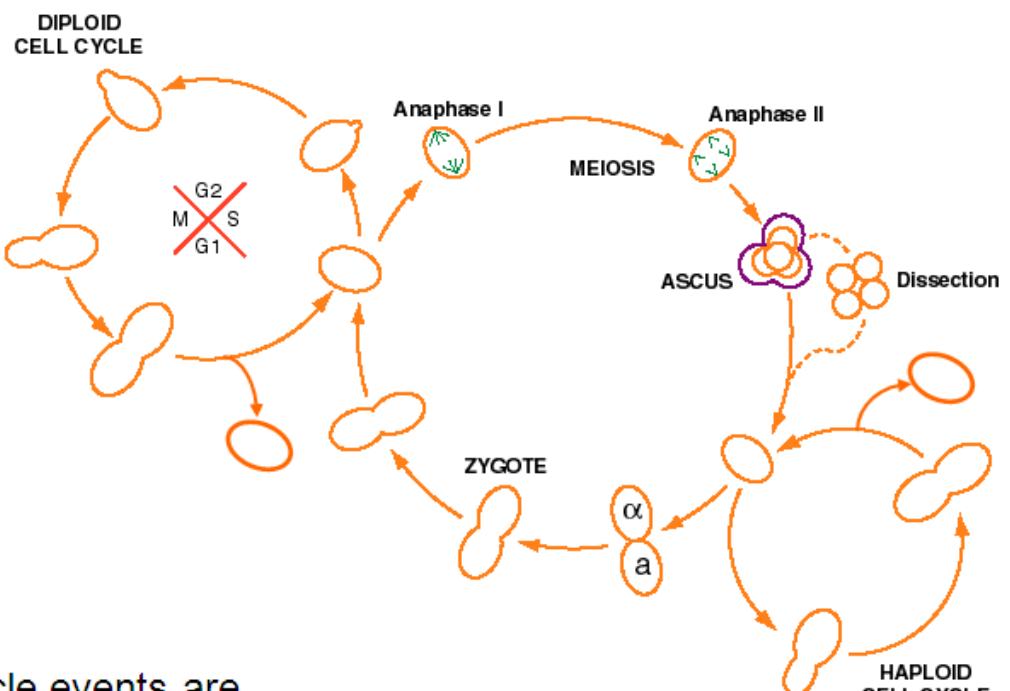


Yeast life cycle



Mitosis or asexual proliferation as a

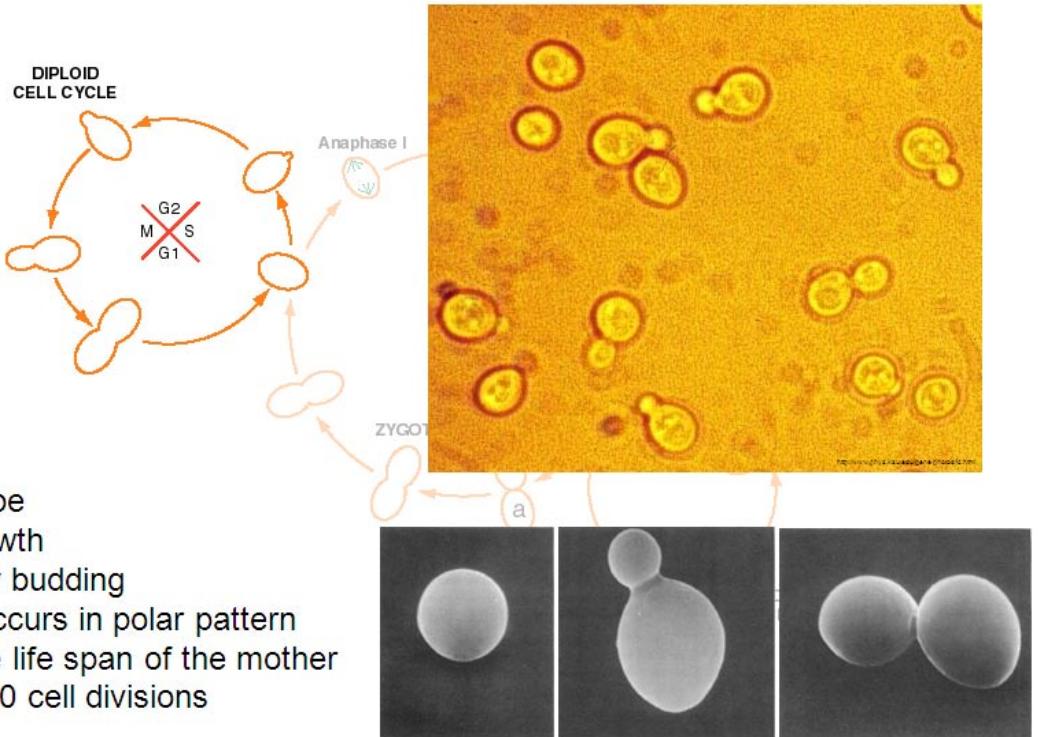
- haploid (1 copy of each chromosome)
- diploid (2 copies of each chromosome)



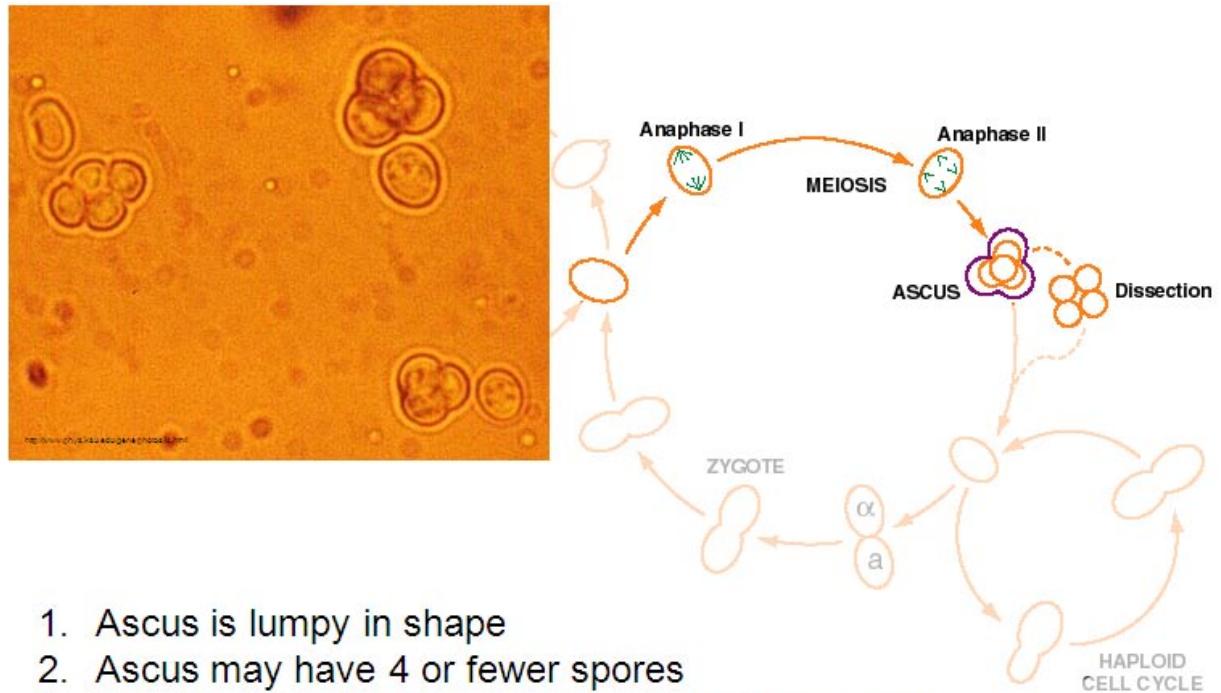
Other life cycle events are

- meiosis (conversion of 1 diploid cell to 4 haploid cells)
- mating (conversion of 2 haploid cells to 1 diploid cell)

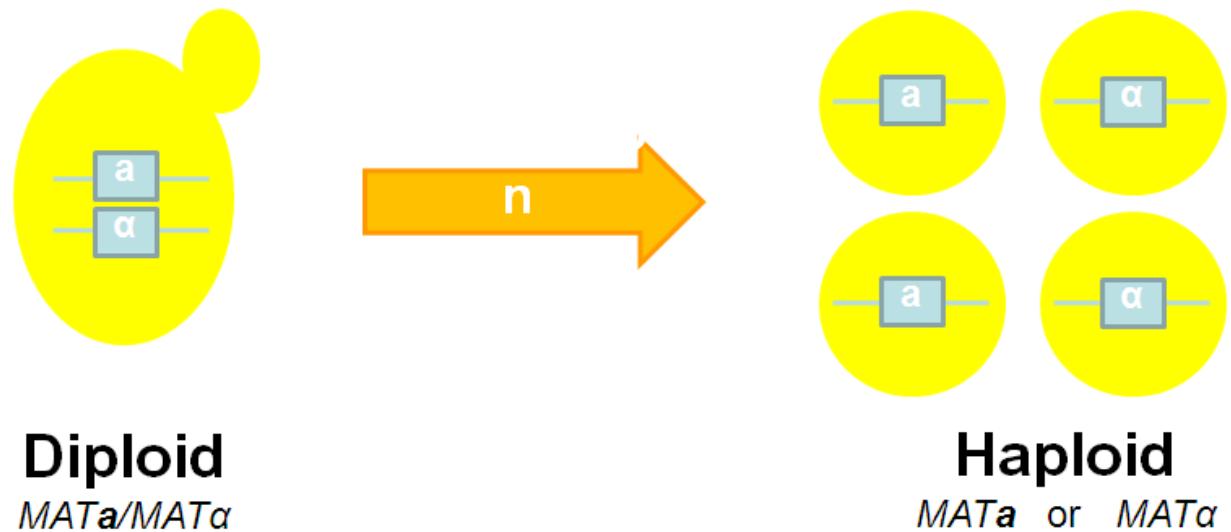
Diploid cells reproduce by mitotic cell division (budding)



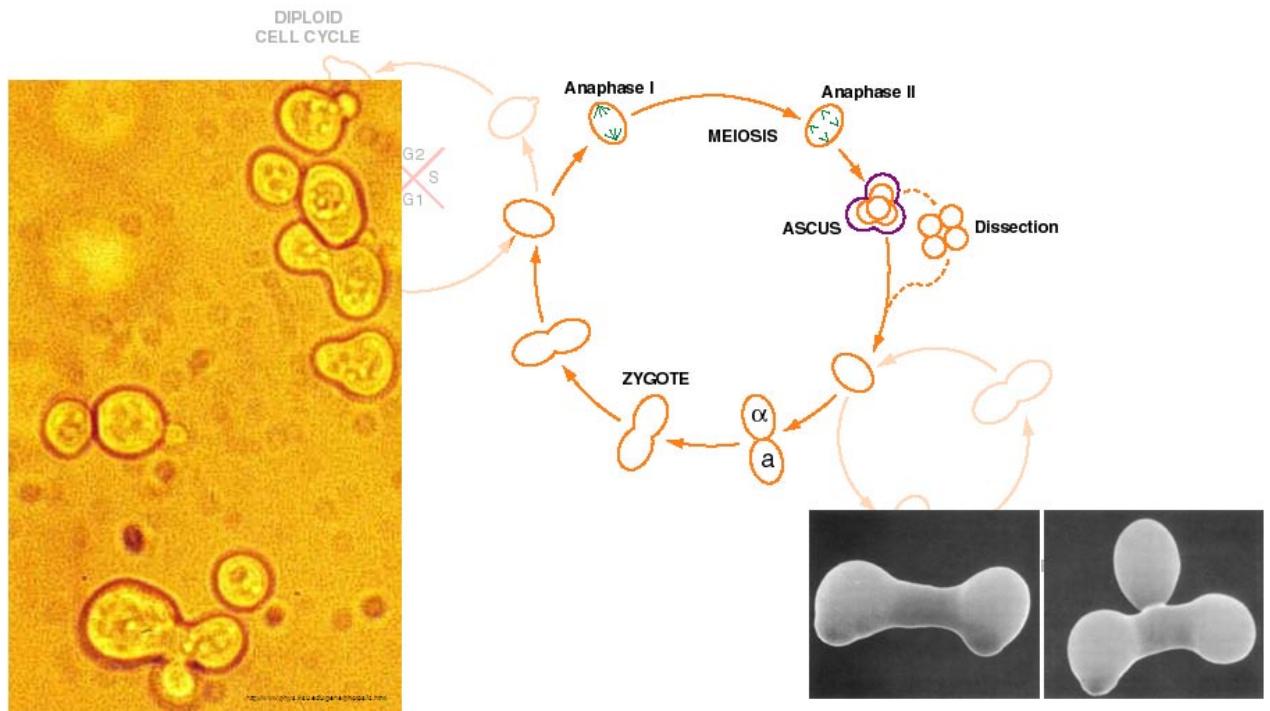
When starved for nitrogen, diploid cells undergo meiosis (sporulation) to produce four haploid spores(tetrad)



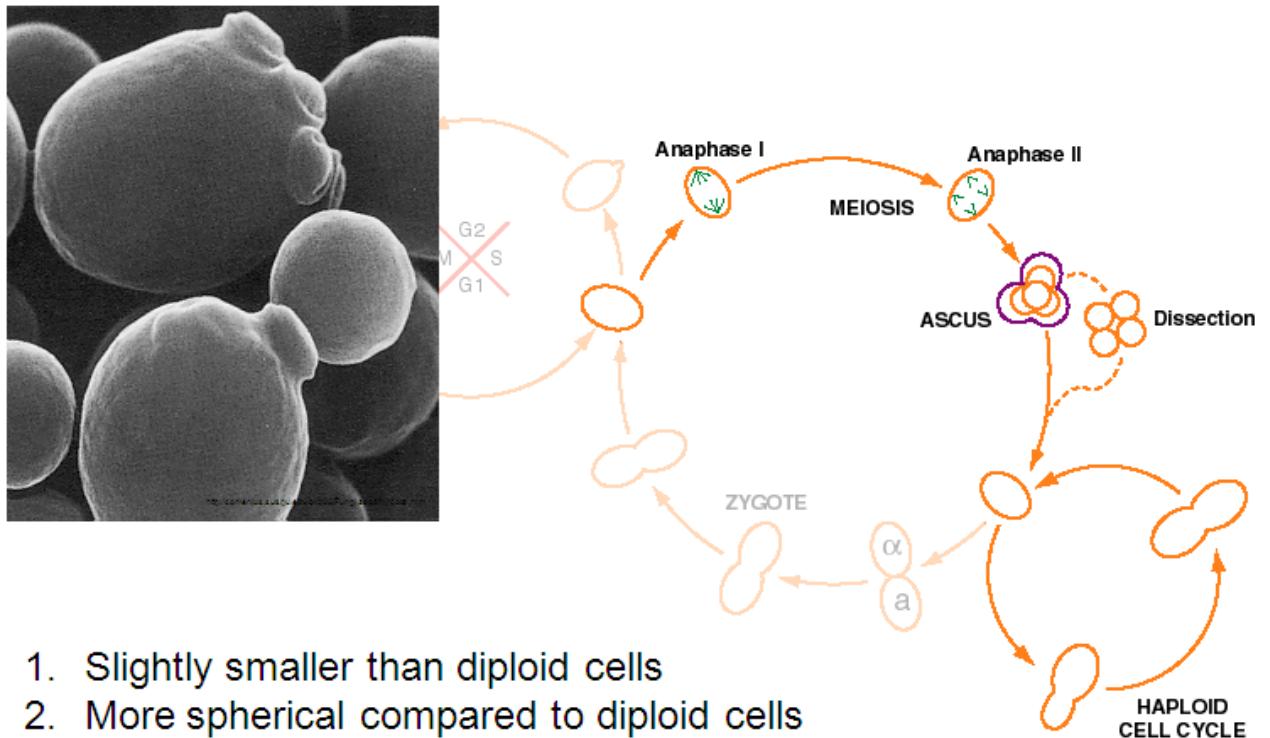
Sporulation produces two a mating type spores and two α mating type spores



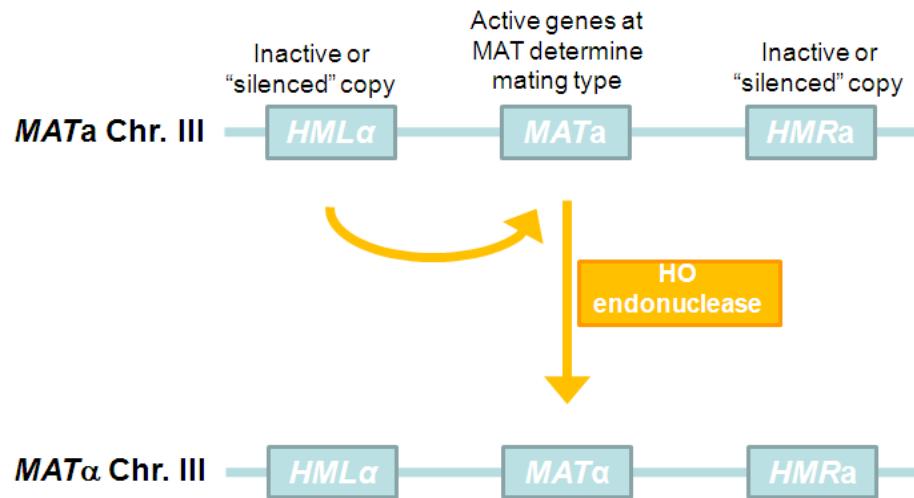
Haploid cells of opposite mating type in close proximity mate to form a diploid cell



In the absence of cells of the opposite mating type, haploid cells divide by mitosis



HO endonuclease initiates a DNA rearrangement that switches the mating type of a haploid cell

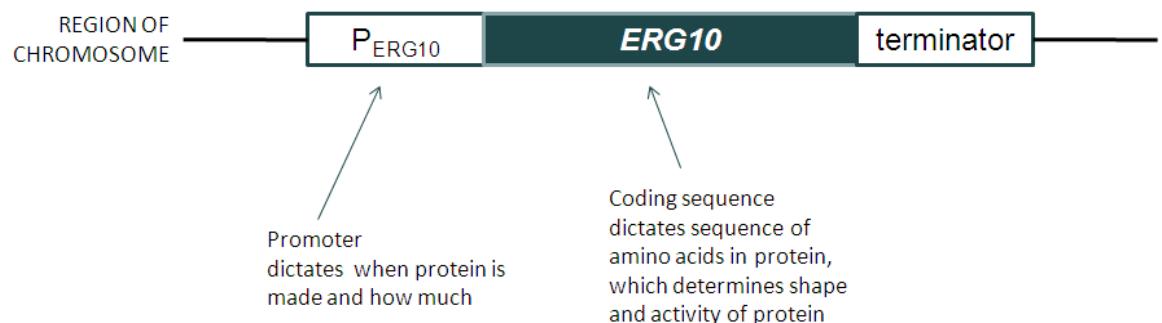


In our strains, HO is deleted, so that we can propagate cells as haploids.

Engineering the *S. cerevisiae* genome

Common engineering we do in *S. cerevisiae*

- Integrating constructs (overexpression, heterologous genes) into the *S. cerevisiae* genome
- Deleting regions from the *S. cerevisiae* genome
- Tuning promoters in the genome (e.g., replacing a strong promoter with a weak promoter, or a constitutive promoter with an inducible promoter)
- Changing individual bases in genes



Gene structure

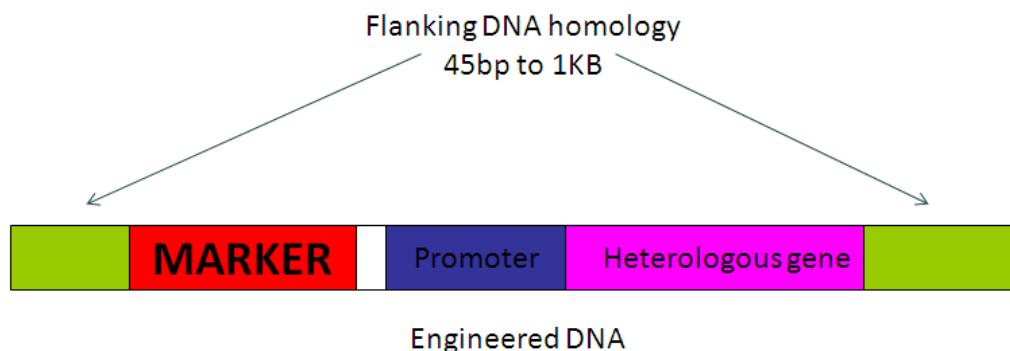
- Promoter (500 bp upstream of the start codon)
 - Often Designated P_{YFG1}
 - Determines when gene is turned on
 - Determines how much transcript is made
 - Inducible – P_{GAL1} or P_{CUP1}
 - Repressible - P_{MET3} or P_{CTR3}
 - Constitutive – P_{TEF1} or P_{CYC1}
- Coding Sequence
 - Codes for protein
 - Always starts with ATG (MET) and ends with TAA, TAG, or TGA
- Terminator (300 bp downstream of the stop codon)
 - Important for determining proper end of transcript

How to get engineered DNA into the cells: Use of Markers

When bathed in foreign DNA, lithium acetate, and PEG, a small fraction of yeast cells take up the DNA, then use homologous recombination to integrate the foreign DNA into its own genome.

We select for this rare event by including a “marker” in the engineered DNA construct. The marker allows us to identify the cells that have properly taken up the DNA.

Example of marker in an engineering construct



After incubating cells with the engineered DNA, they are grown on special media that permits **only the cells that have taken up the marker** to grow.

Common Yeast markers

- Auxotrophic Markers:
 - ***trp1 (trp1-289)* – cannot grow in absence of Tryptophan**
 - ***ura3 (ura3-52)***
 - ***his3 (his3Δ1)***
 - ***leu2 (leu2-1,112)***
 - ***lys2 (lys2 Δ)***
 - ***ade1 (ade1::LEU2)***
- When doing gene replacement with auxotrophic markers, select transformants by plating to medium lacking the amino acid
 - ***ade1::LEU2***
 - transformants would grow in medium containing adenine but lacking leucine
- Dominant Markers
 - ***KanA* – Resistant to G418 (Geneticin)**
 - ***NatA* – Resistant to nourseothricin**
 - ***HygA* – Resistant to hygromycin B**
 - ***DsdA* – D-serine deaminase - can use D-serine as a nitrogen source**
 - only works in prototrophic strains
- When doing gene replacement with dominant markers, transformants must be recovered 3hrs in rich medium (YPD)

Getting the engineered DNA into the right place in the genome: Homologous recombination

Yeast perform homologous recombination with 100 times higher efficiency than non-homologous recombination

All Homologous Recombination is Based on Crossing Over:
How and why do cells do this?

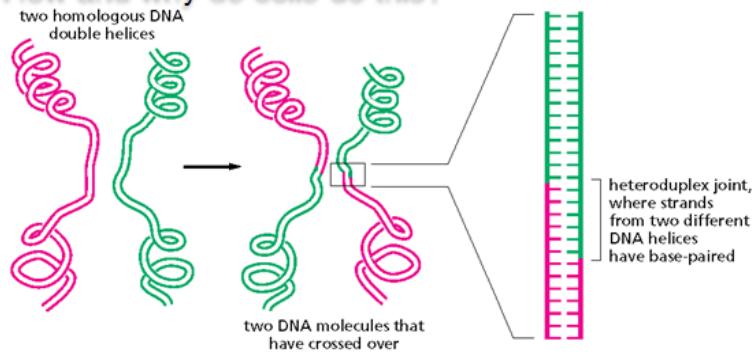


Figure m5-54/5-66 Crossing over.
The breaking and rejoining of two homologous DNA double helices creates two DNA molecules that have "crossed over." The structure that joins two DNA molecules where they have crossed over is called a heteroduplex joint. Such a joint is often thousands of nucleotides long.

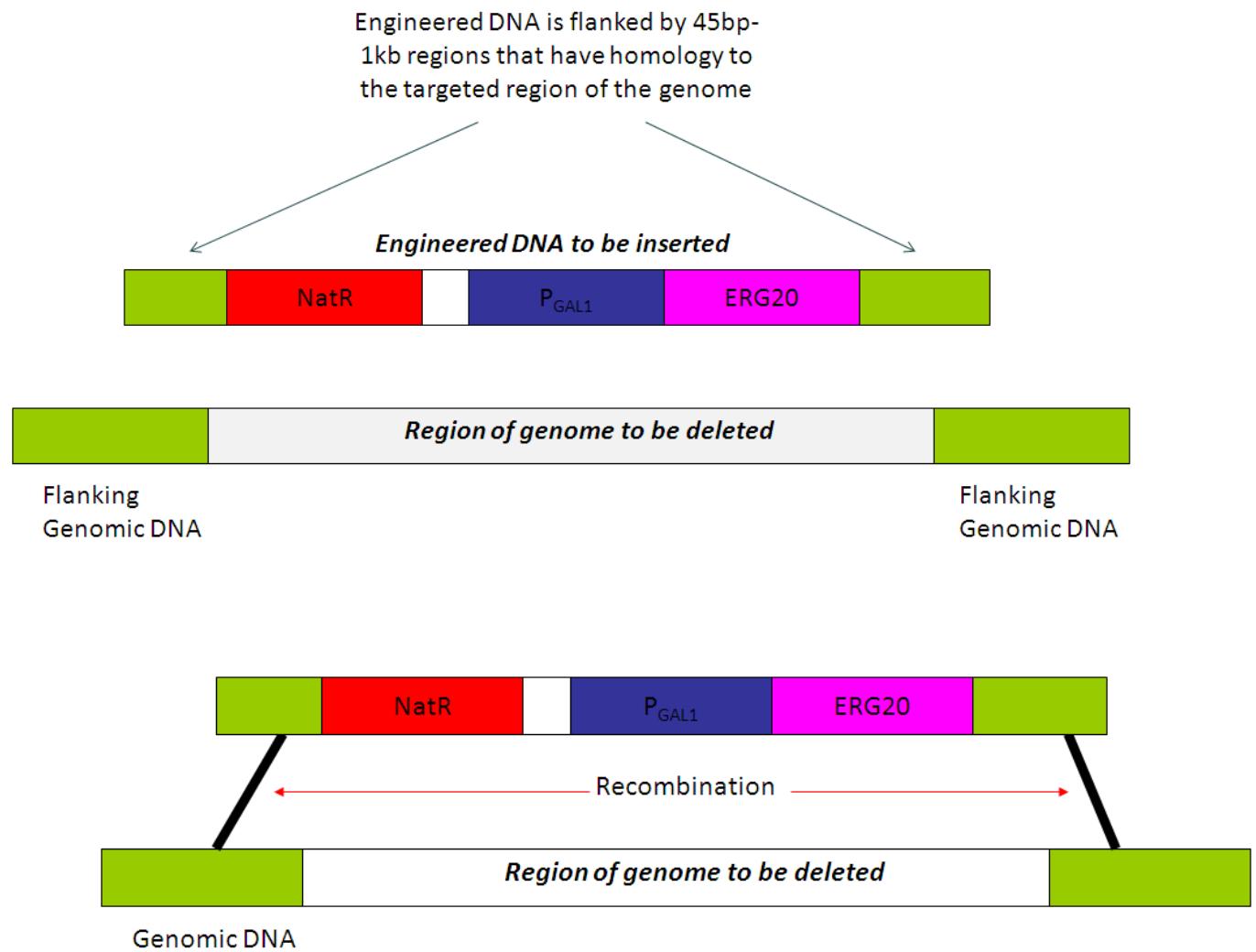
Figure from *Molecular Biology of the Cell*, 5th Edition (2008)
by Alberts, Johnson, Lewis, Raff, Roberts, and Walter

Homologous recombination

- When do organisms naturally perform homologous recombination?
 - To fix broken replication forks during DNA replication
 - To exchange chromosomal regions during meiosis
 - To repair DNA damage, particularly double strand breaks (DSBs), caused by x-rays, cosmic rays, MMS and other chemicals, etc.
 - To switch mating type (specific to *S. cerevisiae*; HO is endonuclease that causes DSB)

- Cells treat transformed linear DNA as damaged DNA
- What triggers homologous recombination?
 - DSBs (or ends of transformed linear DNA) and single-stranded DNA (e.g., freshly boiled carrier DNA added to transformation mix)

Overview of Integrative Transformation



Resulting genomic locus:

Engineered DNA at the correct place in the genome.

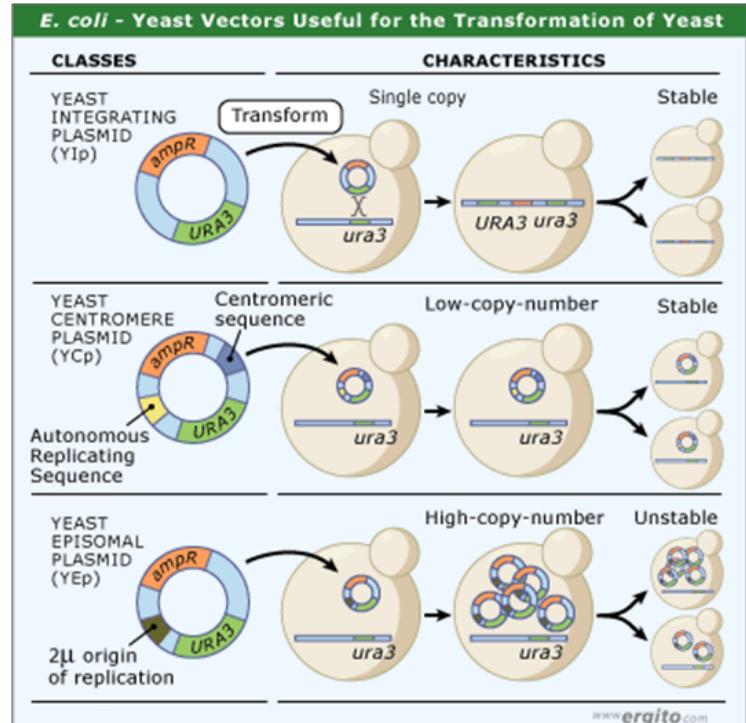
Targeted region of genome deleted.

Integrants are selected on plates containing nourseothricin; only cells that have taken up the NatR gene can survive. We confirm correct integration by PCR.

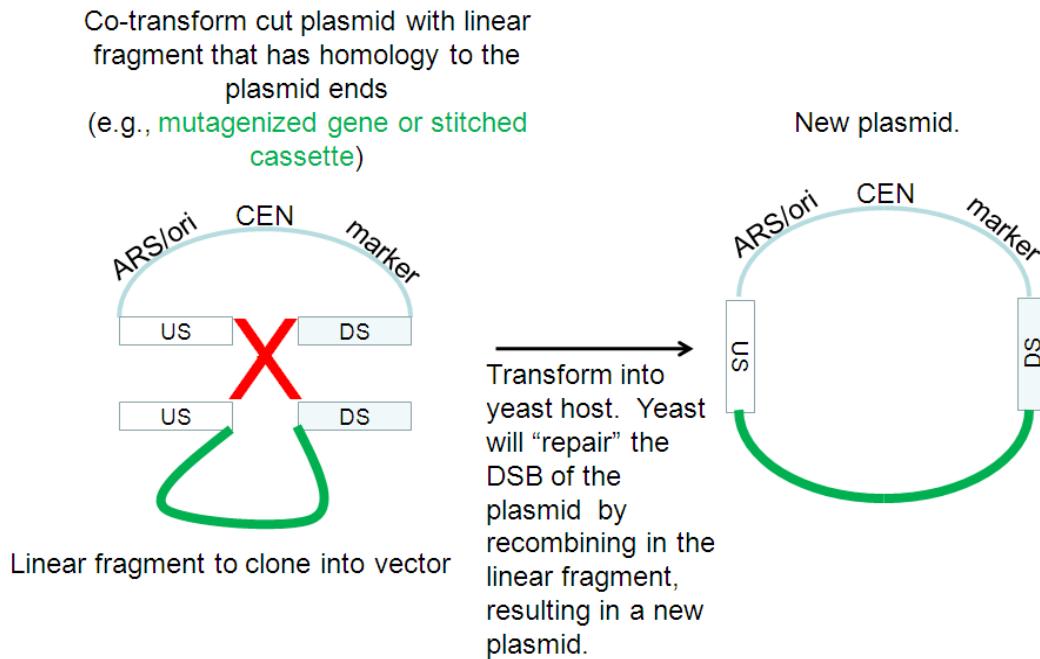


Yeast can also be transformed with self-replicating plasmids that don't integrate into the genome

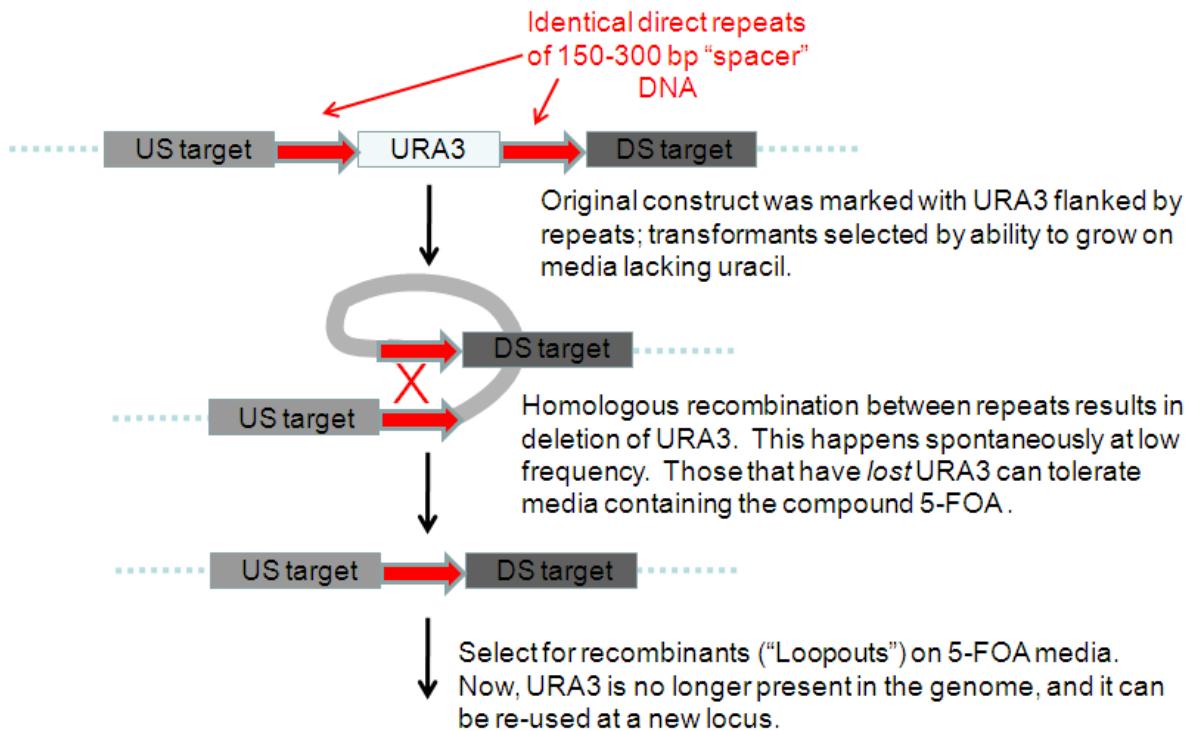
- CEN ARS
 - 1-5 copies/cell
 - Centromeric region (CEN) and a Autonomous Replicating Sequence (ARS)
- 2μ
 - Naturally occurring, DNA episomes
 - Contain 2μ ORI (instead of ARS)
 - 5-20 copies per cell
- 2μ leu2d
 - 20-200 copies/cell



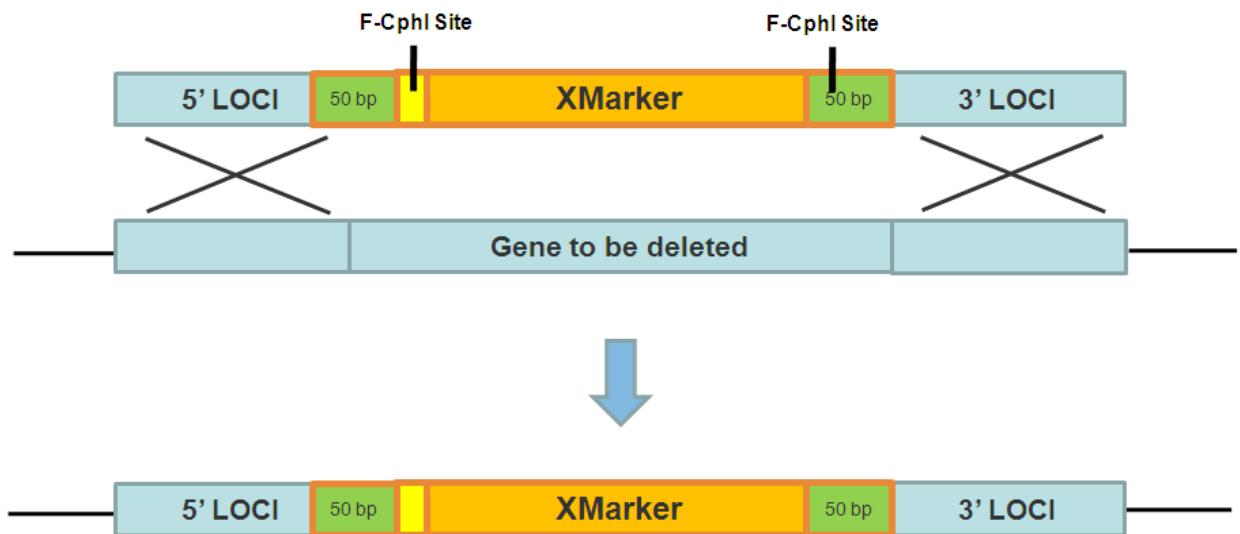
We can clone DNA using yeast plasmids and yeast homologous recombination – “Gap Repair.”



Marker “recycling” prevents us from running out of markers to use in engineering: URA3/5'FOA

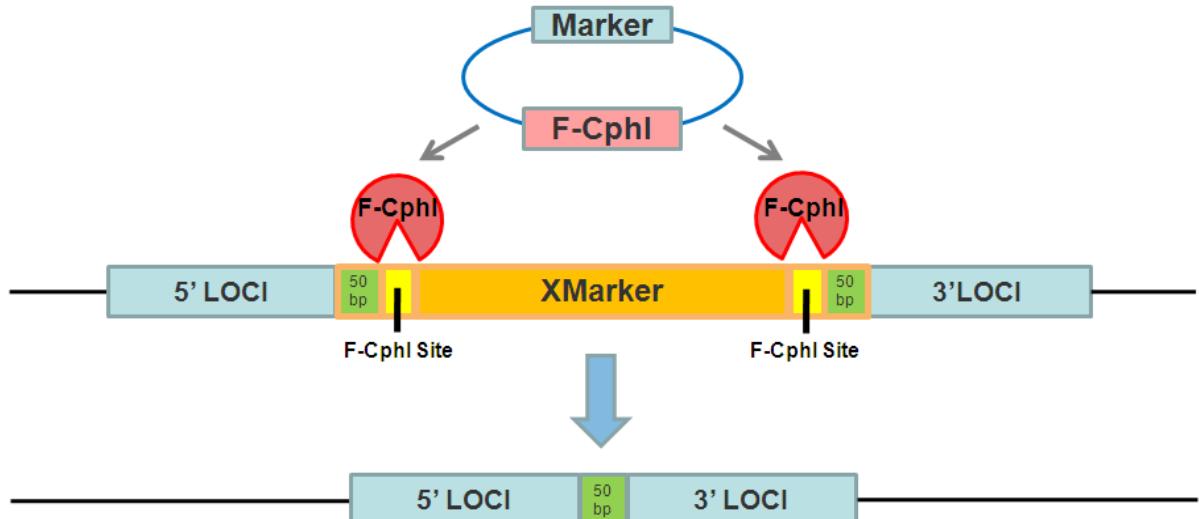


Additional ways to recycle a marker: XMarkers

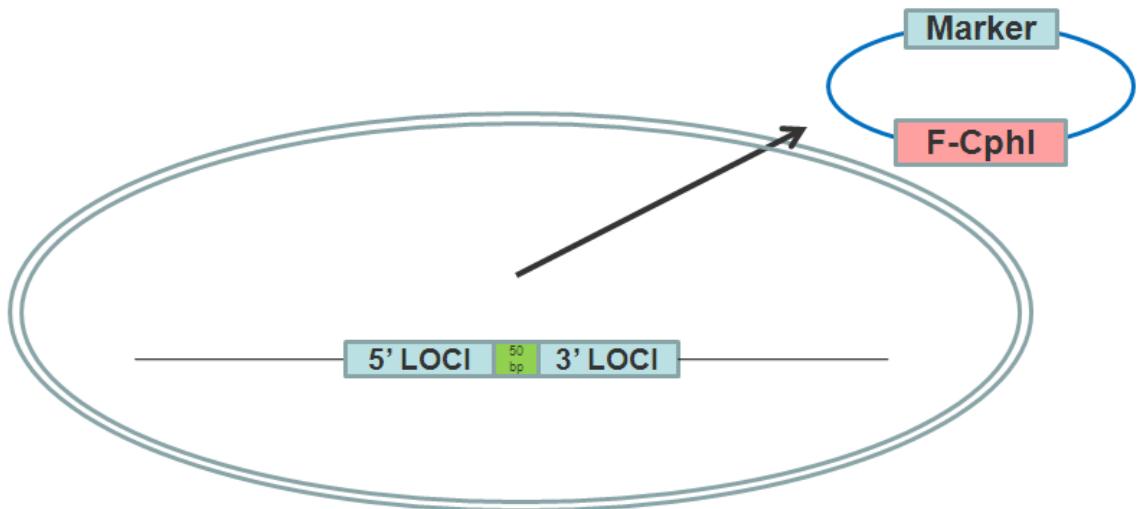


XMarker is a standard marker flanked by recognition sites for a sequence specific DNA cutting enzyme (F-CphI), and 50bp of a repeat sequence. First cells are transformed with the XMarker containing construct, and selected on the appropriate media.

Step 2: Recycling the XMarker



Strains that have incorporated the X-marker are transformed with a plasmid which expresses the F-CphI enzyme. F-CphI cleaves at the recognition sites, leaving double stranded breaks. The cell uses the adjacent homologous 50bp regions to repair the break, thereby excising the XMarker.

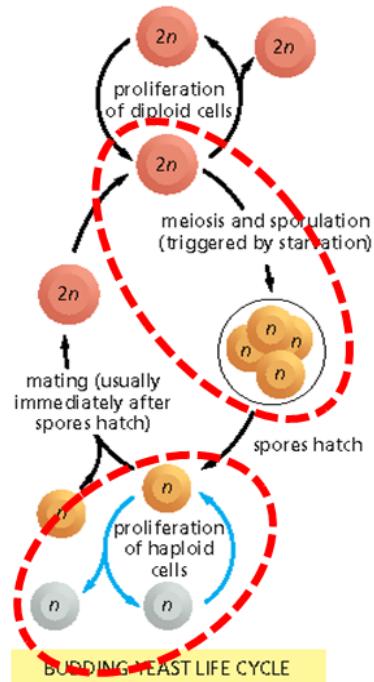


The F-CphI plasmid is subsequently cured, by screening for cells that have lost the marker on the plasmid. The cells are ready for another round of transformations with XMarker constructs.

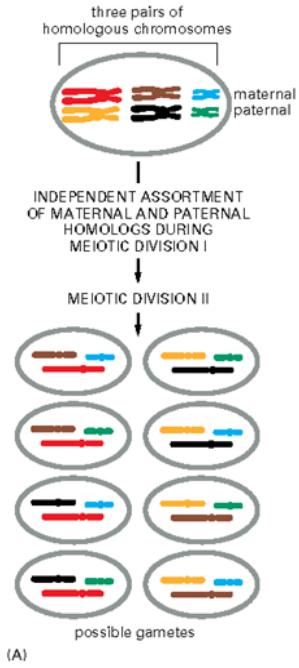
Introduction to how the yeast haploid/diploid cycle can be used in engineering

The Magic of Meiosis (meiosis is part of sporulation in yeast)

S. cerevisiae life cycle

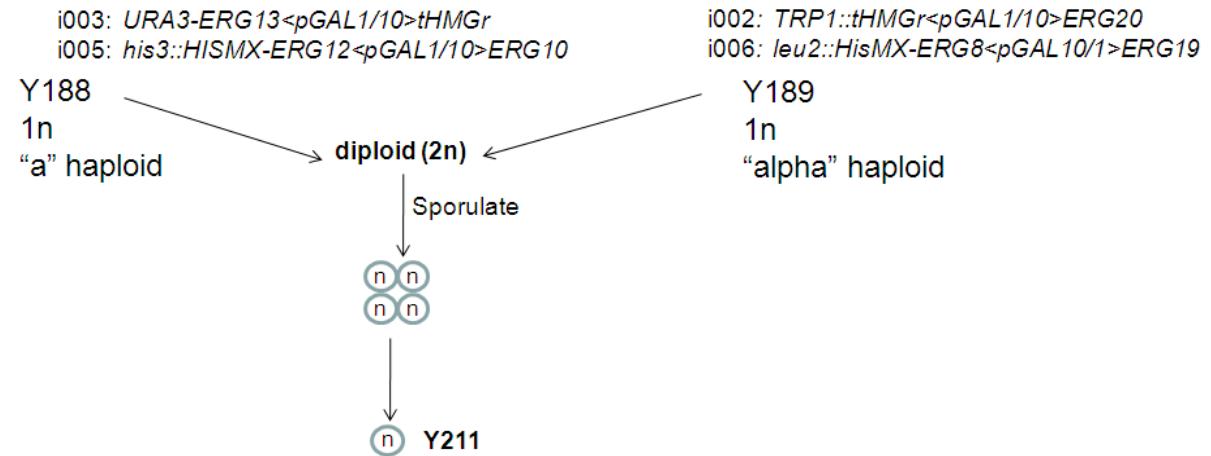


Random assortment of genes in meiosis



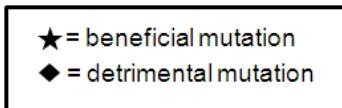
Figures from *Molecular Biology of the Cell* by Alberts, et al. 4th edition, 2002.

Meiosis: Rationally combine traits

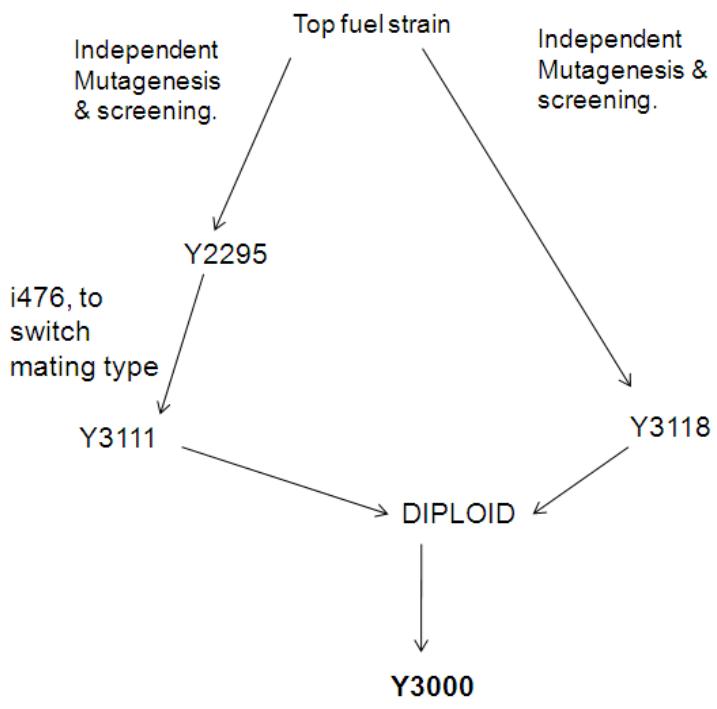
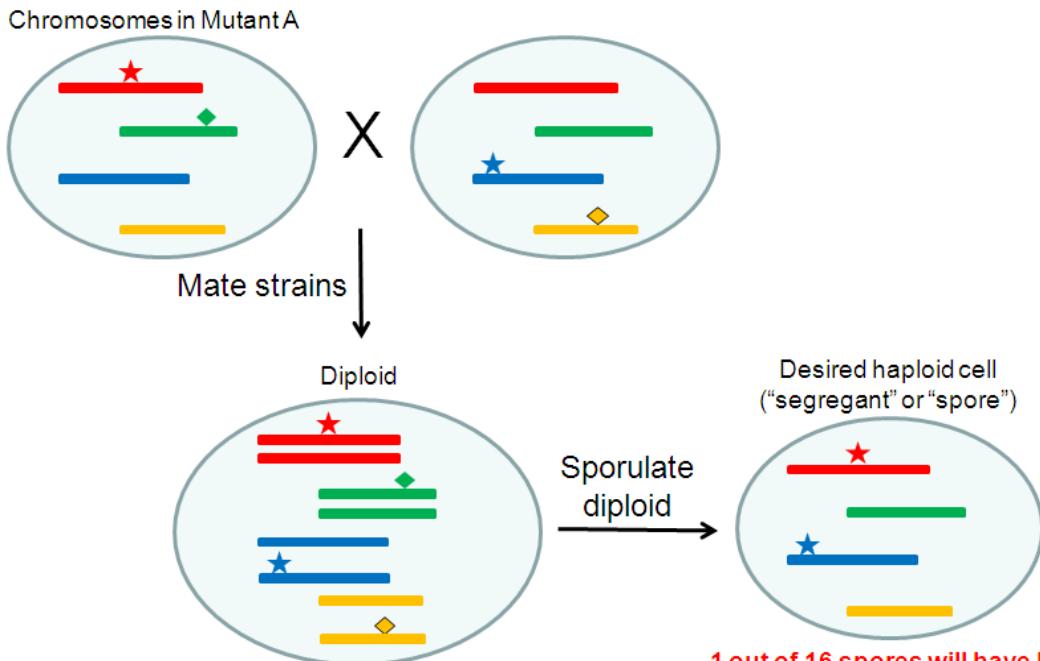


Example: Very early in our top fuels lineage: Y188 has integrations i003 and i005. Y189 has integrations i002 and i006. The strains were mated to form a diploid. The diploid was sporulated, and we screened for a spore that inherited all four integrations.

Meiosis: randomly combine traits and screen for best combination



Imagine two mutant strains, each with a beneficial mutation and a detrimental mutation:



EXAMPLE: Our fuel strains have been mutagenized in many independent lineages.

We can mate independently mutagenized top producers to each other, then sporulate and screen individual spores for even higher production than either parent.

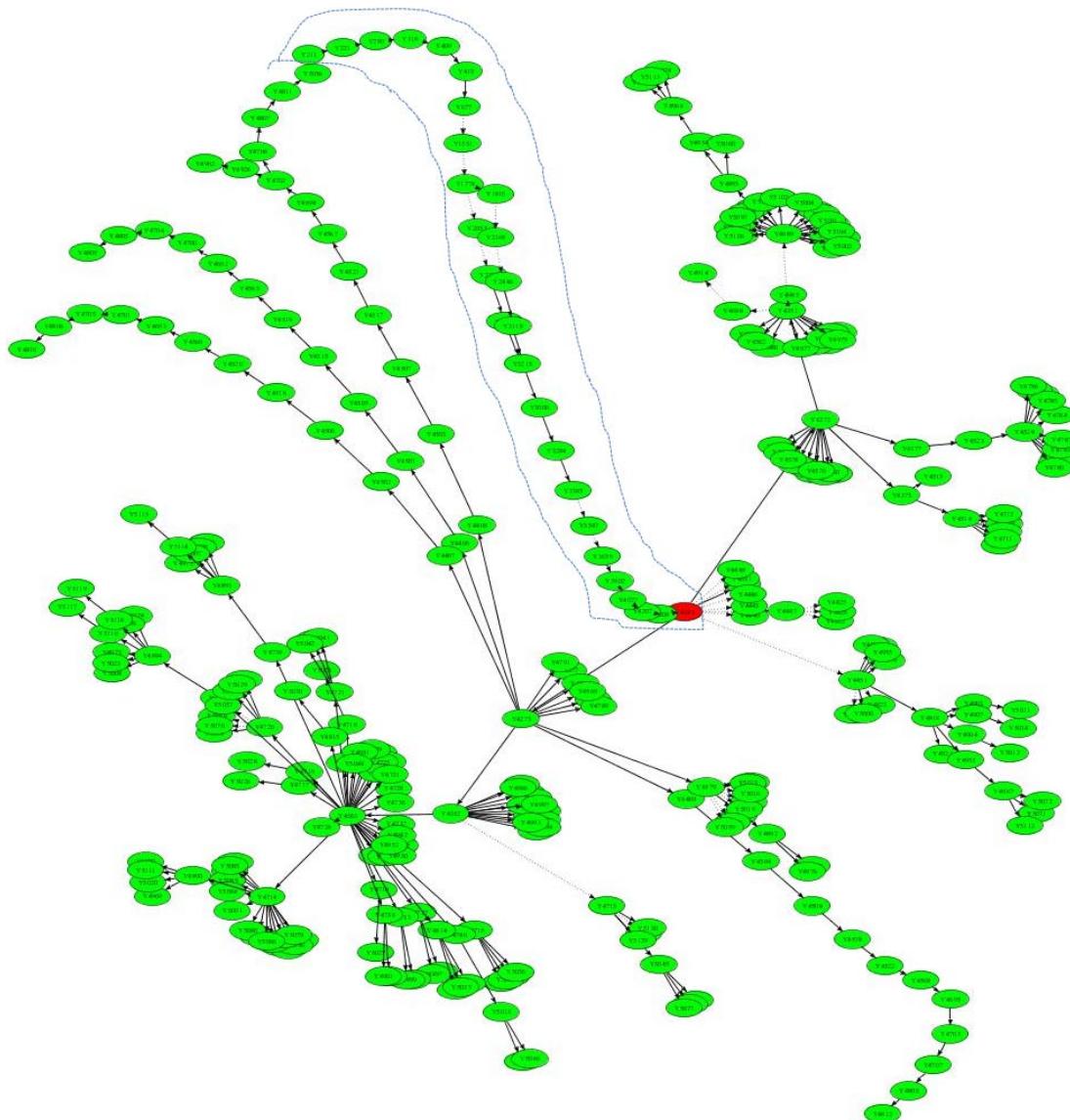
Without even having to identify which mutations are improving fene production, we can combine these improvements into a single strain.

Advanced uses for haploid diploid life-cycle...

- **We can use meiosis/recombination to help us map the mutations responsible for increased fene production in our mutagenesis lineages.**
- **We can cross our strains with industrial strains in order to breed in beneficial industrial traits.**
- **We can sporulate heterozygous industrial diploid strains, and screen for haploids with beneficial traits.**
- **And many more uses!**

2.6 Construction of a yeast farnesene producer (up to Y4242) (Annie Tsong)

The construction path of Y4242 is shown on the next several pages. Refer to strain database section 4.2 for assistance with nomenclature



Construction of Y4242

Stage I = Conversion of naïve strain to a farnesene producer

- Overexpression of native *S. cerevisiae* mevalonate and FPP pathway synthesis pathway
- Overexpression of farnesene synthase

Stage II = Increasing the flux to farnesene

- Multiple rounds of rational engineering (integration and deletion of genes) to increase production of farnesene
- Multiple rounds of mutagenesis and selection for higher farnesene production
- Crossing (mating) strains from independently improved lineages in order to consolidate beneficial genetic features into a single strain
- Integration farnesene synthase that was improved *via* protein engineering, replacing high-copy plasmid bearing unengineered farnesene synthase.

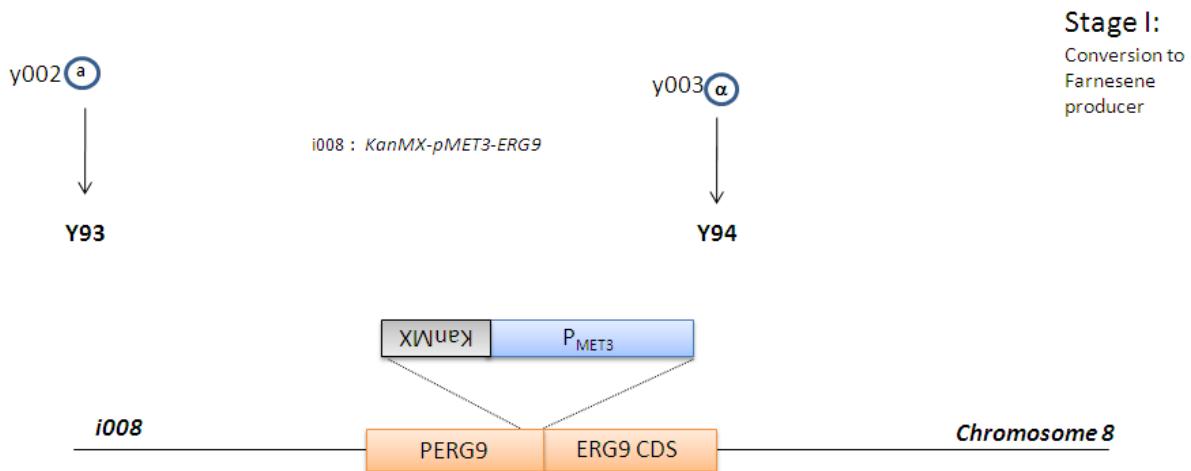
Detail tabulation of steps

Construction and optimization of a Farnesene producer													
From naïve strain to a farnesene producer (to Y977)													
Phase II: Improvement of farnesene producer (to Y4242)													
Red = integration present in the final strain													
Green = non- <i>S. cerevisiae</i> sequence present in final strain													
Phase	Step	Parent strain	Resulting strain	Genetic intervention	Introduced engineering	Comment	id from database	Step	Parent	Strain	Description	id from database	
I	Step 0	Y002	Starting strain	n/a	Starting strain			Step 0	Y003	Starting "alpha" parent			
I	Step 1a	Y002	Y93	insertion	ERG9 US_kanMX_PMET3_ERG9CDS	Promoter swap in front of ERG9	I008	Step 1b	Y003	ERG9 US_kanMX_PMET3(old)_	I008		
I	Step 2a	Y93	Y176	insertion/deletion	"ade1::C _E LEU2	Deletion of ade1	not assigned	Step 2b	Y94	Y177			
I				insertion	URA3 US_hisMX_PGAL1-ERG11_PGA10-ERG12_PURA3_URAS_CDS	Introduction of ERG11, ERG12; restoration of URA3	I003						
I	Step 3a	Y176	Y188	insertion	HIS3 US_hisMX_PGAL1-ERG11_PGA10-ERG12_PURA3_HIS3_D5	Introduction of ERG10, ERG12; restoration of HIS3. HIS3 contains A_gosyphil and S_dalven sequence.	I005	Step 3b	Y177	Y189	LEU2 truncCDS_hisMX_PGAL1-ERG19_PGA10-ERG8_Leu2_D5	I006	
I		Y188 x Y189	no name	mating	n/a	Mating	+I002 (TRP1) +I006 (LEU2)						
I	Step 4	Y238 (diploid)	Y211	insertion	ADE1 US_PGAL1-hMq1_PGA10-ID1_PADE1-ADE1_CDS	Introduction of (D1, hMq1); restoration of ADE1	I004 (ADE1)						
I	Step 5	Y238	V211 (MATα)	sporulation	All integrations segregated into single haploid spore via meiosis	Sporulation to segregate all engineering into one spore	(I002, 5, 4, 5, 6, 8)						
I	Step 6	V211 (MATα)	Y290	insertion/deletion	GAL80 US_hphA_GAL80 DS	Deletion of GAL80	I032						
I	Step 7	Y290	Y409	insertion	pAM404 (skipped V2.0 intermediates w/ amorphous plasmid)	Introduction of plasmid pAM404. FS is from <i>A. annua</i> .	n/a						
I	Step 8	Y409	Y419	insertion/deletion; mutation	ERG9-US_PGA10::GAL4-TGAL4_nata_PMet3-ERG9CDS; also Mx mutation	Introduction of GAL4QC at pMET-ERG9	I008->I033, I005->I688 (HIS3)						
I	Step 9	Y419	Y677	insertion/deletion	GAL80 US_kanR_PGAL1-ERG12_GAL80-DS	Introduction of extra ERG12; deletion of GAL80	I032->I037						
II	Step 10	Y677	Y1778	mutagenesis	mutagenesis	mutagenesis	n/a						
II	Step 11a	Y1778	Y1816	insertion/deletion	HXT2_hph_PTDH3-ERG10-TAP1_TCCW1_PYP01-ERG11(B)u_FTUB2-ERG10_butyrylum	Insertion of trans-genes, knockout of HXT2. (ERG11 is from <i>B. juncea</i> , and ERG10 is from <i>C. butyrylum</i>)	I301	Step 11b	Y1778	Y2295	Mutagenesis		
II	Step 12b	Y1816	Y2446	mutagenesis	mutagenesis	mutagenesis	n/a	Step 12b	Y2295	Y3111	I476_Metapho-hph-metapho (swap mating type)	I476	
II	Step 13	Y2446	Y3118	insertion/deletion	FURA3-URA3 partial-PGAL80-GAL80-TGAL80_URA3 partial-ai-TUGA3	Restoration of GAL80 to facilitate mating; not present in final strain.	I003->I094->I477						
II	Step 14	Y3118 x Y3111 (diploid)	Y3215	mating	mating	mating	->I477, +I476						
II	Step 15	Y3215	Y3000 (MATα/iph)	sporulation	Sporulation	Sporule screen; selected spore is MATα/iph. (I476 is MATα/iph)							
II	Step 16	Y3000	Y3284	insertion/deletion	pu-ura3-hMq1<GALL10>+HMGs-HisG (to rescue URA3 at I003)	Replacement of URA3 from I003 with HisG sequence from <i>Salmonella</i> .	I003->I094 (URA3)						
II	Step 17	Y3284	Y3385	insertion/deletion	I467_Fndt80-URA3_pKT5pAC52-pGK1_pGAL7-POC-Mer-TDH3	Insertion of AC52, POC and deletion of NDT80. zPDC is from <i>Z. mobilis</i> .	I467						
II	Step 18	Y3385	V3639	mutagenesis	mutagenesis	mutagenesis	n/a						
II	Step 19	Y3639	Y4207	insertion/deletion; plasmid loss	pAM404	Insertion of 4xF5, deletion of HO, loss of pAM404. FS is from <i>A. annua</i> .	I797 (HO)						
II	Step 20	Y4207	Y4208	insertion/deletion	I798_ndt80_pGAL4-aaF54-D-ICYC1-pHXT3-AC52-pGK1_pGAL7-POC-2m-tTDH3	Insertion of 4xF5 at NDT80 locus; replacing URA3 marker. FS is from <i>A. annua</i> .	I798 (NDT80)						
II	Step 21	Y4208	Y4242	insertion/deletion	I806_dH1-URA3-pGAL1-AAF5-A-4	Insertion of 4xF5, deletion of dH1	I806						



The **starting strains** were CEN.PK2 haploids of opposite mating types, with the genotype *ura3-52*, *trp1-289* *leu2-3,112* *his3¹* *MAL2-8C SUC2*.

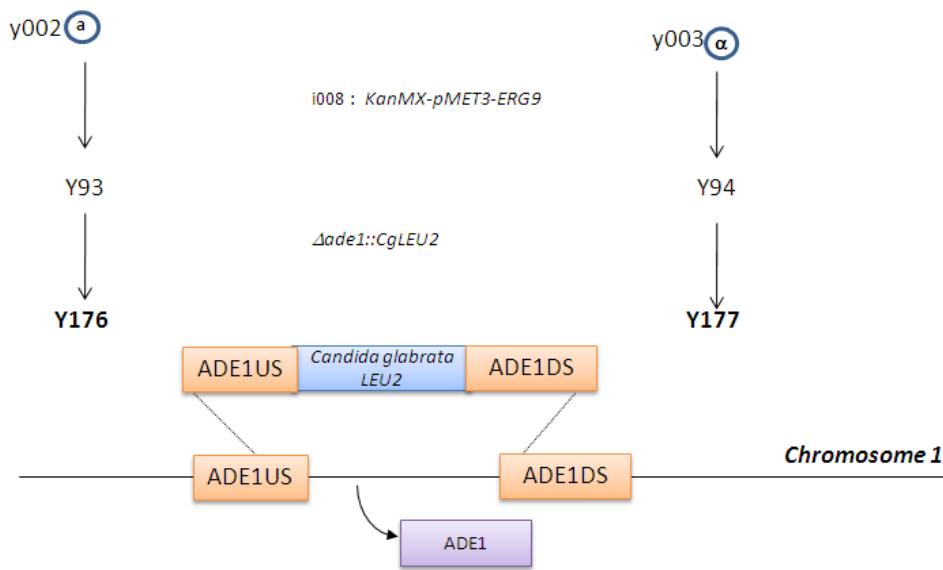
The strains were generated by van Dijken et al. 2000 (Enzyme and Microbial Technology 26:706-714).



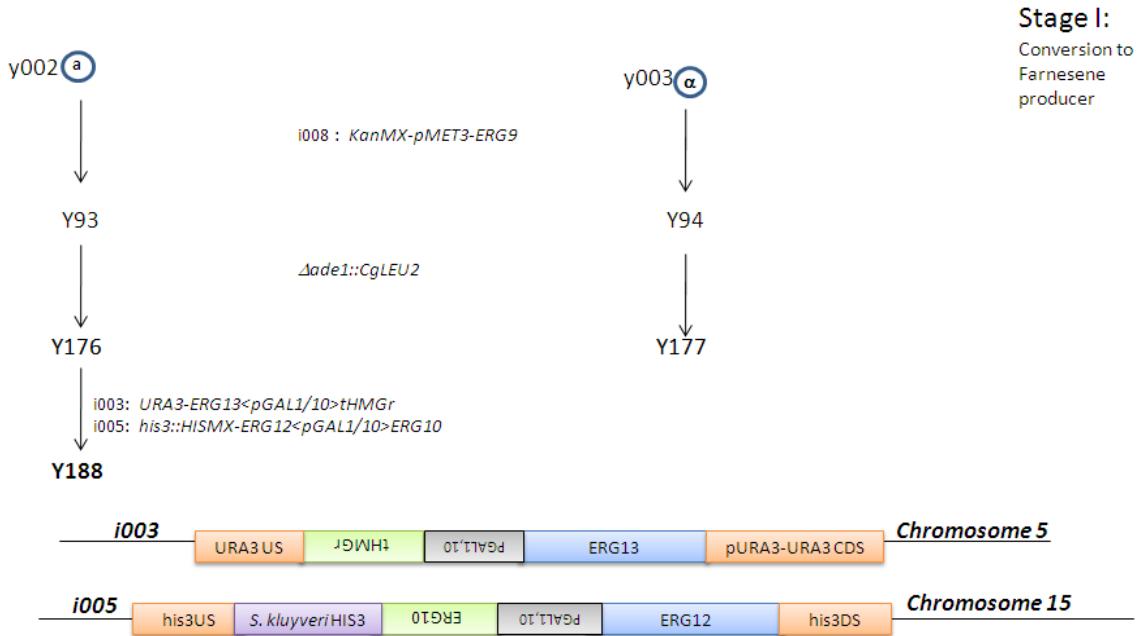
P_{MET3} was integrated in front of the *ERG9* (squalene synthase) gene in Y002 and Y003 (integration i008, resulting in strains Y93 and Y94). This construct results in weak constitutive expression of *ERG9*. Since squalene synthase uses FPP as a substrate, lowering *ERG9* expression is thought to increase the FPP pool available for conversion to farnesene. Due to a truncation, P_{MET3} is not responsive to methionine concentration in this construct.

The integration cassette was generated by using oligos with homology to P_{ERG9} to amplify the KanMX- P_{MET3} region of pAM328.

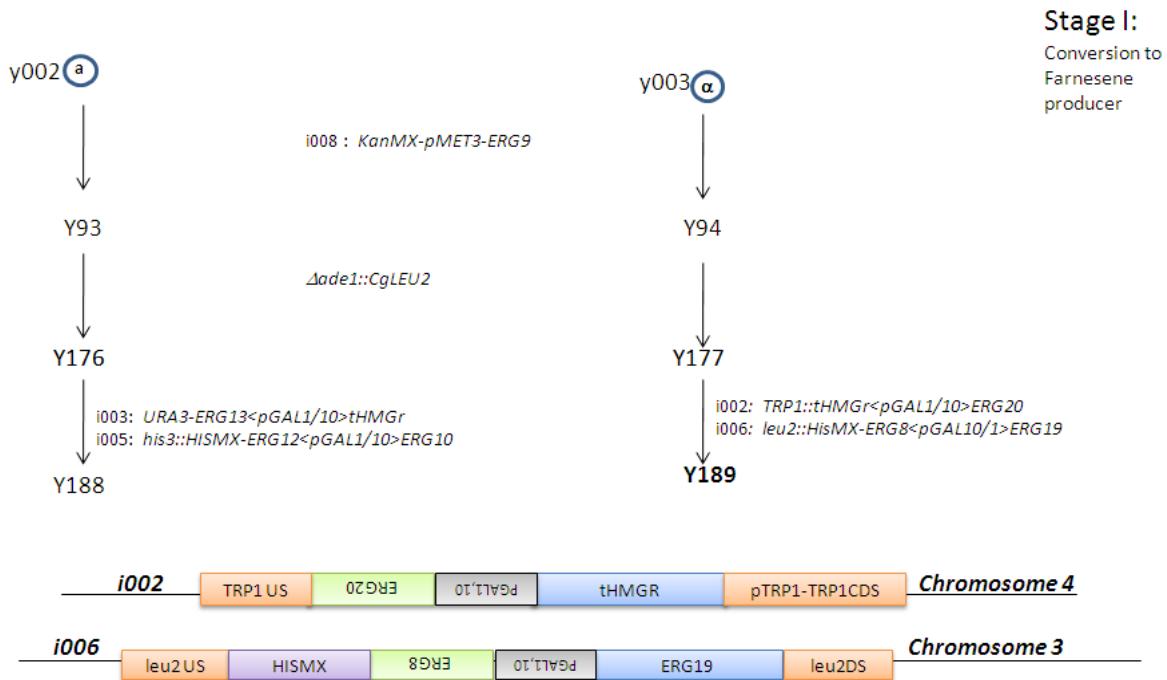
Stage I:
Conversion to
Farnesene
producer



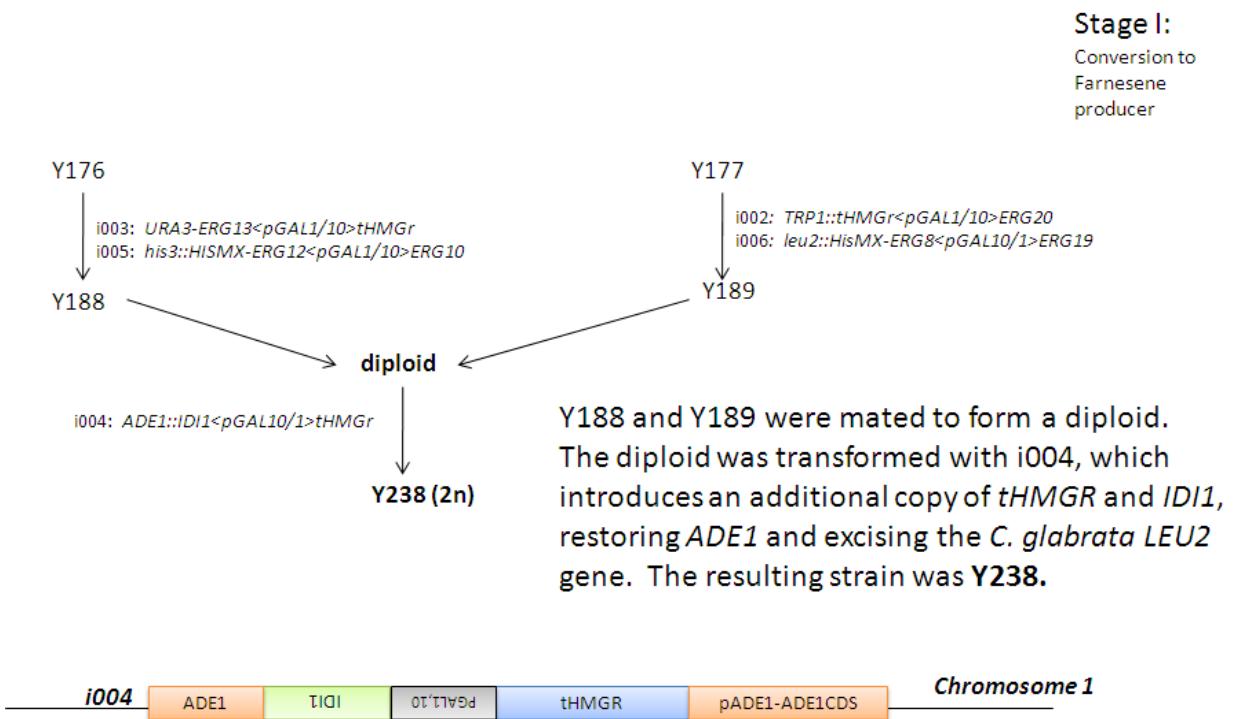
LEU2 was amplified from *Candida glabrata* genomic DNA using primers with homology to *ADE1* upstream and downstream regions. The PCR product was integrated into Y93 and Y94, resulting in $\Delta ade1::CgLEU2$ strains Y176 and Y177.

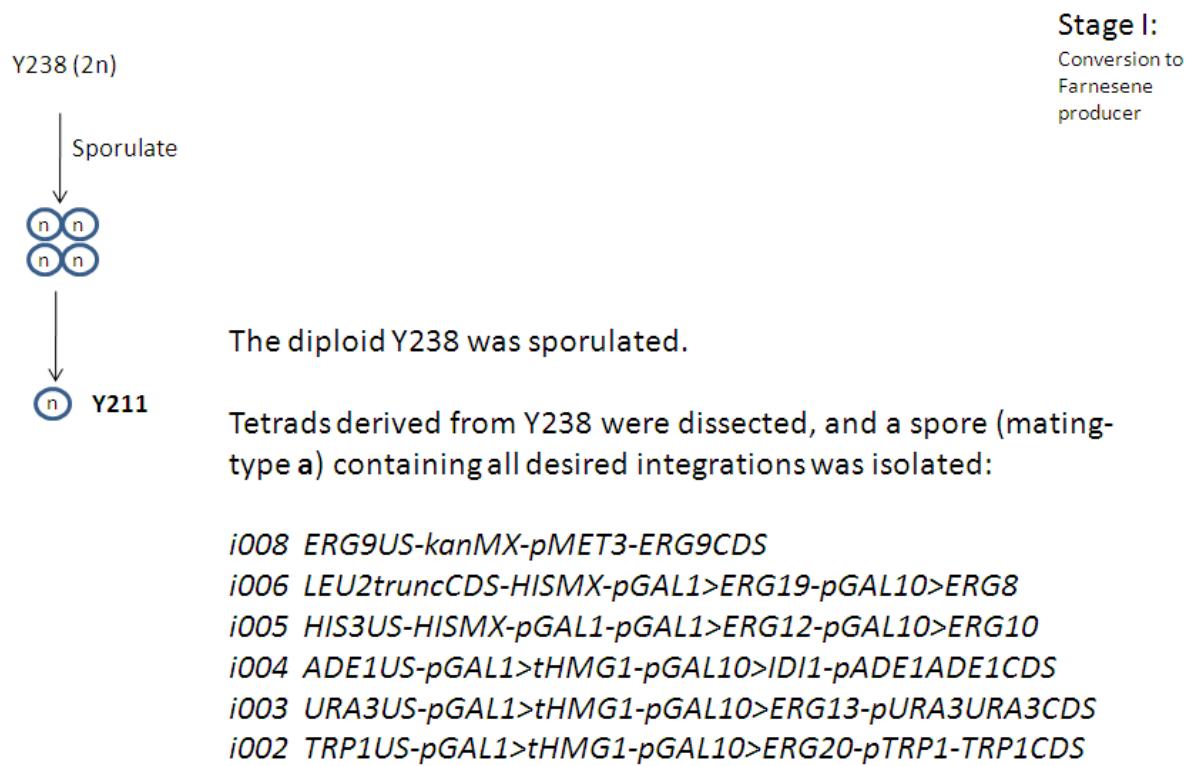


Y176 was transformed with i003 (overexpression of *ERG13* and *tHMGr*, and restoration of the Ura prototrophy at the *URA3* locus) and i005 (overexpression of *ERG12* and *ERG10*, and restoration of His prototrophy at the *HIS3* locus). The resulting strain was **Y188**.

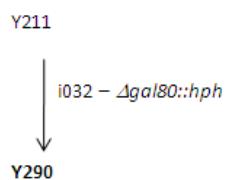


In one step, Y177 was transformed with i002 (overexpression of *tHMGr* and *ERG20*, and restoration of Trp prototrophy at the *TRP1* locus) and i006 (overexpression of *ERG19* and *ERG8*, and restoration of His prototrophy at the *leu2* locus), resulting in Y189.

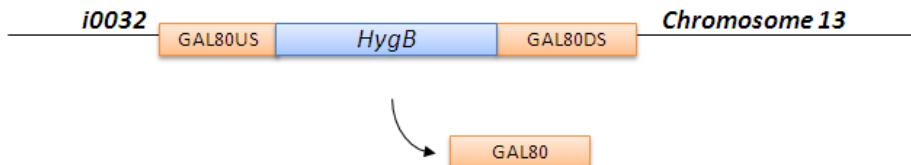


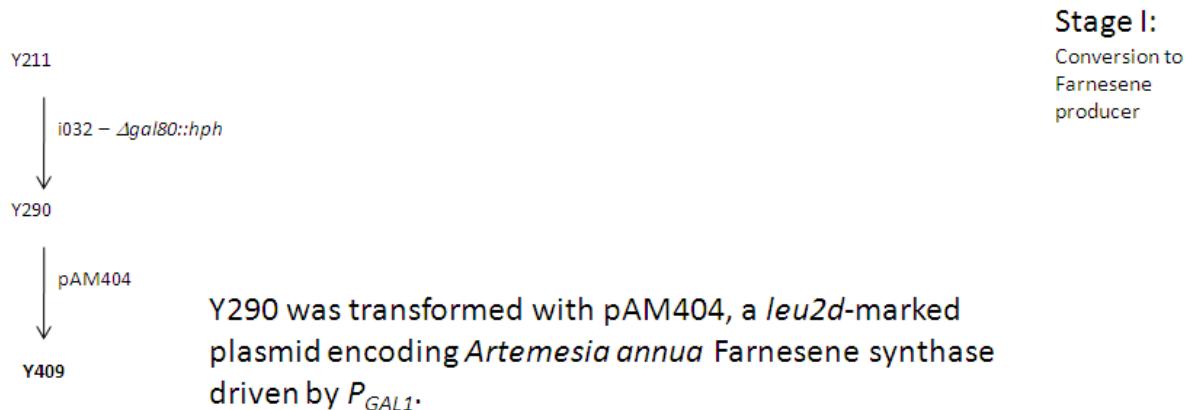


Stage I:
Conversion to
Farnesene
producer

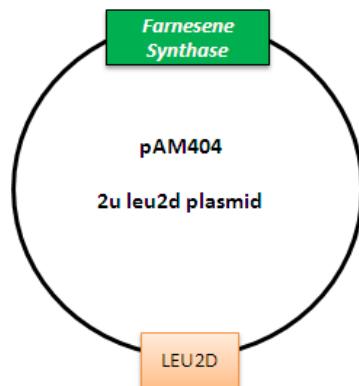


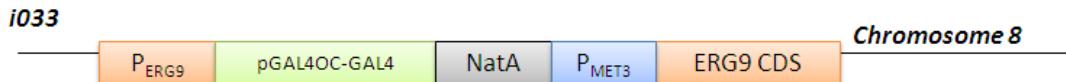
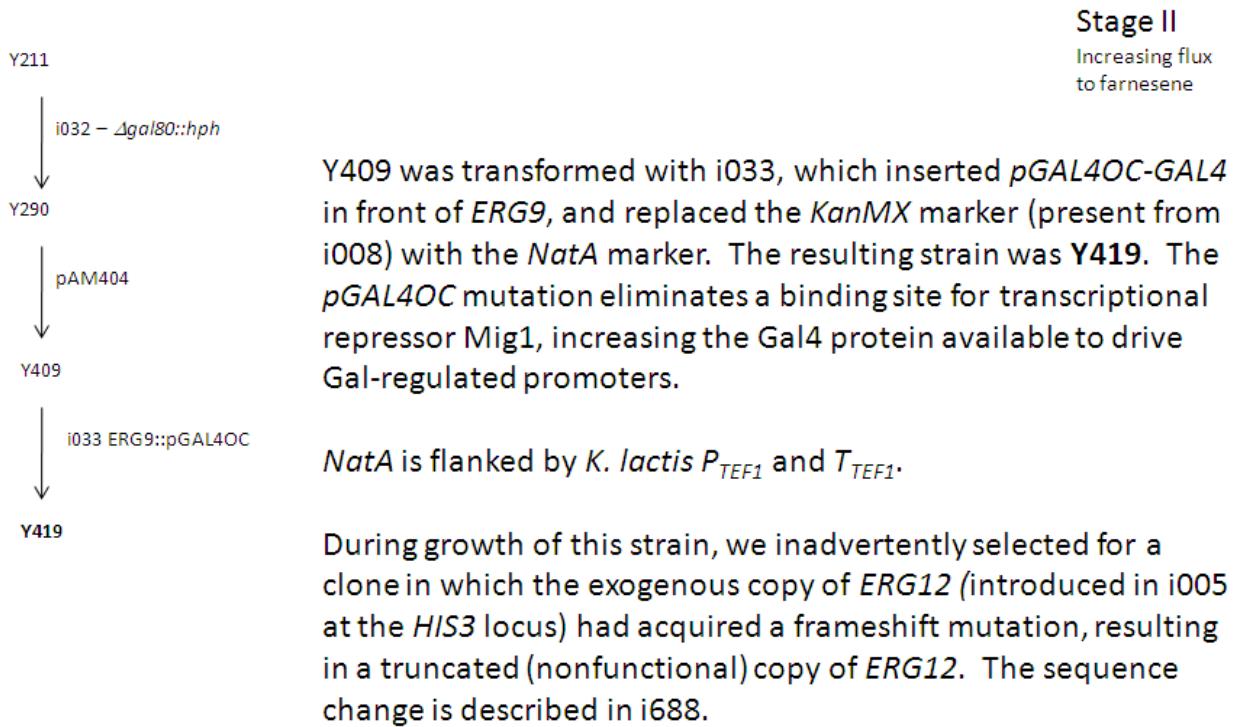
The *GAL80* ORF was precisely deleted in Y211, resulting in strain Y290. Deletion of *GAL80* results in constitutive expression of all pGAL-driven genes, including the engineered mevalonate pathway. The integration construct (i032) was generated by amplifying the *HygB* resistance marker using oligos with homology to the *GAL80US* and *GAL80DS* regions.

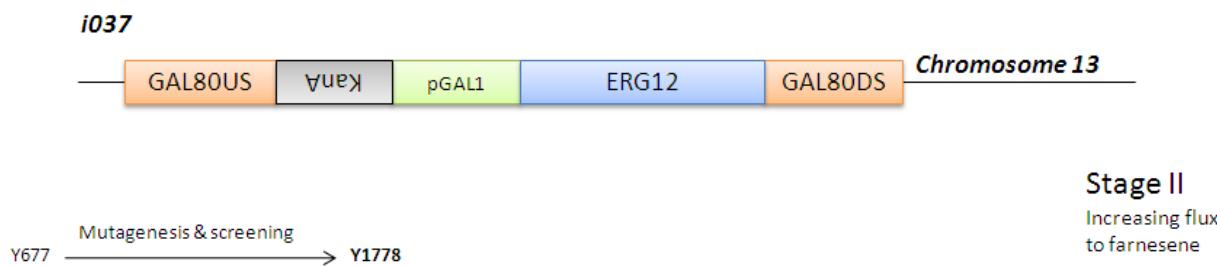
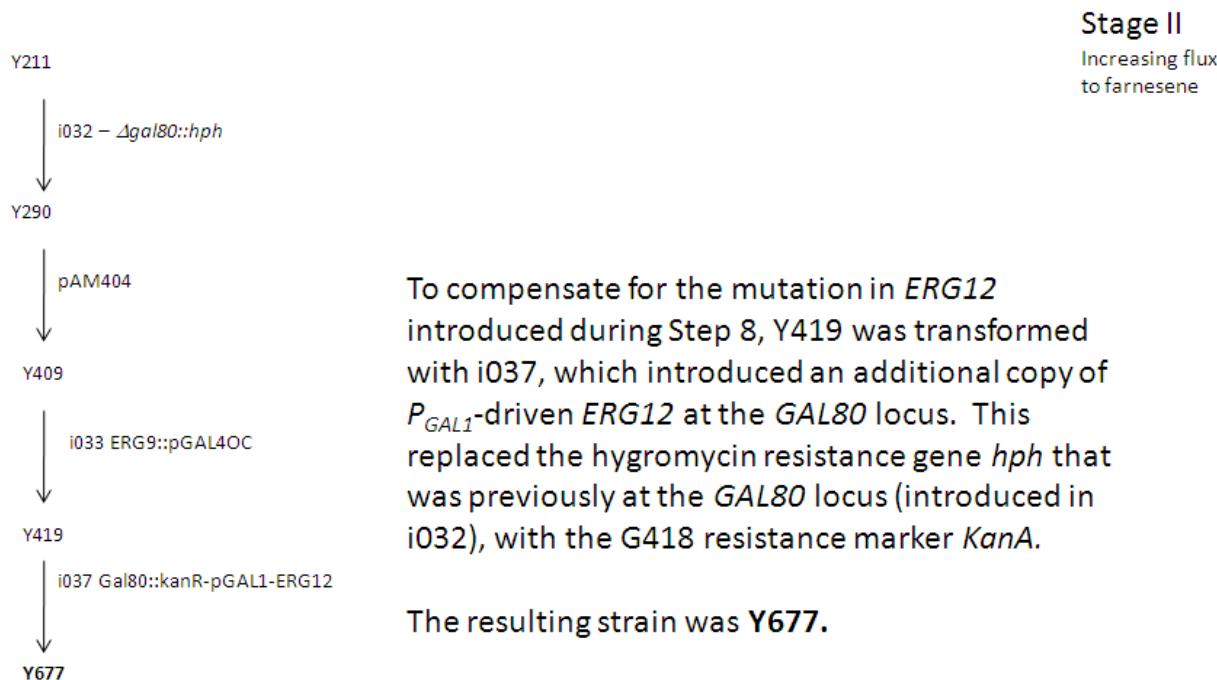




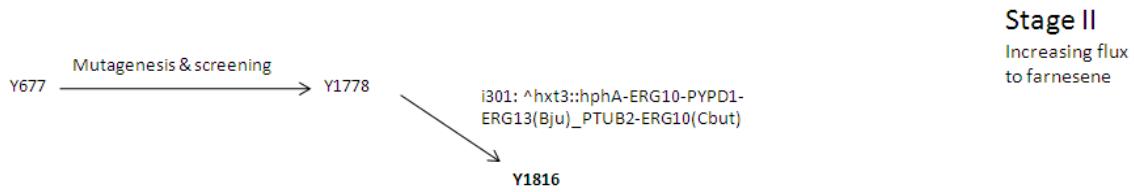
The resulting strain was **Y409**.



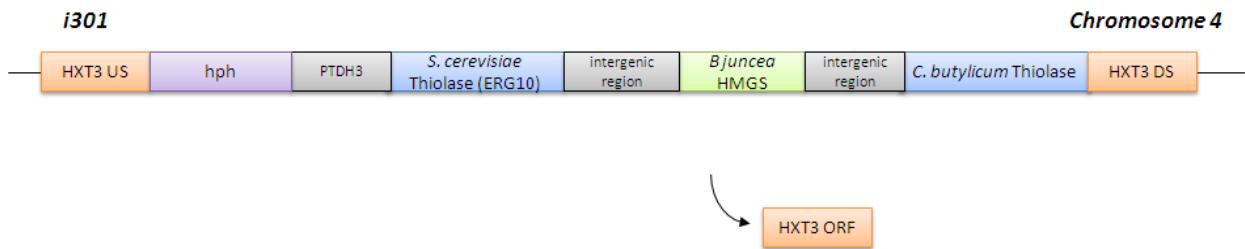


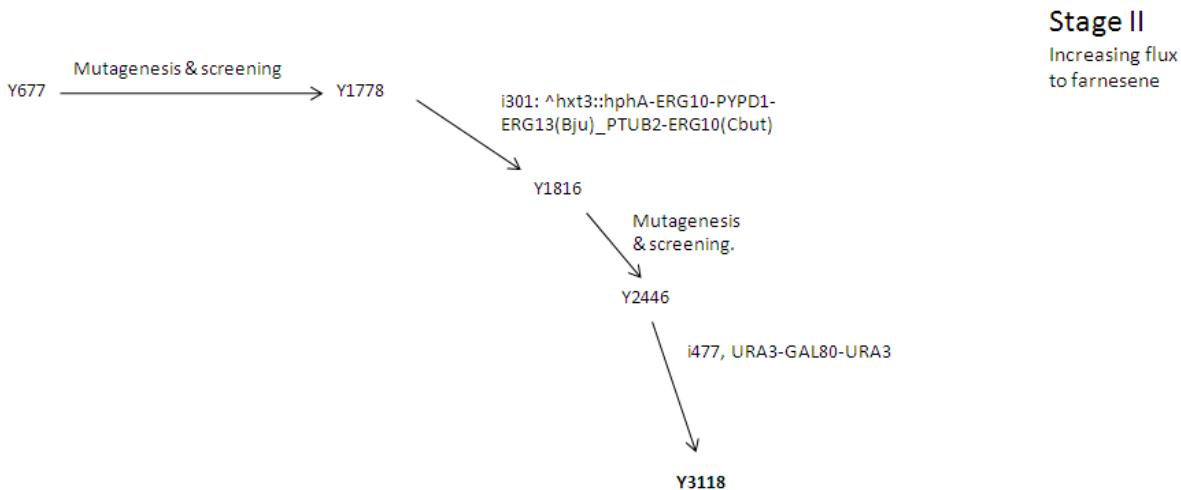


Y677 underwent several rounds of mutagenesis and screening for better farnesene producers, resulting in strain **Y1778**.



Y1778 was transformed with i301, which is comprised of two acetoacetyl-CoA thiolase genes (one derived from *S. cerevisiae*, and the other from *C. butylicum*) and a copy of the *B. juncea* HMGS gene, integrated at the *HXT3* locus. The *HXT3* ORF was deleted by this integration, and the construct was marked with the hygromycin resistance gene *hph*. The resulting strain was Y1816.

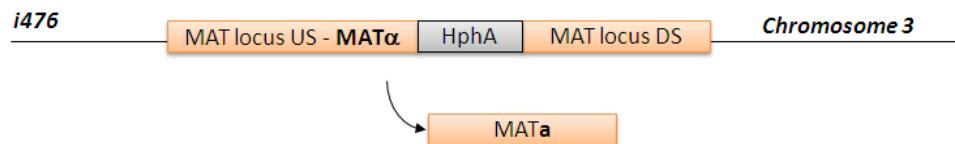
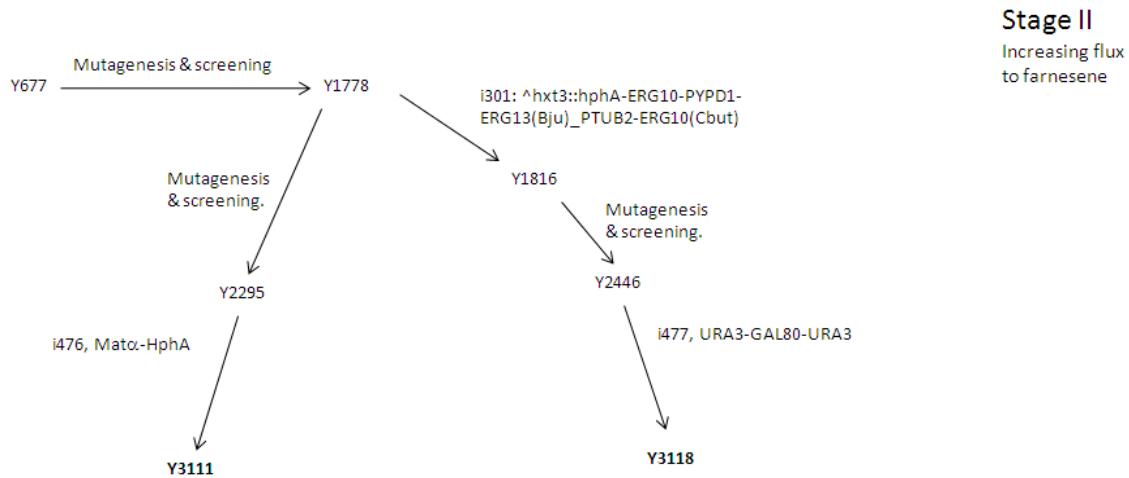




Y1816 was mutagenized and screened for higher farnesene production, resulting in strain **Y2446**.

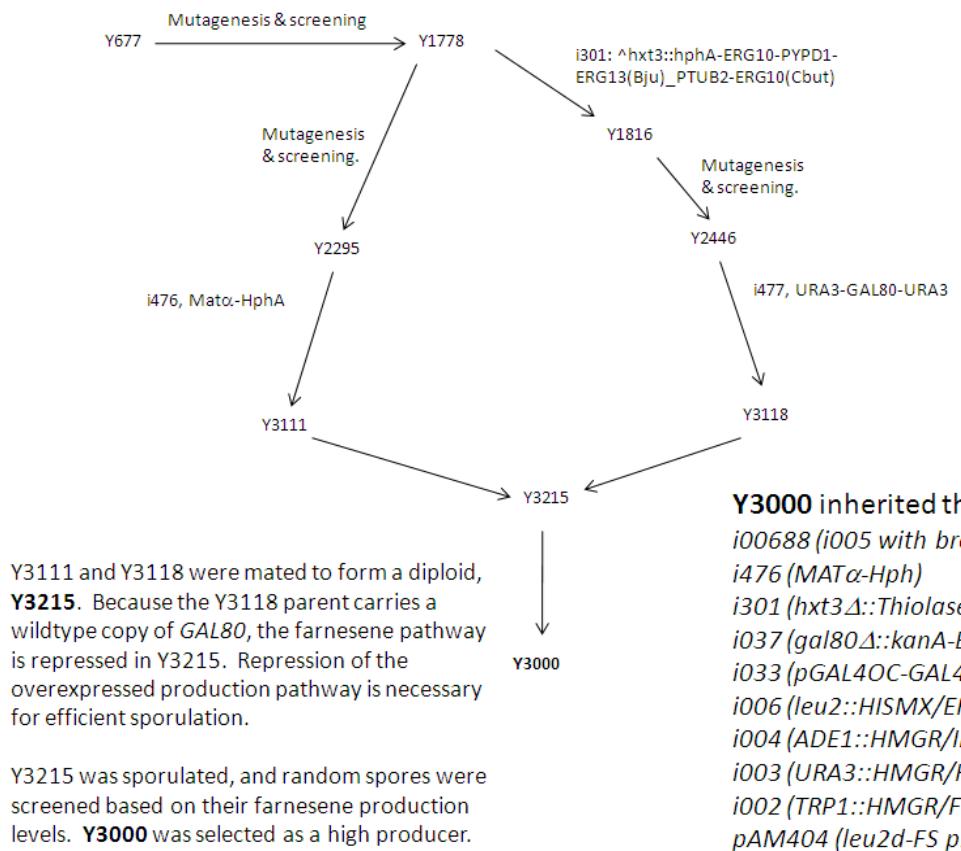
Y2446 was then transformed with i477 which introduced a wildtype copy of *GAL80* while disrupting the *URA3* gene. The resulting strain was **Y3118**. Gal80 represses expression of the engineered farnesene production pathway, most of which is under P_{GAL} regulation.

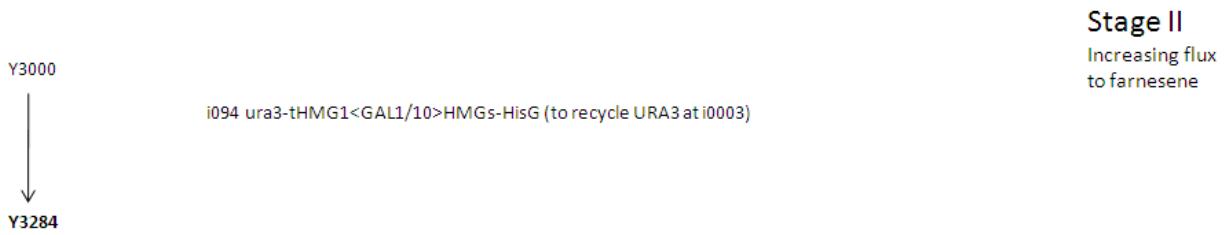




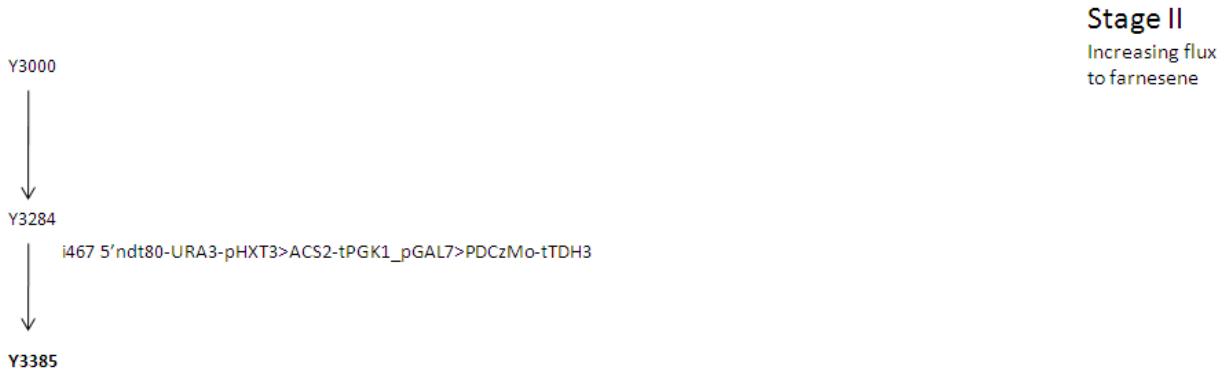
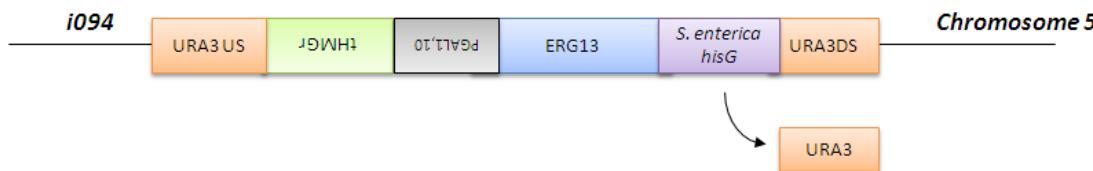
In a separate lineage, Y1778 was further mutagenized, resulting in strain Y2295. Then, Y2295 was transformed with i476, a hygromycin resistance-marked (*hph*) construct which integrates a copy of MAT α at the MAT locus, changing the mating type from a to α . The resulting strain was Y3111.

Stage II
Increasing flux
to farnesene

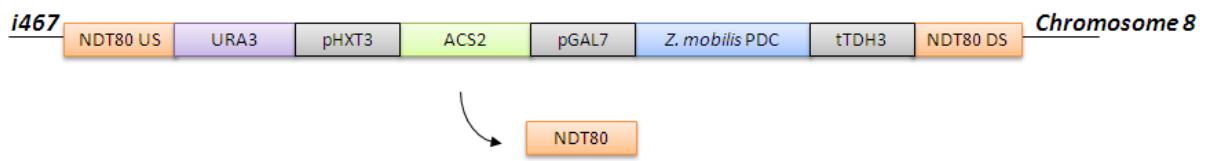


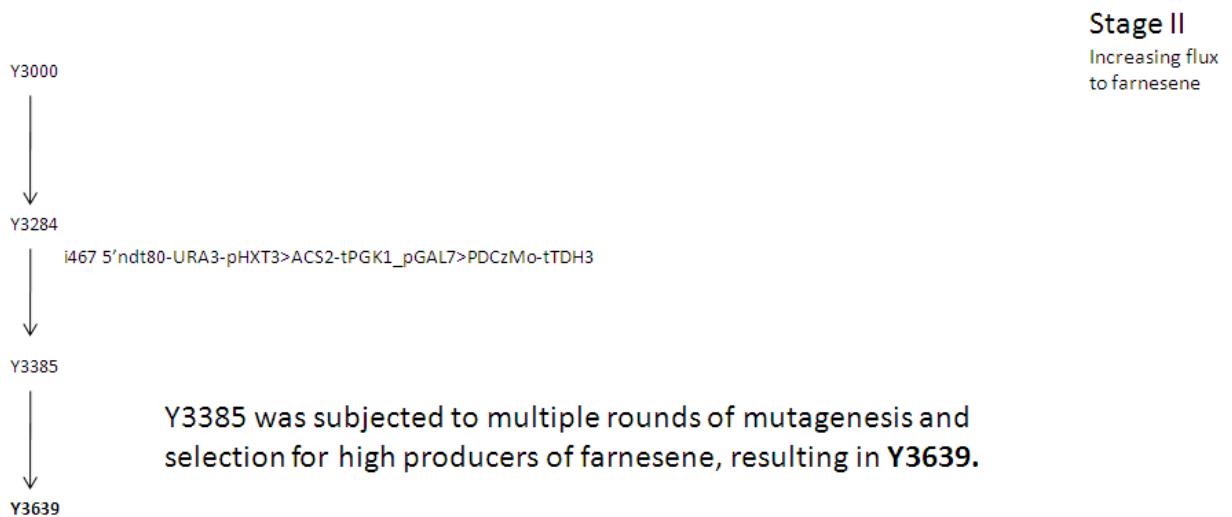


Y3000 was transformed with i094, a construct which recycles the *URA3* marker present at i003 and replaces it with *hisG* sequence from *Salmonella enterica*. The resulting strain was **Y3284**, which is 5'FOA resistant, and ura-.



Y3284 was transformed with i467, which encodes an additional copy of *S. cerevisiae ACS2* under the *P_{HXT3}* promoter, as well as a copy of *Z. mobilis PDC* under the *P_{GAL7}* promoter. This construct deletes *NDT80*, a transcription factor required for sporulation, and is marked with *URA3*. The resulting strain was Y3385.





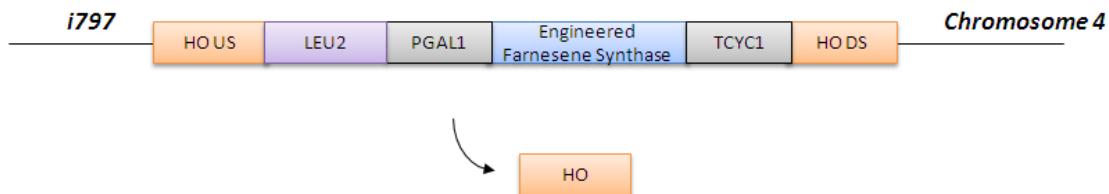
Stage II
Increasing flux to farnesene

Y3639
↓
i797 ho^::LEU2-pGAL-AaFS_A_4.0-tCYC (lose pAM404)
↓
Y4207

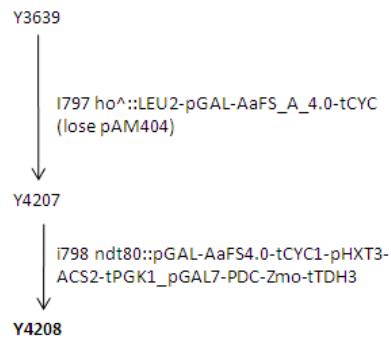
All farnesene production strains up to this point carry FS on pAM404, a high-copy leu2d-marked plasmid. Y3639 was cured of pAM404, resulting in leu auxotrophy.

In the next few steps, multiple copies of mutagenized Farnesene Synthase, screened for higher catalytic activity ("AaFS_A_4.0"), were integrated into Y3639 under the P_{GAL1} promoter.

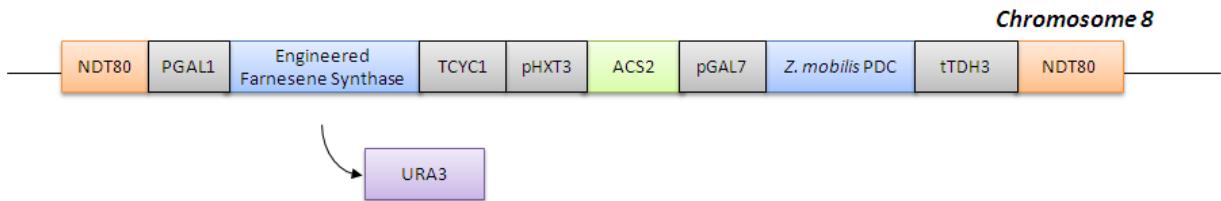
The first copy of AaFS_A_4.0 was marked with LEU2 (i797), and was introduced at the HO locus. The resulting strain was designated Y4207.



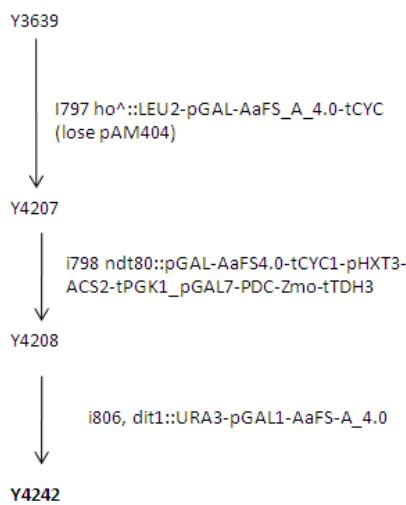
Stage II
Increasing flux
to farnesene



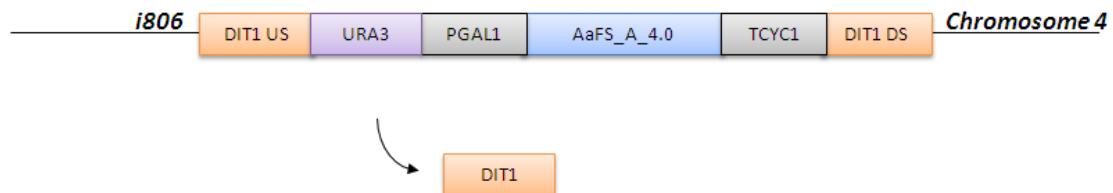
The second copy of *AaFS_A_4.0* was introduced at the *NDT80* locus (i798), replacing the *URA3* marker that was introduced in i467. The other engineering at the *NDT80* locus (*pHXT3-ACS2*, *pGAL7-ZmPDC*) was preserved. FS Integrants were selected on 5'FOA plates and resulting strains were ura- auxotrophs. The resulting strain was **Y4208**.



Stage II
Increasing flux
to farnesene



In i806, a third copy of *AaFS_A_4.0* marked with *URA3* was introduced at the *DIT1* locus, deleting the *DIT1* ORF. *DIT1* encodes an enzyme required for spore wall formation, and null mutants are defective in spore wall maturation. This resulted in strain Y4242.



3. LAB SUPPORT

3.1 Biology Instrumentation and Responsibility (Arthur Salmon)

LOCATION	DEPT	Manufacturer	Model	Description	Person Responsible
202	Biology	Sorval	RT77	Refrigerated low speed Centrifuge	Arthur
202	Biology	Eppendorf	5417R	Refridgerated benchtop Centrifuge	Lab
Chemistry	Biology	Shimadzu	UV-2401 spec	Spectrophotometer with UC probe	Arthur, Shayin and Veronica
Chemistry	Biology	Shimadzu	2040583792	Temp control unit for the Shimadzu spec (for samples)	Arthur, Shayin and Veronica
211	Biology	CBS Scientific	P-036-202	Hood (non laminar, clean box)	Ivan, Tim and Arthur
Microscope Rm	Biology	Singer Instrument Co.	MSM-SYS-300-TSA	Yeast Manipulator/Dissector, with camera	Arthur and Kirsten
211	Biology	Agilent Tech.	G2939AA	2100 electrophoresis bioanalyzer system	Lance
Cold Room	Biology	Bio Spec	50-212-795	Bead beater	Kevin and Arthur
211	Biology	Applied Bio Systems	AP 7300	Real-Time PCR Machine	Arthur
Storage	Biology	Applied Bio Systems	ABI 7900HT	ABI 7900HT RT-PCR machine (not unpacked)	Arthur and Timmy G
-80	Biology	NuAire	Minus 80 times 9	-80 Freezer	Arthur and Facilities
Microscope Rm	Biology	Lecia	Lecia Scope w/ Came	Lecia scope with digital camera, fluroscence	Shayin and Arthur
RAD room, 211	Biology	MP Biomedical / Qiagen	Fast Prep 24	High speed bead beater for DNA, Protein and RNA quantification	Bonny, Sara G and Arthur
202 and 211	Biology	Applied Bio Systems	2700	Thermocycler x28, beige	Arthur
202 and 211	Biology	Genesys	Genesys 10UV	Spectrophotometer (6 Cuvette capacity)	Arthur
202	Biology	NanoDrop Tech	ND1000, x2	Spectrophotometer: small volume (single sample)	Arthur
202	Biology	NanoDrop Tech	ND8000	Spectrophotometer: small volume (8 wells)	Arthur
202	Biology	Eppendorf	05-401-07	Bench centrifuge: 24 place utube rotor	Lab
202	Biology	Eppendorf	5417C	Centrifuge Eppendorf (non-refridgerated) 24 place utube rotor	Lab
202	Biology	Eppendorf	5417C	Centrifuge Eppendorf (non-refridgerated) 24 place utube rotor	Lab
202	Biology	Guava Tech	0500-1980	Flurometer, competent dies and fluroscence	Arthur and Gale
202	Biology	LI-COR Biotechnology	9201.02	Fluorescence imaging machine for Gels	Arthur and Shayin
Storage	Biology	Spiral Biotech	AP4000	Spiral plater	Lab
202	Biology	Eppendorf	05-403-90	Centrifuge: Minispin	Lab
202 and 211	Biology	VWR	Double Stack x8	Incubator, set temp	Arthur
202	Biology	BTX	45-0422	Electroporator, for 2mm gap disposable cuvettes	Arthur and Jed3
Centrifuge Hallway	Biology	Beckman Coulter	JE:369001	Floor Centrifuge	Arthur
Centrifuge Hallway	Biology	Beckman Coulter	JE:369002	Floor Centrifuge	Arthur
Centrifuge Hallway	Biology	Beckman Coulter	JS 5.3	Rotor for JE Centrifuge	Arthur
Gel Room	Biology	Fotodyne	60-7000	FotoDyne Gel Imager UV and VIS; with photo printer and PC	Arthur
MBMP	Biology	Ohaus	Adven Pro	Analytical Balance	Jen and Arthur
MBMP	Biology	Denver	APX-200	Analytical Balance	Jen and Arthur
MBMP	Biology	Ohaus	AP205AD	Analytical Balance	Jen and Arthur
Centrifuge Hallway	Biology and Chemistry	Beckman Coulter	J/A-20 (we have two)	50ml x8 adapter (w/ 15ml inserts) Centrifuge Rotor	Arthur
Centrifuge Hallway	Biology and Chemistry	Beckman Coulter	J/S-5.9 (we have two)	96 well x4 swing arm adapter Centrifuge Rotor	Arthur
Centrifuge Hallway	Biology and Chemistry	Beckman Coulter	J/A-16.250	250ml x6 Centrifuge Rotor	Arthur
Centrifuge Hallway	Biology and Chemistry	Beckman Coulter	J/A-9.1000	1L x4 Centrifuge Rotor	Arthur
Shaker Hallway	Biology	NBS	Innova 4900 x 5	Incubator / Shaker (Humidified, 30' four universal platforms 2" throw)	Arthur and Facilities
RAD Room	Biology	NBS	C25	Incubator / Shaker (Floor top loader, 30' and 2" throw)	Arthur
211	Biology	Lab Line (Carmet)	Lab-Line MaxQ 4000	Refrigerated Benchtop Incubator / Shaker (Humid, 30 and 2" throw)	Arthur
MBMP	Biology	Yamato	sm150	Media Autoclave / Portable Sterilizers	Jen, Brett and Arthur
202	Biology	Capliver Instruments	Caiper EX	Fluid electrophoresis machine	Jed
202	Bio	Canon	Canon ez scan	Colro scanner	Shayin
202	Biology	Coy Industries	Vinyl Bag	Anaerobic chamber for O2-less protein purification	Kevin and Arthur
202	Biology	NuAire	NUAire BSC	Biological Safety cabinet	Arthur and Facilities
202	Biology	NBS	NBS Coffin	Refrigerated Cofflin shaker, 1" throw	Arthur
Tecan room	Biology	Tecan	Tecan EVO 200	Liquid handler, Span 8 and 96 head (200UL), Integrated thermalcyclers and plate reader	Dan, Don, William and Arthur
Gel room	Biology	Unknown	Safelight imager	Safe LED blue gel illuminator	Jeff U and Arthur
202	Biology and Fermentation	YSI	YSI 7100	Bioanalyzer; probes for ETOH/GLU/SUC	Brandon and Arthur
Shaker Hallway	Biology	ATR (Infors)	ATR Multitron	Plate shaker for growing cultures in 96 well plates. 2mm orbit, refrigerated and humidified.	Arthur and HTS
Minus 80 Hallway	Biology	Misonix	Misonix plate	Indirect bath sonicator for sonicating plates (must be PS plates)	Arthur
Minus 80 Hallway	Biology	VWR	VWR horn	Horn style sonicator. Various horn sizes	Arthur
211	Biology	Molecular Devices	Molecular Devices M	UV/VIS/Flu plate reader. Temp controlled and shaker	Arthur and HTS
211	Biology	Molecular Devices	Molecular Devices Stak	Automatic plate loader to a M5. 100 plate capacity	Arthur and HTS
211	Biology	Beckman Coulter	Beckman NXp x3	Liquid handler, 96 tip head, integrated plate reader, shaker and tip loader	Arthur and HTS
211	Biology	Beckman Coulter	Beckman NXp x1	Liquid handler, 96 tip head. Shaker and tip loader only	Arthur and HTS.
211	Biology	GE HealthCare	ACTA FPLC	FPLC for protein purification	Jim
Purification room	Biology	Avestin	Avestin C5	Tank driven homogenizer, for Ecoli and Yeast	Arthur, Sheela and Kevin

3.2 Media and reagent support (Brett and Arthur)

Liquid and Plate Media

Contact:

Jennifer Lau	lau@amyris.com	(510) 597-4784 desk (ext. 567)
Brett Gellman	gellman@amyris.com	(303) 518-4237 cell
Nicole Klinkner	klinkner@amyris.com	(510) 597-4897 desk (ext. 591)

Liquid Media – Stock and Location

Media Type	Bottle Type	Location
450g/L Galactose	1L filter units	Media kitchen shelf
1M NaOH	1L filter units	Media kitchen shelf
100mM NaOH	50mL conical tubes	Media kitchen shelf
9g/L NaCl	1L filter units	Media kitchen shelf
50% Glycerol	500-100mL bottles	Media kitchen shelf
20% Galactose	500-250mL, 1L filter units	Media kitchen shelf, Downstairs media room
20% Glucose	250mL, 1L filter units	Media kitchen shelf, Downstairs media room
0.2% Adenine Sulfate	1L filter units	Media kitchen shelf, Downstairs media room
1% Histidine	500mL filter units	Media kitchen shelf, Downstairs media room
1% Leucine	500mL filter units	Media kitchen shelf, Downstairs media room
1% Lysine	500mL filter units	Media kitchen shelf, Downstairs media room

1% Methionine	500mL filter units	Media kitchen shelf, Downstairs media room
0.2% Uracil	1L filter units	Media kitchen shelf, Downstairs media room
YP Liquid Base	500mL bottles	Media kitchen shelf
10x CSM50mL conical tubes, 500mL	Media kitchen shelf, Downstairs media room	filter units
Sterile Water	500-100mL bottles	Media kitchen shelf
50% PEG15mL conical tubes	Media kitchen shelf	
Luria Broth (LB)	500-100mL bottles	Media kitchen shelf
SOC	15mL conical tubes	Media kitchen shelf
1x PBS	500-250mL filter units	Media kitchen shelf
LB Agar	500mL bottles	Media kitchen shelf
YP Agar	500mL bottles	Media kitchen shelf
Agar Bottle	500mL bottles	Media kitchen shelf

Media Descriptions:

E. Coli Flask Media:

Luria Broth

Storage: On shelves in Media Room – 100-500mL bottles

Request Type: Keep stocked

Components Summary:

- 25g/L Luria Broth

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Flask Media - BFL

E.Coli Media Components:

20% Galactose

Storage: On shelves in Media Room – 250-500mL filter units

Request Type: Keep stocked

Components Summary:

- 200g/L (20%) Galactose

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Media Components -BMC

20% Glucose

Storage: On shelves in Media Room – 250mL filter units

Request Type: Keep stocked

Components Summary:

- 200g/L (20%) Glucose

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Media Components -BMC

50% Glycerol

Storage: On shelves in Media Room – 100-500mL bottles

Request Type: Keep stocked

Components Summary:

- 500mL/L (50%) Glycerol

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Media Components -BMC

CARB

Storage: In common stock freezer upstairs, media mini-fridge downstairs – 500uL aliquots

Request Type: Keep stocked

Components Summary:

- 100mg/mL CRB

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Media Components -BMC

CLM

Storage: In common stock freezer upstairs, media mini-fridge downstairs – 500uL aliquots

Request Type: Keep stocked

Components Summary:

- 34g/mL CLM

- Made in 99.9% ethanol instead of DI water

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Media Components –BMC

E.Coli Plate Media:

CARB plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 25g/mL Luria Broth
- 1mL/L of 100mg/mL (0.1%) CRB
- 1.5% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Plate Media - BPL

CLM plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 25g/L Luria Broth
- 1mL/L of 34g/mL (0.034%) CLM
- 1.5% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Plate Media - BPL

No Ab (LB) plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 25g/L Luria Broth
- 1.5% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\E. coli Media\E. coli Plate Media - BPL

Yeast Flask Media:

DXP-B + 2% Glucose

Storage: Given to requester

Request Type: Through media request system

Components Summary:

- 6.7g/L YNB without amino acids
- 10g/L Succinic Acid
- 6g/L NaOH
- 1.92g/L CSM-U
- 10mL of 0.2% Adenine Sulfate (0.002%)
- 100mL of 20% Glucose (2%)

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Flask Media -YFL

DXP-B + 2.7% Galactose

Storage: Given to requester

Request Type: Through media request system

Components Summary:

- 6.7g/L YNB without amino acids
- 10g/L Succinic Acid
- 6g/L NaOH
- 1.92g/L CSM-U
- 10mL of 0.2% Adenine Sulfate (0.002%)
- 65mL of 450g/L Galactose (2.925%)

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Flask Media –YFL

DXP-B + 4% Galactose

Storage: Given to requester

Request Type: Through request system

Components Summay:

- 6.7g/L YNB without amino acids
- 10g/L Succinic acid
- 6g/L NaOh
- 1.92g/L CSM-U
- 10mL 0.2% Adenine

- 96.3mL/L of 450g/L Galactose (4.33%)

YP Liquid Base

Storage: On shelves in Media Room – 500mL bottles

Request Type: Keep stocked

Components Summary:

- 11.12g/L Yeast Extract
- 22.23g/L Bacto Peptone

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Flask Media -YFL

Yeast Media Components:

1M LiAC

Storage: On shelves in Media Room – 50mL conical tubes

Request Type: Keep stocked

Components Summary:

- 102.02g/L LiAc (1M)

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

10X CSM

Storage: On shelves in Media Room – 500mL filter units downstairs, 50mL conical tubes upstairs

Request Type: Keep stocked

Components Summary:

- 20g/L CSM
- 67g/L YNB without amino acids

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

10X CSM-L

Storage: On shelves in downstairs Media Room – 500mL filter units

Request Type: Keep stocked

Components Summary:

- 20g/L CSM-L
- 67g/L YNB without amino acids

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

10X CSM-L-U

Storage: On shelves in downstairs Media Room – 500mL filter units

Request Type: Keep stocked

Components Summary:

- 20g/L CSM-L-U
- 67g/L YNB without amino acids

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

10X CSM-U

Storage: On shelves in downstairs Media Room – 500mL filter units

Request Type: Keep stocked

Components Summary:

- 20g/L CSM-U
- 67g/L YNB without amino acids

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

G418

Storage: In common freezer upstairs, media mini-fridge downstairs – 500uL aliquots

Request Type: Keep stocked

Components Summary:

- 200g/L G418

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

NAT

Storage: In common freezer upstairs, media mini-fridge downstairs – 500uL aliquots

Request Type: Keep stocked

Components Summary:

- 100g/L NAT

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Media Components - YMC

Yeast Plate Media:

5FOA Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 0.5g/500mL 5FOA
- 2% Glucose
- 100mL/L 10x CSM
- 0.01% Leucine
- 0.005% Uracil
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

Agar Bottle

Storage: On shelf in media room

Request Type: Keep stocked

Components Summary:

- 2.5% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L + 1x CRB Plates/Q-trays

Storage: Plates in labeled cold room bin; Q-trays in deli fridge

Request Type: Keep stocked

Components Summary:

- 1mL/L of 100mg/mL CRB (0.1%)
- 2% Glucose
- 100mL/L 10x CSM-L
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L + Ethanol Plates

Storage: Given to requester

Request Type: Through media request system

Components Summary:

- 2% Glucose
- 26.66mL/L Ethanol
- 100mL/L 10x CSM-L
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L + Nile Red Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 2% Glucose
- 100mL/L 10x CSM-L
- 10mL/L of 50mg/500mL Nile Red (10%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 2% Glucose
- 100mL/L 10x CSM-L
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L-U + 1x CRB Plates/Q-trays

Storage: Plates in labeled cold room bin; Q-trays in deli fridge

Request Type: Keep stocked

Components Summary:

- 2% Glucose
- 100mL/L 10x CSM-L-U
- 1mL/L of 100mg/mL CRB (0.1%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-L-U Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 2% Glucose
- 100mL/L 10x CSM-L-U
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-M-L-U-W-H-A-K Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- 2% Glucose
- 100mL/L 10x CSM
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

CSM-U + 2% Galactose Plates

Storage: Given to requester

Request Type: Through media request system

Components Summary:

- 2% Galactose
- 2% agar
- 100mL/L 10x CSM-U

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YP + 2-Deoxygalactose Plates

Storage: Given to requester

Request Type: Through media request system

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 0.2g/L 2-Deoxygalactose
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YP Base Agar

Storage: On shelf in media room

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD + 1x G418

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 500uL/500mL of 200g/L G418 (0.2%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD + 1x G418 + 1x NAT

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 500uL/500mL of 200g/L G418 (0.2%)
- 500uL/500mL of 100g/L NAT (0.1%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD + HygB

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 1.5mL/500mL of 100mg/mL HygB (0.3%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD + 1x HygB + 1x G418 + 1x NAT

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 1.5mL/500mL of 100mg/mL HygB (0.3%)
- 500uL/500mL of 200g/L G418 (0.2%)
- 500uL/500mL of 100g/L NAT (0.1%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD + 1x NAT

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 500uL/500mL of 100g/L NAT (0.1%)
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media -YPL

YPD Plates

Storage: In labeled cold room bin

Request Type: Keep stocked

Components Summary:

- YP Liquid Base (11.12g/L yeast extract and 22.23g/L bacto peptone)
- 2% Glucose
- 2% agar

BR Link: S:\Departments\Lab Services\Media Prep\Current Batch Records\Yeast Media\Yeast Plate Media –YP

3.3 Antibiotics: Mechanisms of Action and Resistance

Carbenicillin (CRB/Carb)

- An inhibitor of the last step in cell wall synthesis, the cross-linking of different peptidoglycan strands.
- Carbenicillin exerts its antibacterial activity by interference with final cell wall synthesis of susceptible bacteria. Penicillins acylate the penicillin-sensitive transpeptidase C-terminal domain by opening the lactam ring. This inactivation of the enzyme prevents the formation of a cross-link of two linear peptidoglycan strands, inhibiting the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that carbenicillin interferes with an autolysin inhibitor.
- Resistance: The bla gene codes for a β-lactamase enzyme which inactivates the antibiotic.

Chloramphenicol (CLM)

- A bacteriostatic agent that binds to the 50S ribosomal subunit and inhibits ribosomal peptide bond formation. It is sometimes used as a way of "amplifying" plasmid production by shutting down protein synthesis in cultures, while allowing plasmid replication to continue.

Kanamycin (KAN)

- A bactericidal agent that binds to 70S ribosomes and causes misreading of the messenger RNA.
- The aminoglycosides irreversibly bind to the 30S ribosome and freeze the 30S initiation complex (30S-mRNA-tRNA), so that no further initiation can occur. They also slow down protein synthesis that has already initiated and induce misreading of the mRNA.

- Resistance: Kanamycin is inactivated by bacterial aminophosphotransferases (APHs). The APHs inactivate kanamycin by transferring the γ -phosphate of ATP to the hydroxyl group in the 3' position of the pseudosaccharide. The *Kan^R* gene codes for kanamycin resistance.

G418

- “Aminoglycoside similar to Kanamycin (“Eukaryotic version of Kanamycin”).
- Irreversibly binds to 80S ribosomal subunit, disrupting proofreading.
- Inhibits protein synthesis by binding to L6 protein of the 50S ribosomal subunit.
- Resistance: see Kanamycin

Nourseothricin-dihydrogen sulfate (NAT)

- The mechanism of action of nourseothricin is comparable with that of other aminoglycoside antibiotics: Specific partial steps of the protein synthesis are inhibited by the antibiotic.
- Resistance: Development of resistance is based on monoacetylation of β -amino groups of the β -lysyl moiety of the streptothricin molecules.

HygB

- Hygromycin B is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 80S ribosome.
- Hygromycin B is an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes (1,2). Hygromycin B strongly inhibits protein synthesis through a dual effect on mRNA translation (3,4). Like other aminoglycoside antibiotics, hygromycin B induces misreading of aminoacyl-tRNA by distorting the ribosomal A site (decoding center) (3,5,6,7). Hygromycin B also affects the ribosomal translocation process (3,8,9). In the presence of the antibiotic, mRNA is often mistranslocated, being moved more or less than the three necessary bases.
- Resistance to hygromycin is conferred by hygromycin B phosphotransferase (Hph). Hph catalyzes the phosphorylation of the 4-hydroxyl group on the cyclitol ring (hyosamine), thereby producing 7"-O-phosphoryl-hygromycin B which totally lacks biological activity both *in vivo* and *in vitro*.

Tetracycline (TET)

- Tetracyclines exert their bacteriostatic effect by inhibiting protein synthesis in bacteria. This antibiotic prevents transfer-RNA (tRNA) molecules (a type of nucleic acids which transport amino acids) from binding to the 30S subunit of bacterial ribosomes.

- Resistance: Tet resistance is mediated by efflux. Several tetracycline resistance determinants are currently used in molecular biology. The most encountered are the *tetA* genes of classes A (RP1, RP4 or Tn1721 derivatives), B (Tn10 derivatives) and C (pSC101 or pBR322 derivatives) encoding a tetracycline efflux system. These genes are regulated by a repressor protein (TetR). This feature has also been exploited to construct tightly regulated, high level mammalian expression systems by using the regulatory elements of the Tn10 tetracycline operon (Tet-OffTM and Tet-OnTM Expression Systems & Cell Lines, Clontech).

Antimetabolites:

Canavanine (LCan)

- Canavanine is a non-protein amino acid antimetabolite. It acts primarily as an arginine antagonist and gets incorporated into proteins and disrupts their normal function.
- Resistance in yeast: Resistance is conferred by spontaneous mutants that affect arginine uptake

5-fluororotic acid (5-FOA)

- 5-FOA is a nucleotide analog that is used in yeast genetics to select against a functional URA3 gene. URA3 codes for orotidine decarboxylase which catalyzes the decarboxylation of orotidine monophosphate to deoxyuridine monophosphate (dUMP). URA3 also catalyzes the decarboxylation of FOA to FdUMP, which is the true antimetabolite suicide inhibitor via its action on thymidylate synthase.
- Irreversible inhibition of thymidylate synthase affects DNA synthesis by lowering the intracellular pool of available thymidine monophosphate. Molecules like FOA that are themselves not inhibitors but are activated to inhibitors are sometimes referred to as “Trojan horse” inhibitors.

3.4 Sample Drop Boxes

<u>Desc</u>	<u>Owner</u>	<u>Location</u>	<u>Temp 'C</u>	<u>Box/Bin descriptor</u>
Frozen Stocks for Archival:	Rachae:	-80 Freezer #6:	-80'C	Drop Off - Red
Seed Vials; analysis on ldr plt:	HTS:	-80 Freezer #6:	-80'C	Regimentation - Orange
Strains for Strain Banking:	Rachae	Deli #3:	4'C	Strain Banking Drop Off - Clear
Culture drop off for Minipreps:	Rachae:	Deli #3:	4'C	Miniprep Drop off - Clear

3.5 Amyris Antibodies

KEY Freezer boxes containing antibodies are color coded by pathway or species e.g. <i>S. cerevisiae mevalonate</i> pathway antibodies are in yellow chipboard boxes.											
Aliquots color coded red have been tested and shown to work well for Western blotting.											
Single blacked out antibody shown not to work for HMGR detection (alternative available).											
Antibody #	Item #	Label	Amyris antigen Name	Open Bio antigen name	bleed	Rabbit	Open Bio Rabbit #	Project	date	Volume	Working Volume
DXP pathway (Eco)											
1	A	1A	dxs	DXS_DXP	DDS	Test	?	4064	?	8ml test only	
1	B	1B	dxs	DXS_DXP	DDS	Production	1	6667	4064	10.3.07	15ml
1	C	1C	dxs	DXS_DXP	DDS	Production	2	6668	4064	10.3.07	15ml
1	D	1D	dxs	DXS_DXP	DDS	Final	1	6667	4064	12.11.07	25ml
1	E	1E	dxs	DXS_DXP	DDS	Final	2	6668	4064	12.11.07	25ml
2	A	2A	dxr	DXR_DXP	DDR	Test	?	4060	?	8ml test only	
2	B	2B	dxr	DXR_DXP	DDR	Production	1	5036	4060	10.3.07	15ml
2	C	2C	dxr	DXR_DXP	DDR	Production	2	5037	4060	10.3.07	15ml
2	D	2D	dxr	DXR_DXP	DDR	Final	1	5036	4060	12.18.07	25ml
2	E	2E	dxr	DXR_DXP	DDR	Final	2	5037	4060	12.18.07	25ml
3	A	3A	ispE	ispE_DXP	DDispE	Test	?	4038	?	8ml test only	
3	B	3B	ispE	ispE_DXP	DDispE	Production	1	5020	4038	8.28.07	15ml
3	C	3C	ispE	ispE_DXP	DDispE	Production	2	5021	4038	8.28.07	15ml
3	D	3D	ispE	ispE_DXP	DDispE	Final	1	5020	4038	12.11.07	25ml
3	E	3E	ispE	ispE_DXP	DDispE	Final	2	5021	4038	12.11.07	25ml
4	A	4A	ispG	ispG_DXP	DDiG	Test	?	4084	?	8ml test only	
4	B	4B	ispG	ispG_DXP	DDiG	Production	1	6686	4084	10.18.07	15ml
4	C	4C	ispG	ispG_DXP	DDiG	Production	2	6687	4084	10.18.07	15ml
4	D	4D	ispG	ispG_DXP	DDiG	Final	1	6686	4084	12.18.07	25ml
4	E	4E	ispG	ispG_DXP	DDiG	Final	2	6687	4084	12.18.07	25ml
5	A	5A	ispD	ispD_DXP	DDispD	Test	?	4061	?	8ml test only	
5	B	5B	ispD	ispD_DXP	DDispD	Production	1	5038	4061	10.3.07	15ml
5	C	5C	ispD	ispD_DXP	DDispD	Production	2	5039	4061	10.3.07	15ml
5	D	5D	ispD	ispD_DXP	DDispD	Final	1	5038	4061	12.10.07	25ml
5	E	5E	ispD	ispD_DXP	DDispD	Final	2	5039	4061	12.10.07	25ml
6	A	6A	ispA	ispA_DXP	DDispA	Test	?	4063	?	8ml test only	
6	B	6B	ispA	ispA_DXP	DDispA	Production	1	6665	4063	10.03.07	10ml
6	C	6C	ispA	ispA_DXP	DDispA	Production	2	6666	4063	10.03.07	10ml
6	D	6D	ispA	ispA_DXP	DDispA	Final	1	6665	4063	12.18.07	25ml
6	E	6E	ispA	ispA_DXP	DDispA	Final	2	6666	4063	12.18.07	25ml
7	A	7A	IDI	IDI_DXP	DDIDI	Test	?	4042	?	8ml test only	
7	B	7B	IDI	IDI_DXP	DDIDI	Production	1	5028	4042	8.28.07	10ml
7	C	7C	IDI	IDI_DXP	DDIDI	Production	2	5029	4042	8.28.07	10ml
7	D	7D	IDI	IDI_DXP	DDIDI	Final	1	5028	4059	12.12.07	25ml
7	E	7E	IDI	IDI_DXP	DDIDI	Final	2	5029	4059	12.12.07	25ml
Yeast											
8	A	8A	ERG10	THL_Sce	DDRG10	Test	?	4040	?	8ml test only	
8	B	8B	ERG10	THL_Sce	DDRG10	Production	1	5024	4040	8.28.07	15ml
8	C	8C	ERG10	THL_Sce	DDRG10	Production	2	5025	4040	8.28.07	10ml
8	D	8D	ERG10	THL_Sce	DDRG10	Final	1	5024	4040	12.11.07	25ml
8	E	8E	ERG10	THL_Sce	DDRG10	Final	2	5025	4040	12.11.07	25ml
9	A	9A	mvaA	HMGR_Sce	DDMVA	Test	?	4062	none		
9	B	9B	mvaA	HMGR_Sce	DDMVA	Production	1	6663	4062	10.3.07	15ml
9	C	9C	mvaA	HMGR_Sce	DDMVA	Production	2	6664	4062	10.3.07	15ml
9	D	9D	mvaA	HMGR_Sce	DDMVA	Final	1	6663	4062	12.18.07	25ml
9	E	9E	mvaA	HMGR_Sce	DDMVA	Final	2	6664	4062	12.18.07	25ml
10	A	10A		PMD_Sce	DDPEMD	Test	?	4037	?	8ml test only	
10	B	10B		PMD_Sce	DDPEMD	Production	1	5018	4037	8.28.07	15ml
10	C	10C		PMD_Sce	DDPEMD	Production	2	5019	4037	8.28.07	10ml
10	D	10D		PMD_Sce	DDPEMD	Final	1	5018	4037	12.11.07	25ml
10	E	10E		PMD_Sce	DDPEMD	Final	2	5019	4037	12.11.07	25ml
11	A	11A	ERG20	FPPS_Sce	DDRG20	Test	?	4041	?	8ml test only	
11	B	11B	ERG20	FPPS_Sce	DDRG20	Production	1	5026	4041	8.28.07	10ml
11	C	11C	ERG20	FPPS_Sce	DDRG20	Production	2	5027	4041	8.28.07	15ml
11	D	11D	ERG20	FPPS_Sce	DDRG20	Final	1	5026	4041	12.12.07	25ml
11	E	11E	ERG20	FPPS_Sce	DDRG20	Final	2	5027	4041	12.12.07	25ml
12	A	12A	ERG8	PMK_Sce	PMATK	Test	?	4046	?	8ml from unknown rabbit	
12	B	12B	ERG8	PMK_Sce	PMATK	Production	1	5034	4046	9.07.09	15ml
12	C	12C	ERG8	PMK_Sce	PMATK	Production	2	5035	4046	9.07.07	10ml
12	D	12D	ERG8	PMK_Sce	PMATK	Final	1	5034	4046	12.12.07	25ml
12	E	12E	ERG8	PMK_Sce	PMATK	Final	2	5035	4046	12.12.07	25ml
13	A	13A	IDI1	IDI_Sce	DDID1	Test	?	4059	?	7ml from unknown rabbit	
13	B	13B	IDI1	IDI_Sce	DDID1	Production	1	8257	4059	10.03.07	10ml
13	C	13C	IDI1	IDI_Sce	DDID1	Production	2	8254	4059	10.03.07	10ml
13	D	13D	IDI1	IDI_Sce	DDID1	Final	1	8257	4059	12.18.07	25ml
13	E	13E	IDI1	IDI_Sce	DDID1	Final	2	8254	4059	12.18.07	25ml

Do not use. Bad antibodies

acetoacetyl-CoA thiolase

acetoxycarbonyl-CoA thiolase

acetoacetyl-CoA thiolase

acetoacetyl-CoA thiolase

FPP synthase

FPP synthase

FPP synthase

FPP synthase

IPP:DMAPP Isomerase

IPP:DMAPP Isomerase

IPP:DMAPP Isomerase

IPP:DMAPP Isomerase

Amyris Antibodies (con't)

14	A	14A	ERG13	HMGS_Sce	DDHGS	Test	1	5016	4036	?	4ml from 5016 only	
14	B	14B	ERG13	HMGS_Sce	DDHGS	Production	1	5016	4036	8.28.07	10ml	8ml from unknown rabbit
14	C	14C	ERG13	HMGS_Sce	DDHGS	Production	2	5017	4036	8.28.07	15ml	
14	D	14D	ERG13	HMGS_Sce	DDHGS	Final	1	5016	4036	12.11.07	25ml	4ml from unknown rabbit
14	E	14E	ERG13	HMGS_Sce	DDHGS	Final	2	5017	4036	12.11.07	25ml	
15	A	15A	ERG12	MK_Sce	MATK	Test	?		4045	?	8ml from unknown rabbit	HMGCoA-synthase
15	B	15B	ERG12	MK_Sce	MATK	Production	1	5082	4045	9.07.07	10ml	8ml from unknown rabbit
15	C	15C	ERG12	MK_Sce	MATK	Production	2	5038	4045	9.7.07	15ml	
15	D	15D	ERG12	MK_Sce	MATK	Final	1	5032	4045	12.12.07	25ml	8ml from unknown rabbit
15	E	15E	ERG12	MK_Sce	MATK	Final	2	5033	4045	12.12.07	25ml	mevalonate kinase
16	A	16A	thMGR	thMGR	IS120801	Final	1	F4095	D2038-8807	11/26/2008	50	
												1HMGR
			Staph aureus									
17	A	17A	mva5	HMGS_Sau	DDMVS	Test	?	6688	4085	?	NONE	16ml from unknown rabbit
17	B	17B	mva5	HMGS_Sau	DDMVS	Production	1	6688	4085	10.28.07	15ml	16ml from unknown rabbit
17	C	17C	mva5	HMGS_Sau	DDMVS	Production	2	6689	4085	10.18.07	10ml	
17	D	17D	mva5	HMGS_Sau	DDMVS	Final	1	6688	4085	12.18.07	25ml	16ml from unknown rabbit
17	E	17E	mva5	HMGS_Sau	DDMVS	Final	2	6689	4085	12.18.07	25ml	
18	A	18A	mvaR	HMGR_Sau		Test	?				NONE	16ml from unknown rabbit
18	B	18B	mvaR	HMGR_Sau		Production	1				NONE	16ml from unknown rabbit
18	C	18C	mva5	HMGR_Sau		Production	2				NONE	
18	D	18D	mva5	HMGR_Sau		Final	1				NONE	
18	E	18E	mva5	HMGR_Sau		Final	2				NONE	
			E coli									
19	A	19A	atoB	THL_Eco	DDATB	Test	?		4039		NONE	none
19	B	19B	atoB	THL_Eco	DDATB	Production	1	5022	4039	8.28.07	15ml	
19	C	19C	atoB	THL_Eco	DDATB	Production	2	5023	4039	8.28.07	15ml	
19	D	19D	atoB	THL_Eco	DDATB	Final	1	5022	4039	12.12.07	25ml	
19	E	19E	atoB	THL_Eco	DDATB	Final	2	5023	4039	12.12.07	25ml	
20	A	20A	mvaA	HMGR_Pme	DDHGR-Pmev	Test	?		4048		NONE	none
20	B	20B	mvaA	HMGR_Pme	DDHGR-Pmev	Production	1	5030	4048	8.28.07	10ml	none
20	C	20C	mvaA	HMGR_Pme	DDHGR-Pmev	Production	2	5031	4048	8.28.07	15ml	
20	D	20D	mvaA	HMGR_Pme	DDHGR-Pmev	Final	1	5030	4048	12.12.07	25ml	
20	E	20E	mvaA	HMGR_Pme	DDHGR-Pmev	Final	2	5031	4048	12.12.07	25ml	
			FS antibodies									
21			FS	FS_Annua	antiFS42607	Production	?	?	?	?	9ml	9
22			ADS	ADS_Annua	antiADA42607	Production	?	?	?	?	10ml	10
			Other antibodies									
23			alpha peptide 2									
24			ubiquitin									
25			T7 TAG									

3.6 Blanket Purchase Order numbers (POs)

IDT: Oligos - PO 16011 Do not put the gene name in the description

IDT: Gene Synthesis - PO 15996 Please put the length of the gene in the description so track price and turnaround time can be tracked.

Elim: Sequencing – PO 16010

Quintara: Sequencing – PO 16027

3.7 Archiving

3.7.1 Strains and seed vial preparation(Chi-Li, Rachae, Shayin)

- Strain banking services to preserve, archive and maintain the strains developed throughout the Biology group. Strain banking team also prepares small and large batches of seed vials for fermentation process development in Emeryville and Campinas.



- The current SOPs are in the following links.
- <S:\Departments\Biology\HTP Project\Protocols\100529 Seed Vials Preparation Protocol for HTS and Biology.docx>
- <M:\100531 SOP for making a large batch of seed vials for Y strain.docx>
- A video tape is also available for reviewing the procedure (S:\Departments\Biology\HTP Project\Protocols\seed vial prep.wmv).

3.7.3 Plasmid archiving (Rachae)

Plasmid archiving is done to preserve and maintain a working stock and archive of the plasmids developed here in the Biology department. There are a few key steps when archiving a new plasmid:

1. Adding the new plasmid to the Strain database to receive an official pAM number.

2. Transforming into a bacterial host for long term storage, adding the new bacterial strain to the strain database, and archiving the strain by submitting a request to the strain archiving team.
3. Preparing the plasmid DNA using one of the various plasmid prep/miniprep protocols available and dropping off an aliquot for the general use plasmid stock.

3.7.3 Oligo ordering and archiving (Shayin)

Ordering and Storage of Oligonucleotides from Integrated DNA Technologies (IDT)

How to create an IDT account

Every biology researcher needs to create their own account for ordering oligos.

The website for oligonucleotide ordering through IDT is: www.idtdna.com

On the IDT home page:

Hit **Login**, then **setup new account**. Fill out the section for contact information. Under personal information there is a field for **Initial PO** (purchase order number.) PO numbers change quarterly – you can get the current number from purchasing (purchasing@amyris.com) or from Arthur Salmon (Biology Lab Manager.)

For payment on future orders select **Purchase order**. Use the current quarters purchase order number for every order (this is a blanket PO for all of biology's orders.)

Use the delivery addresses below

Delivery

AMYRIS Attn: **your name**

5885 Hollis St, suite 100

Emeryville, CA 94608

Billing address

Amyris Attn: A/P

5885 Hollis St, suite 100

Emeryville, CA 94608

To see your order history

After logging into the IDT website, an order history can be obtained by selecting the “Order History” button located on the left-side of the webpage. The most recent orders are listed by order number, date, and number of oligos per order. However, past orders can be searched by number of days, order number, PO number (not applicable), and reference number.

Naming Oligonucleotides

Personal oligonucleotides must be named according to the following format:

Your initials-sequential ID#-notebook # - page # —*description** (*optional*)

example: “SSG210-91-10-pGal3F” = Shayin S. Gottlieb, oligo 210, notebook 91, page 10, pGal3F.

(The next oligo ordered at that time would be “SSG211-91-10-pGal3” = Shayin S. Gottlieb, oligo 211, notebook 91, page 10, pGal3”)

**The description should not include information confidential to Amyris. Use abbreviations when necessary.*

Project Oligonucleotides must be named according to the following format

Project Code-your initials-sequential ID#-notebook # - page # —*description** (*optional*)

Example: “ISO-SSG210-91-10-pGal3F” =Isoprene project, Shayin S. Gottlieb, oligo 210, notebook 91, page 10, pGal3F.

Ordering Oligonucleotides

IDT offers several synthesis and delivery options for oligonucleotides. Information can be obtained at <https://www.idtdna.com/Catalog/Catalog.aspx> All orders will automatically be billed to Amyris' account and the necessary fields will be filled-in for your use.

Note: Do not use the overnight service if you do not really need the oligo's 10:30am the next day!

Instructions for Storing Oligonucleotides

Oligonucleotides should be resuspended in sterile TE buffer (pH 8.0) at a concentration of 100 µM and stored at -20°C as master stocks. Working stocks at the desired concentration may be made from these master stocks using aseptic technique. Oligos should be stored in clearly labeled boxes in either the common oligo freezer or your personal freezer (indicate on the outside of the storage box the oligos contained within the box (e.g. SG_oligo box 1_ID#s 1-200). Please keep oligos organized and arranged in ascending order so that your colleagues can easily locate your oligos day or night.

Saving Oligo Files in Clone Manager

A clone manager file must be created for each oligo. Alternately, a list of oligos can be saved in an excel file for import into Clone Manager (see instructions below.) Oligo files should be saved to the oligonucleotide folder <S:\Departments\Biology\Molecular Biology\Oligonucleotides\Oligo Files>.

There are multiple methods for entering oligos into Clone Manager – see the help files for instructions for entering single oligos.

The steps below will save your primers as a .px5 file. This will group your primers into collections, but that is ok, as each group of primers in the .px5 file is still searchable. Saving your primers as a .px5 file makes searching for "primers that bind" MUCH, MUCH faster.

- 1) Create and save an excel spread sheet with primers as a .txt (tab delimited) file
 - (1) the Excel spreadsheet should have three columns in the following order:

Name, Description, Sequence
 - (2) The sequence can have NO spaces or other characters besides the bases

- 2) To Import Primers into Clone Manager
 - a) Open Clone Manager
 - b) Select "Primer List" from the "PRIMER" drop down option at top
 - c) Click "my collections"
 - d) A folder icon with a drop down menu will now appear in the upper left corner
 - e) Click open the drop down menu and select "import"
 - f) Find the .txt (tab delimited) excel file you want to import
 - i) Name the .px5 file
 - ii) Click OK

You have just saved all those primers in a searchable format for other clone manager users. But you have to make sure the new .px5 file is in the right location!

Where is your new .px5 primer file? That depends on where your default save is for clone manager

- a. In clone manager, select "preferences" in the FILE drop option at top
- b. Under the BASIC tab
 - i. The first line is the location of the default place to save files
 - ii. This is where your new .px5 primer file is located
- c. Copy/Cut and paste YOUR .px5 file into the Amyris Oligo folder
(S:\Departments\Biology\Molecular Biology\Oligonucleotides\INDEXED OLIGO FILE (px5))
- d. OR change your preferences in clone manager so that your default save is the Amyris Oligo folder: (S:\Molecular Biology\Oligonucleotides\Oligo Files)

What about your old .pd4 files?

You can convert all those individual .pd4 primer sequences into a single folder of .px5 sequences; this makes searching through all the current primers MUCH faster. We did this for the ENTIRE oligo folder as it currently stands, and then used it to look for primers that bind, and it did it in a fraction of the time it used to take!

1. Convert all .pd4 files to a .px5 file
 - a. In clone manager, under FILE, select "Multiple File Conversion"
 - i. Click the primer button
 - ii. Select the folder containing the .pd4 files
 - iii. Name your new .px5 file
 - iv. It will save this "condensed" .px5 oligo file into your default save locations (see above on how to change this location if you want)
 - b. Next time you search for primers, use this .px5 file, and it will be so much faster!

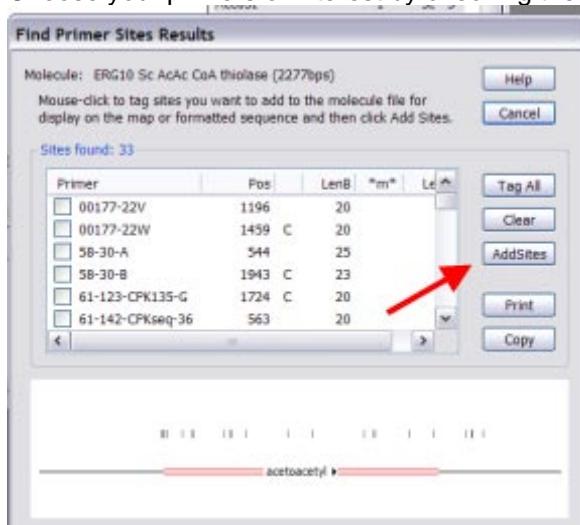
Searching for Oligonucleotides using Clone Manager

- i. Load your sequence of interest into Clone Manager
- ii. Go the primer menu at the top of the screen and select **Find Primers That Bind**.

- iii. Click the folder icon to select the oligo folder and choose your binding temperature and binding cutoff. Alternately primer lists or single files can be selected.



- iv. Click **OK**
v. Choose your primers of interest by checking the box next to the primers and click **Add Sites**



4. CAPABILITIES

4.1 Construction (ASE and RYSE)

Introduction

The need for a new revolution in writing DNA

A revolution in molecular biology in the twentieth century catalyzed the development of powerful tools for “reading” and “writing” DNA. On the reading side, advances in DNA sequencing technology led to the elucidation of the human genome—the human book of life. At the same time, game changing technologies for “writing” or “constructing” DNA were advanced, including automated oligonucleotide synthesis, recombinant DNA technology and PCR.

The first decade of the 21st century has seen a second revolution in DNA reading technology-- “next generation” DNA sequencing platforms have significantly expanded sequencing capacity and increased “reading” speed. As a result, a large library of “life books” detailing the DNA sequences of numerous and myriad species is rapidly being assembled. The content of these books is the material used by modern metabolic engineer plagiarists who borrow sentences and paragraphs as they seek to write their own stories of metabolism in their preferred microbial hosts.

In the last decade, a powerful new concept for “writing” DNA has also emerged: Synthetic biology. Synthetic biology promises to do for DNA writing what next gen sequencing has done for DNA reading. So far, however, synthetic biology—or perhaps a better term is “next gen molecular biology”—has remained largely an abstract concept and has yet to be translated into a useful tool or technology. As a result, the writing of DNA remains a major bottleneck for the metabolic engineer. *The game changing DNA writing platform that we are developing at Amyris will remove the universal strain construction bottleneck and will lead to rapid advances in strain improvement.*

Our standardized DNA construction platform is based on the PCR assembly of DNA elements stored in plasmid vectors called RABits. A series of oligonucleotide linkers flank each element such that the elements can be combined in a modular fashion. The automation of the process, from design through strain construction, is known as automated strain engineering (ASE).

What is ASE?

- High-throughput rational strain construction
- Enable modular strain construction from a user-defined genotype
- Maintain a capacity of 1000 strains/4 weeks/4 FTE's

- Utilize automation and computational tools/databases
- Employs modular, reproducible molecular biology tools and validated reagents

ASE is a high-throughput strain construction pipeline that relies on automation and integrated database tracking / worklist management to generate rationally-designed DNA constructs in a specific, modular format, and combine them into large numbers of potentially high-value yeast strains.

Our strength is the facile and parallel construction of "bistitch" combinations from a smaller number of stitches and RABits, using optimized protocols and a standard workflow. While we are open to the specific needs of our Amyris collaborators, custom work that falls outside our automated workflow will be considered on a case-by-case basis.

The entry point to our pipeline is a collaborative conversation about how we can fit your constructs into our format (see next pages), how many combinations we should aim for, what level of redundancy is correct for your essential parts, and what base strains we should target.

Currently, we run about 1000 yeast strains per cycle, with a 4-week delivery time (colonies on plates).

While we aim to produce as many of the strains that are requested as possible, our high throughput workflow does not allow dropped or difficult clones to be constructed, if they drop out of the current processing cycle. If certain constructs are essential to your project, we will keep you in the loop and may ask you to help us by trouble shooting any failed clones and /or providing us with gel-purified PCR products. Additionally, some collaborators may want to jump in during our process and do extra QC steps on the parts going into their strains.

We can deliver:

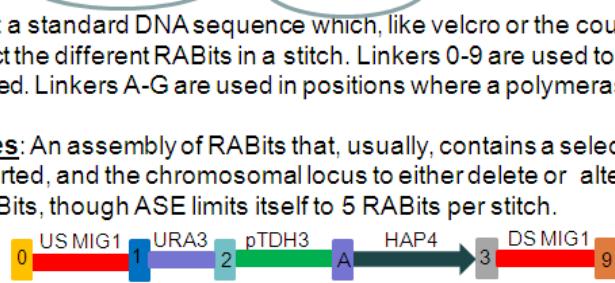
1. cloned RABits or DNA
2. cloned stitches or DNA, which is appropriate if HTS does not yet support libraries associated with your project, or
3. transformants in a limited list of yeast hosts (usually two “top” strains recommended by Lead Consolidation and 1 control strain), colony PCR verified and with Tier-1 Nile red scores (e.g. all strains will proceed through the HTS pipeline and ranked based on their performance).

We want to develop ASE into a tool that is useful to the community, so your feedback is essential.

Please don't hesitate to bug our staff with crazy or obvious ideas.

Glossary of parts used in ASE

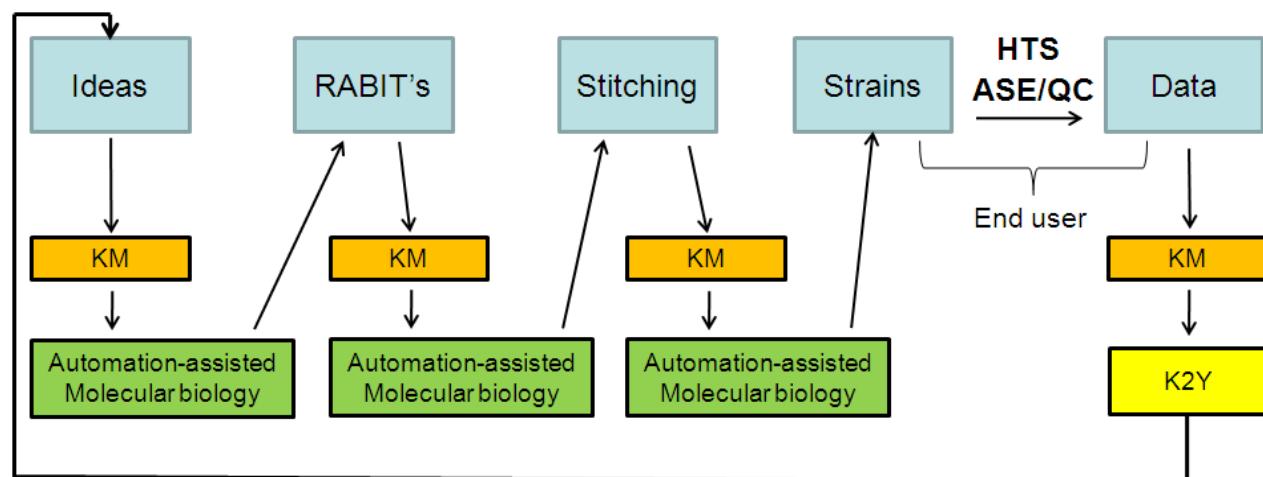
- RYSE:** Rapid Yeast Strain Engineering. A system that turns DNA into modular, interchangeable, connectable parts. Individual pieces are **RABits** which get connected through **linkers** into biologically functional **stitches** and **bistitches**.
- RABit:** RYSE Associated Bit. Any piece of DNA, usually a promoter, gene, terminator, etc. cloned into a vector and flanked by two linkers which allow interconnection. The linker-flanked DNA piece in a RABit can always be cleanly excised from the vector using a *SapI* restriction enzyme digest.
- Linker:** a standard DNA sequence which, like velcro or the couplers between railroad cars, is used to connect the different RABits in a stitch. Linkers 0-9 are used to connect bits where no read-through is expected. Linkers A-G are used in positions where a polymerase will read through.
- Stitches:** An assembly of RABits that, usually, contains a selectable marker, any genes and promoter to be inserted, and the chromosomal locus to either delete or alter. Stitches are usually made from two to ten RABits, though ASE limits itself to 5 RABits per stitch.



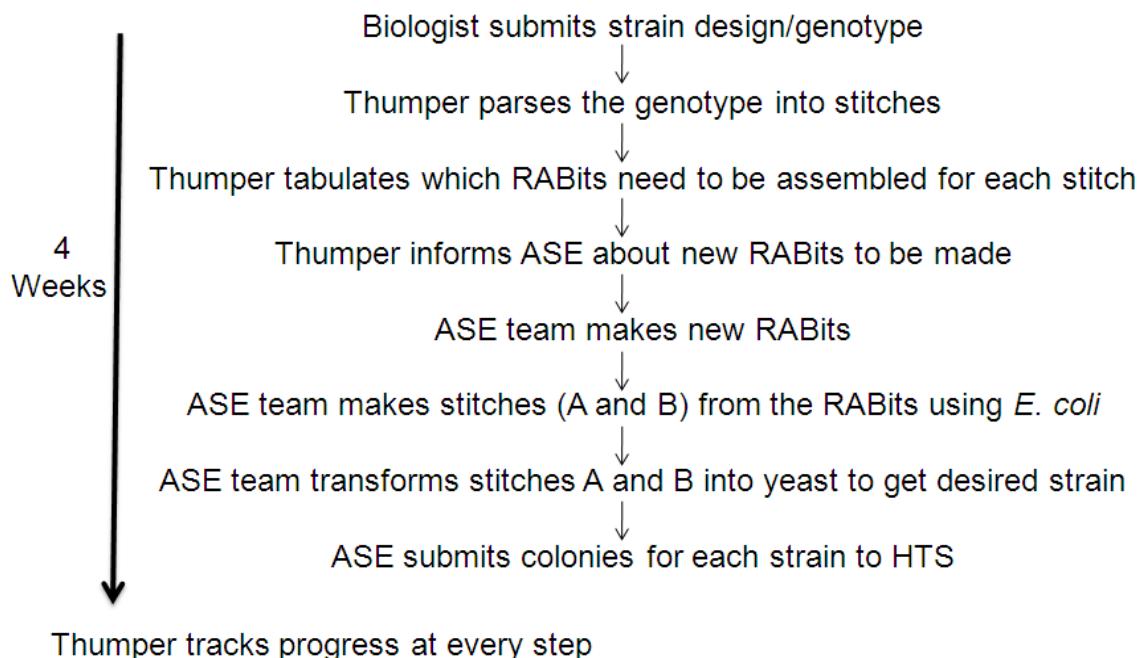
- Bistitches:** Two stitches, usually combined during yeast transformation, and usually with a split marker to provide overlap. Using bistitches both allows 10 RABits per integration and allows more combinations to be tested.



Overview of ASE Workflow

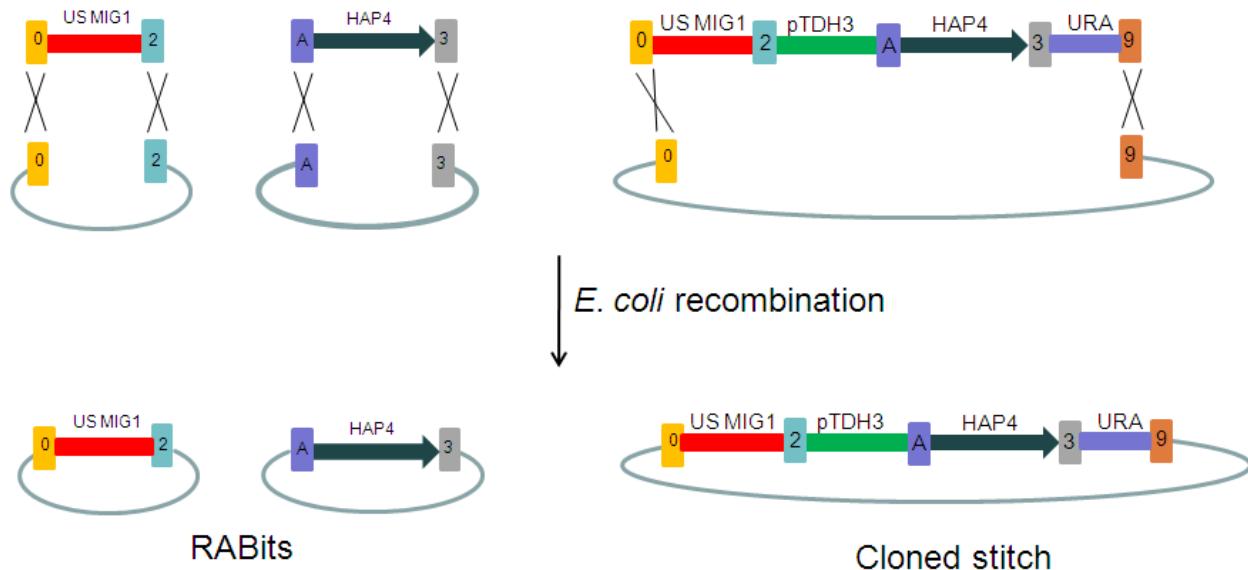


From Ideas to Constructs



Building a DNA Library

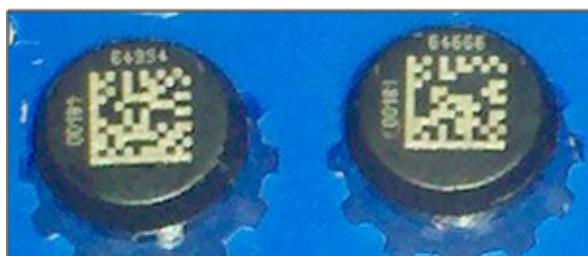
All DNA fragments (promoters, genes, resistance markers, stitches) are cloned into vectors



Curation and Storage of the Library (The RABit Bank)



- Plasmids are stored in laser-tagged tubes
- Thumper maintains catalog of plasmids



Unique bar code
etched at the bottom

Submitting Ideas to ASE for strain construction:

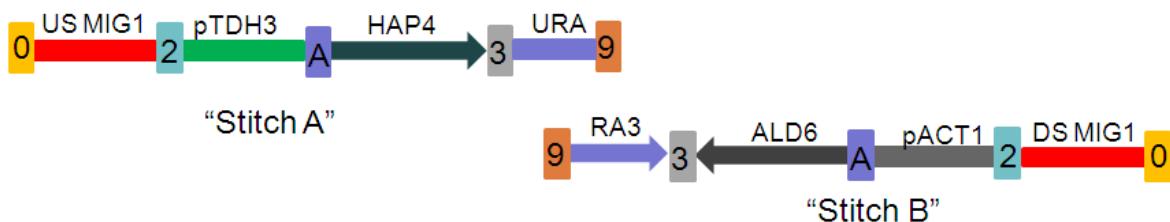
Currently ideas for strains are submitted to Sunil Chandran (Chandran@amyris.com) . In the future, ideas will be submitted automatically through a user interface built into the thumper database.

Example: From Idea to Bistitch

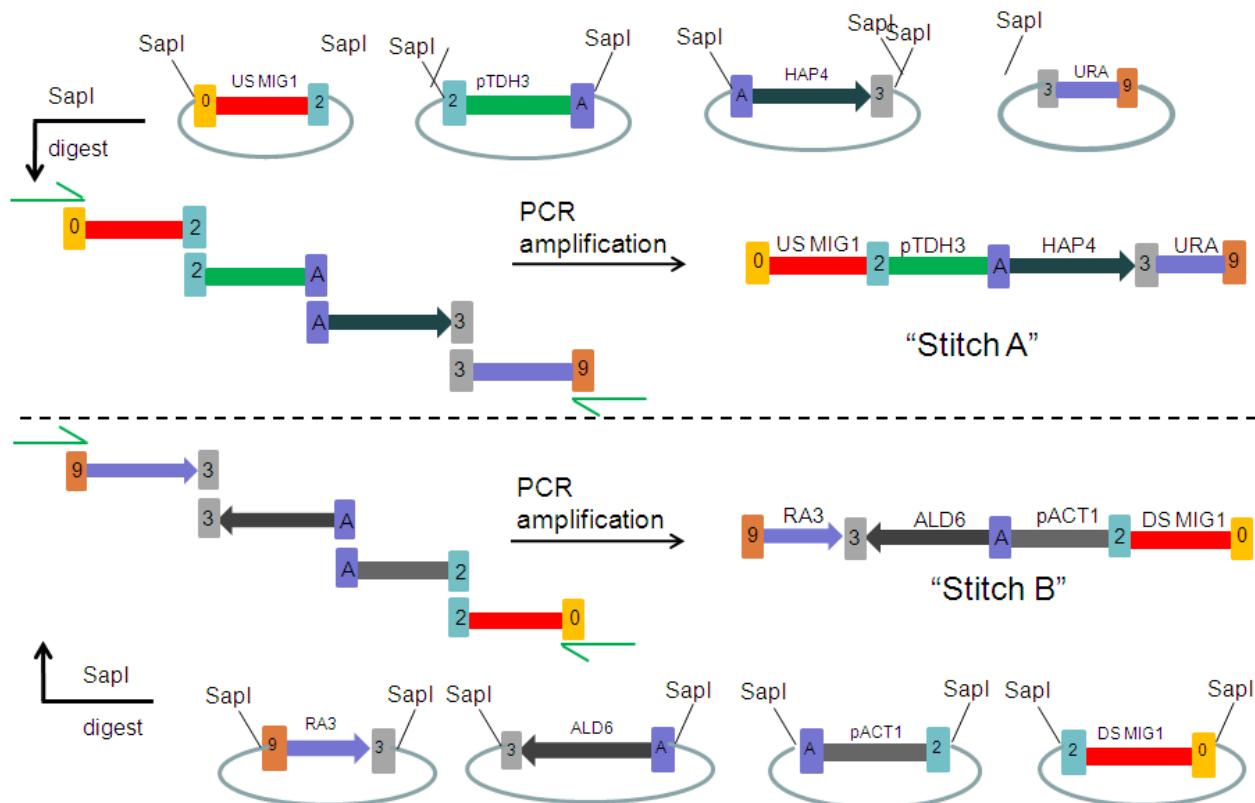
"Would like to knockout MIG1 and overexpress HAP4 and ALD6"

- Generated using:
 • Standard format
 • Generic parts
 • Promoter selection software

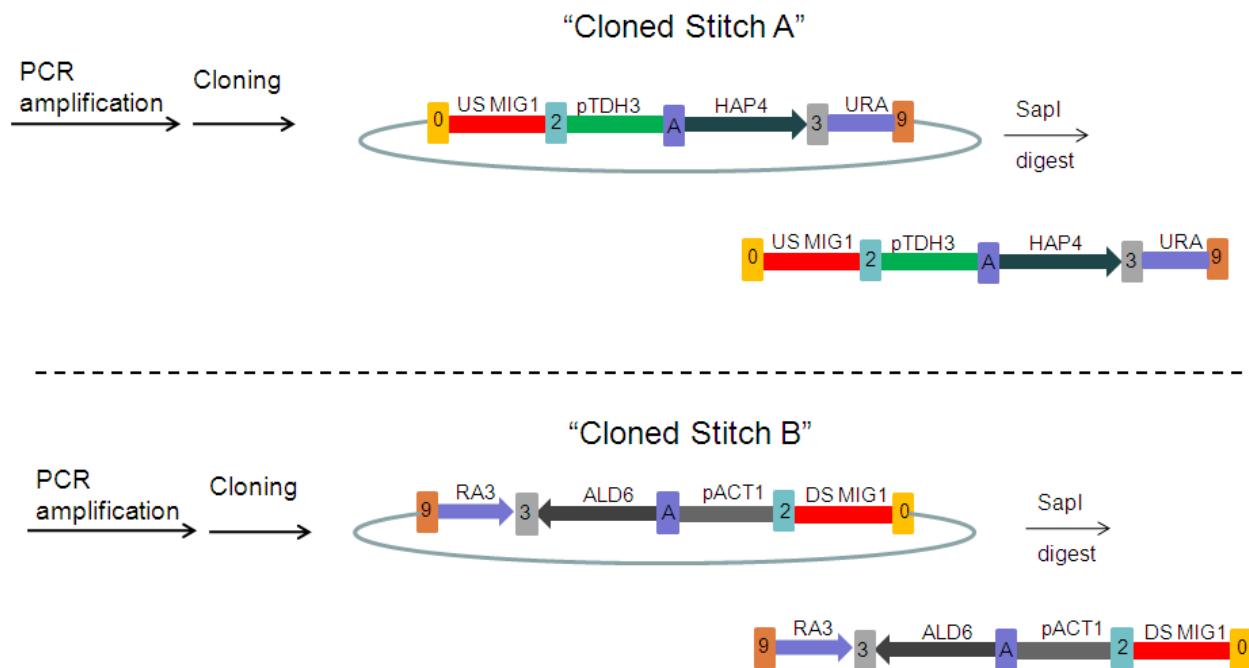
MIG1⁺::pTDH3>HAP4::pACT1>ALD6



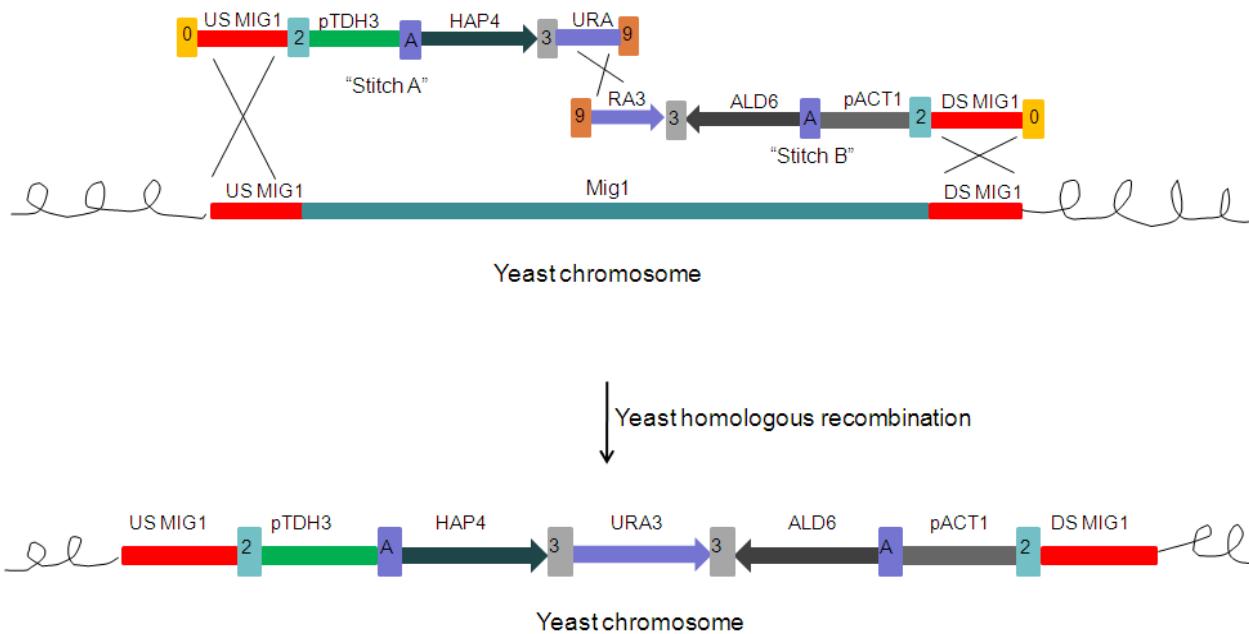
RABits are combined to make stitches



Stitches are cloned, then excised



Two stitches become a bistitch in the chromosome



4.2 Automation (Chance, Dan, Don, William)

The biology department has made a significant commitment to automation and maintains a large collection of automated liquid handlers and assorted robotics. The objective of the automation group is to provide researchers with facile access to automation to drive innovation, accuracy, efficiency, and health.

Our philosophy is as follows:

Be flexible, anticipate future needs, but don't overbuild with complicated solutions that sacrifice responsiveness.

Continually monitor research automation needs versus capability. Stay ahead of demand.

Empower the team (and end users) to innovate—don't settle for clunky solutions.

Don't be afraid to change processes if they are inefficient.

Minimize development time.

- Organize scripts to enable facile development of new scripts based on existing templates.
- Actively engage community and promote partnership between developers and end users.
- Establish a training program to educate end users.

To learn more about our robotics, what procedures we have automated, and/or to request that your procedure be automated (script request), visit the Automation website:

<http://superdog/display/AUTO/Automation>

4.3 High Throughput Screening (HTS) (Candace, Sarah, Jericho)

What We Do

- Screen libraries for improved production
 - Funnel (Tier-based) screening
 - Up to 30,000 strains/week
- Provide standardized strain testing (CORE) for entire community
- Nimble: need to quickly adapt and add improvements/new assays as they are developed



Terminology

- HTS – High throughput Screening group
- CORE – Plate growth & assay service

What We Screen

- Mutagenesis Libraries and GEMS sorted versions
- ASE (automated strain engineering)
- Spores from out- and backcrosses
- Directed-Mutagenized Targets
- Other Random Libraries (ORFeome overexpression, cDNA, etc)
- PV Tank Surveillance

HTS Drives Yield Improvements in Fermenters

Screening Throughput and Yield Progress

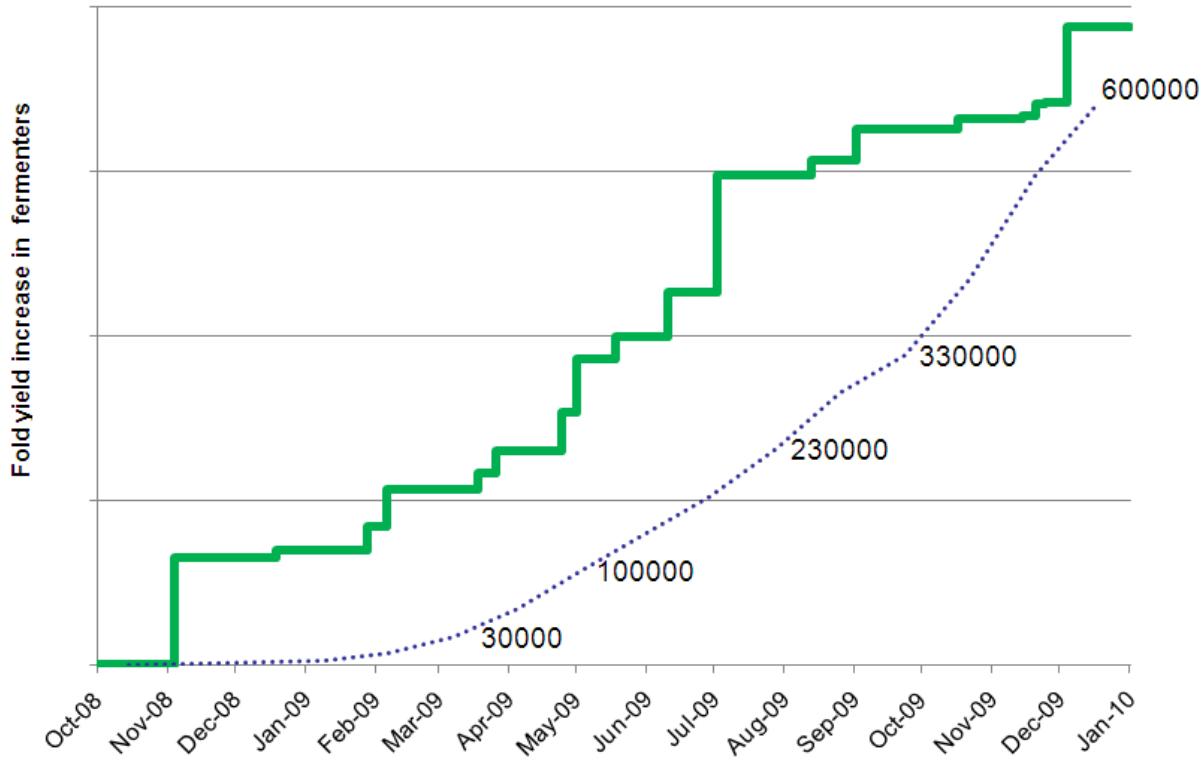
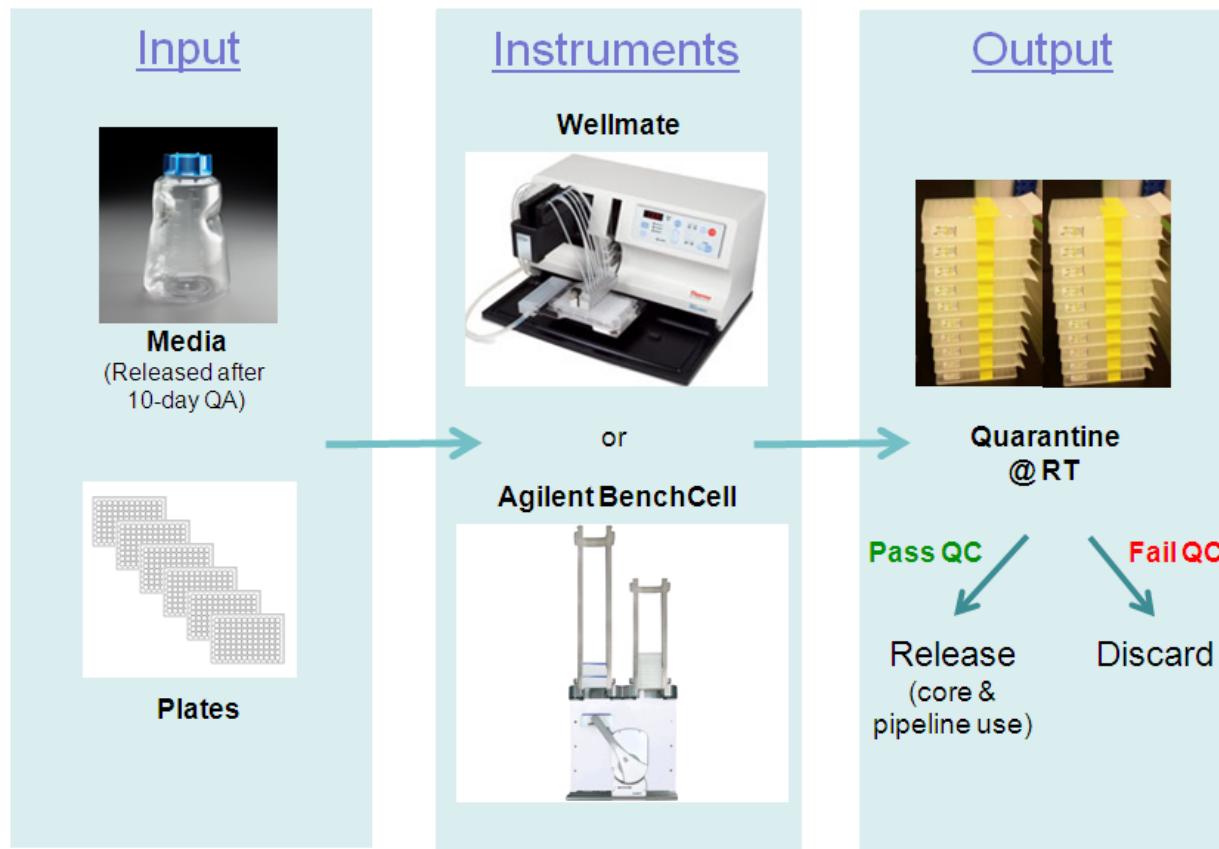


Plate-Filling Process

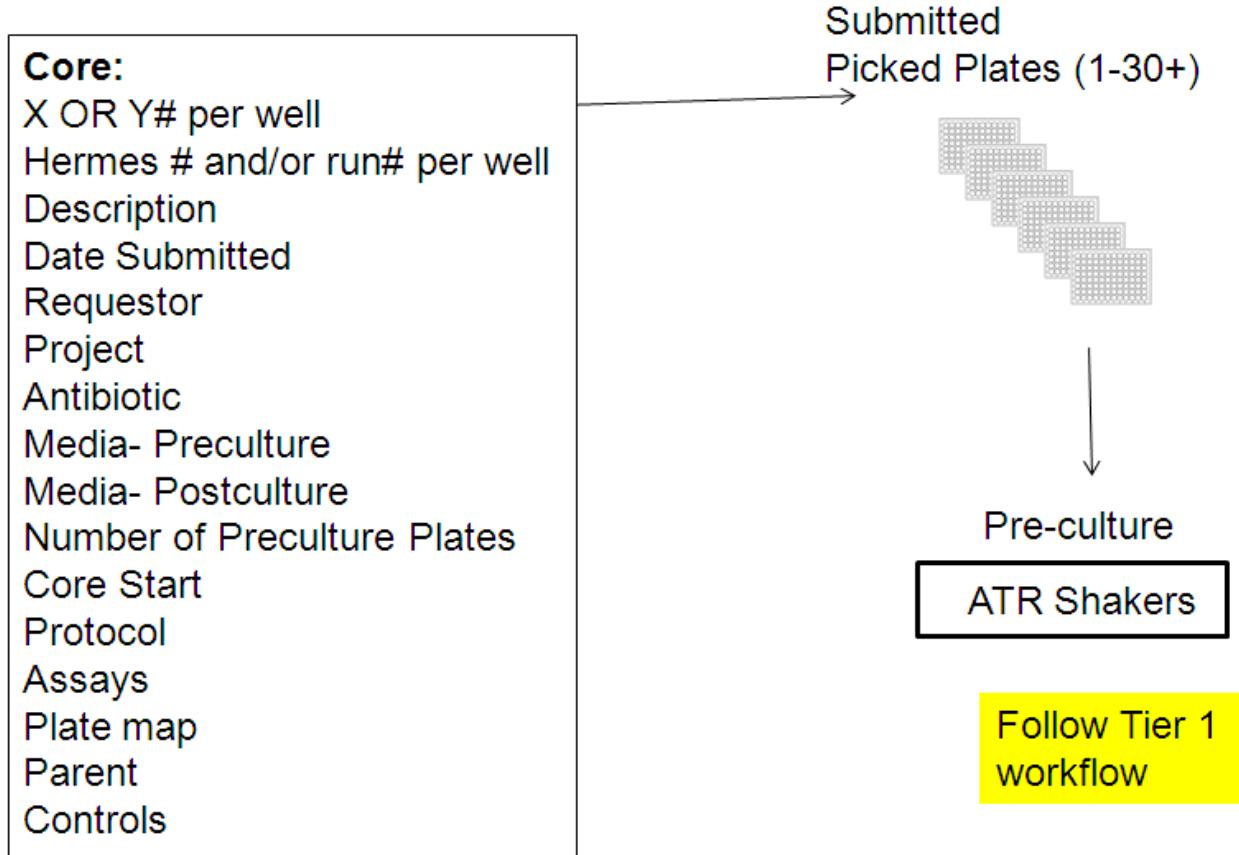


Core Overview

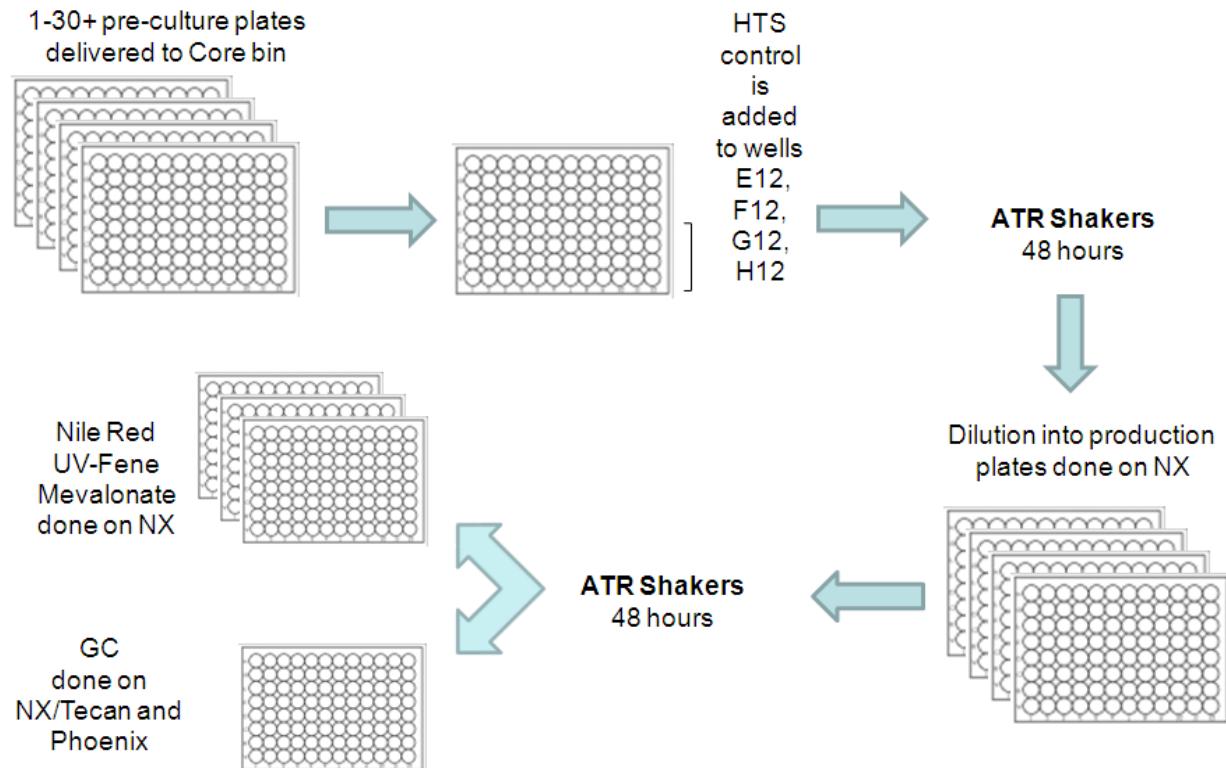
- Service allows requestor to have samples run using HTS process
- Assay options provided: Nile Red, UV-Fene Assay, Farnesene extraction for Gas Chromatography and Mevalonate
- Core schedule - 3 batches run each week (~30 assays run/batch)
 - Submission days are Monday, Wednesday and Friday
- Limit to 30 NR or Mevalonate plates and 10 GC plates OR 13 UV-Fene per start date- check how many plates are already submitted!

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Start CORE		Dilute CORE		Process CORE (NR/GC/UV/ Mev)		
		Start CORE		Dilute CORE		Process CORE (NR/GC/UV/ Mev)
	Process CORE (NR/GC/UV/ Mev)			Start CORE		Dilute CORE

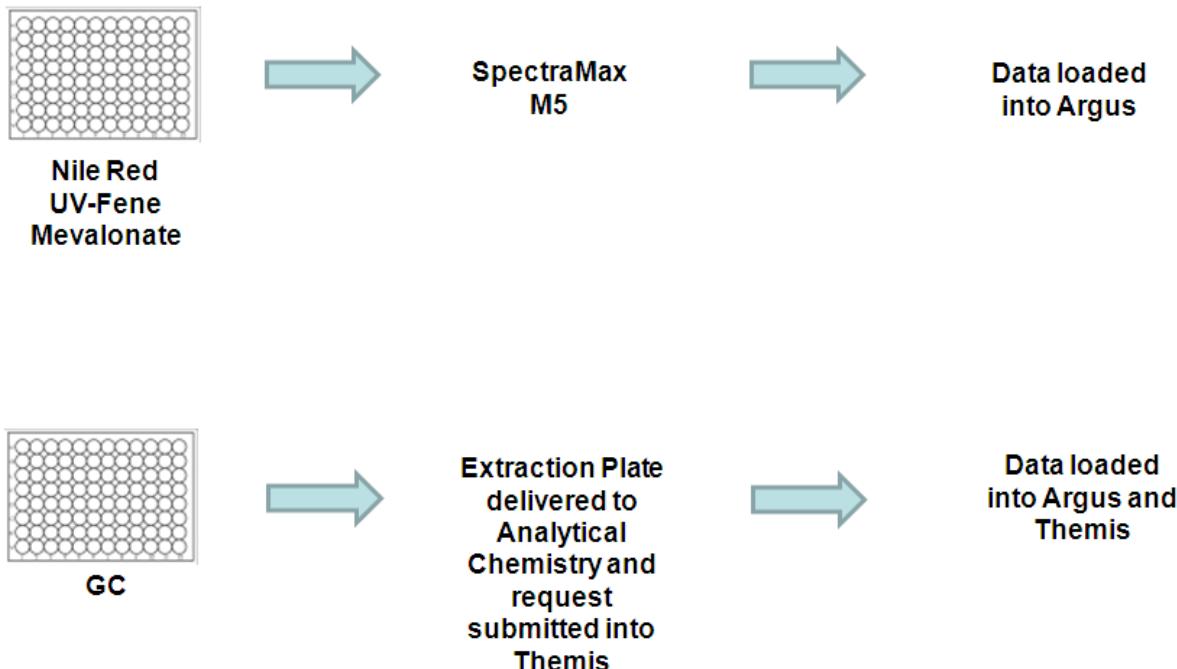
Core Submissions



Core work flow



Data Generation/Upload



New Core Shake Request

Argus - New Core Shake Request - Mozilla Firefox
File Edit View Bookmarks Tools Help
http://argus.amryns.local/request/home.php
User: Jenicho Pantaleon [Logout] Advanced Search or Select Saved Search
Argus - New Core Shake Request

New Core Shake Request

Main Menu

- Summary Views
 - List All Plates
 - Mutagenesis
 - List Plate Groups
 - List Protocols
 - List Schedules
 - Active Schedules
 - List Well Groups
 - New Root Plate
 - Preferences
 - Administration

Description

Requestor: Jenicho Pantaleon

Project:

PreCulture Media Type:

Production Media Type:

Antibiotic:

Number of PreCulture Plates:

Core Start: Friday, 26 Mar 2010

Apply Protocol:

Assays	Dilution	Plates Per PreCulture Plate
Nile Red		0
Fene		0
Mev		0
Tri		0

Leave blank for "Growth Only"

Add

Core Schedule

A total of 20 plates can be run on each day, only 8 of them can be GC.

Friday, 3/26/2010 - Total: 34 Gc: 10

ID	Project	Plates	Assays	Owner
219	08- Lead consolidation	2	Nr Gc	Hanxiao Jiang
220	28- FS Engineering	6	Gc Tri	Andrew Main
221	05- Mevalonate to...	2	Nr Gc	Anna Tai
222	05- Mevalonate to...	1	Nr	Anna Tai
223	01- Performance...	16	Nr	Sarah Leng
224	01- Performance...	1	Nr	Sarah Leng
225	10- Fermentation...	2	Nr	Elisa Porcel

Monday, 3/29/2010 - Total: 0 Gc: 0

None Scheduled

Friday, 4/2/2010 - Total: 0 Gc: 0

None Scheduled

Monday, 4/5/2010 - Total: 0 Gc: 0

None Scheduled

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Done

Processing Requests

The screenshot shows a web browser window titled "Argus - Core Shake Request Schedule - Mozilla Firefox". The URL is <http://argus.amryns.local/request/list/basic.php>. The user is Jencho Pantaleon. The main content area is titled "Core Shake Request Schedule". A sidebar on the left contains a "Main Menu" with links like "List All Plates", "List Plate Groups", "List Requests", "Active Schedules", "List Well Groups", "New Root Plate", "Preferences", and "Administration". Below this is an "Actions" section with "New Request", "Bulk Operations", "Process Requests", and "Export Options". A red arrow points from this section to a blue callout box containing the text: "Process Requests – assigns Argus numbers to plates". The main table lists requests with columns: Id, Description, Created On, Scheduled For, Requested By, Processed By, Plates, Nr, Fene, Mev, Tri, Started, Data. The table shows several entries, with the first one being "Friday, 3/19/2010 - Total: 31 Gcs 3". A modal dialog box titled "Process 14" is open at the bottom, showing details for a request involving "Combination experiment 2" on Mar 10, 2010, scheduled for Mar 15, 2010, requested by Andrew Main, processed by Eva Wong, and assigned plate numbers C-602411-T1 through C-602416-T1.

Request Details

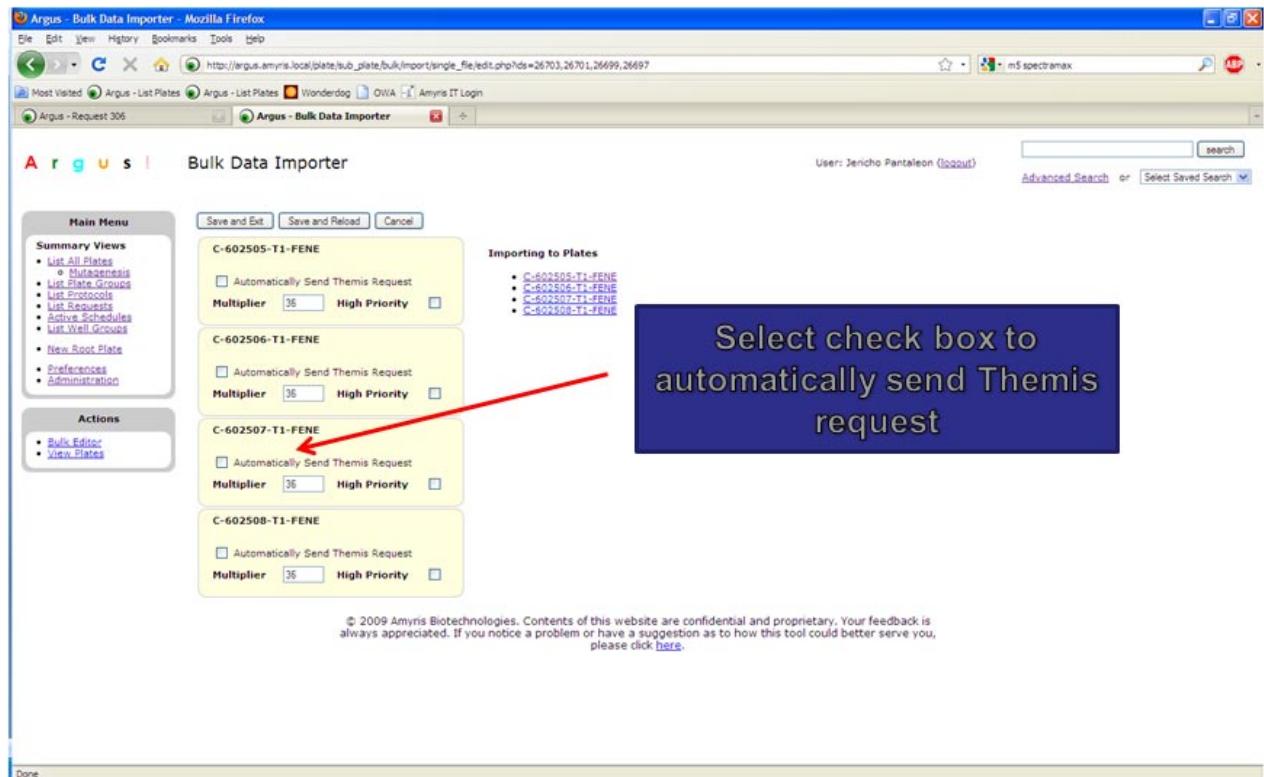
The screenshot shows the Argus software interface in Mozilla Firefox. The title bar reads "Argus - Request 314 - Mozilla Firefox". The main content area displays the details of Request 314. On the left, there is a navigation menu with sections like Main Menu, Summary Views, and Actions. The main content area includes fields for Description, Requestor, Project, PreCulture Media Type, Production Media Type, Antibiotic, Number of PreCulture Plates, Core Start, Assays, Total Plate Count, and Dilution. Below this, there is a section for Plates with two entries. A large blue callout box with white text in the center says "Review details of request for additional instructions". A red arrow points from the top right towards this callout box. At the bottom of the page, there is a copyright notice: "© 2009 Amyris Biotechnologies. Contents of this website are confidential and proprietary. Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please click [here](#)".

Core Shake Request Schedule (Printed hard copy of Core schedule helps organize all requests of the day)

Core start 3.19.2010												
id	requestor	started	scheduled	preculture_media	production_media	nr	fene	mev	tri	staff	count	plates
296	Kirsten Benjamin	3/15/2010	Friday	BSM 2%Suc	BSM 4%Suc	2	0	0	1	Jericho Pantaleon	1	C-602505-T1
298	Elisa Porcel	3/16/2010	Friday	BSM 2%Suc	BSM 4%Suc	2	0	0	0	Jericho Pantaleon	2	C-602439-T1;C-602440-T1
299	Sarah Leng	3/17/2010	Friday	BSM 2%Suc	BSM 4%Suc	6	0	0	0	Jericho Pantaleon	6	C-602441-T1;C-602442-T1;C-602443-T1;C-602444-T1;C-602445-T1;C-602446-T1
300	Sarah Leng	3/17/2010	Friday	BSM 2%Suc	BSM 4%Suc	3	0	0	0	Jericho Pantaleon	3	C-602447-T1;C-602448-T1;C-602449-T1
301	Sarah Leng	3/17/2010	Friday	BSM 2%Suc	BSM 4%Suc	2	0	0	0	Jericho Pantaleon	2	C-602450-T1;C-602451-T1
302	Sarah Leng	3/17/2010	Friday	BSM 2%Suc	BSM 4%Suc	3	0	0	0	Jericho Pantaleon	3	C-602452-T1;C-602453-T1;C-602454-T1
303	Kevin Dietzel	3/17/2010	Friday	BSM 2%Suc + Ura	BSM 4%Suc + Ura	1	1	0	0	Jericho Pantaleon	1	C-602455-T1
304	Michele Pleck	3/17/2010	Friday	BSM 2%Suc	Custom	3	0	0	0	Jericho Pantaleon	3	C-602456-T1;C-602457-T1;C-602458-T1
305	Hiroko Tsuruta	3/17/2010	Friday	BSM 2%Suc	BSM 4%Suc	6	0	0	0	Jericho Pantaleon	6	C-602459-T1;C-602460-T1;C-602461-T1;C-602462-T1;C-602463-T1;C-602464-T1
306	Tim Geistlinger	3/18/2010	Friday	BSM 2%Suc	BSM 2%Suc	2	0	0	2	Jericho Pantaleon	2	C-602465-T1;C-602466-T1
307	Tim Geistlinger	3/18/2010	Friday	Custom	Custom	2	2	0	0	Jericho Pantaleon	2	C-602467-T1;C-602468-T1
308	Christopher Reeves	3/18/2010	Friday	BSM 2%Suc	BSM 4%Suc	5	5	0	0	Jericho Pantaleon	5	C-602506-T1;C-602507-T1;C-602508-T1;C-602509-T1;C-602510-T1
309	Sarah Leng	3/18/2010	Friday	BSM 2%Suc	BSM 4%Suc	3	0	0	0		3	C-602513-T1;C-602514-T1;C-602515-T1
310	Sarah Leng	3/18/2010	Friday	BSM 2%Suc	BSM 4%Suc	1	0	0	0		1	C-602516-T1

41 8 0 3 0 40

Submitting GC plates into Themis



Themis Database

The screenshot shows a Mozilla Firefox browser window displaying the Analytical Chemistry Sample Running Database. The title bar reads "Analytical Chemistry Sample Running Database - Jericho Pantaleon - Mozilla Firefox". The address bar shows the URL "http://themis/tray/view.php?id=8565". The main content area is titled "Tray: 10299".

Tray edit

Label on Tray: 10299
Notes: Auto Submission
Tray Type: Titer Plate 96 wells 1.1 mL **High Priority?** No
Method: Trichodiene-HTP
Project: 03- High-throughput screening **Finished on:**
Tray location: von Bellingshausen - Shelf B - High Thru-Put Fene Assay **Requested By:** Jericho Pantaleon on 03-23-10
Progress: Ready to be Processed **Processed by:**
Export to: Argus
Plate Barcode: 10299
Argus Plate Id: 10299

Samples

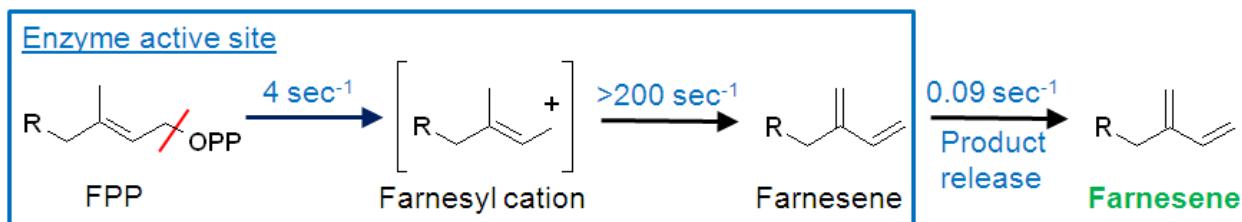
Well Position	Multiplier	Notes
A1	36	
A2	36	
A3	36	
A4	36	

Done

4.4 Enzymology and Protein Engineering (Lishan and Lan)

The objective or protein engineering is to improve the properties of enzymes in key metabolic pathways relevant to Amyris' renewable products. Our work on Farnesene synthase (FS) serves as the prototypical example of the utility of enzyme engineering and illustrates our protein engineering capability.

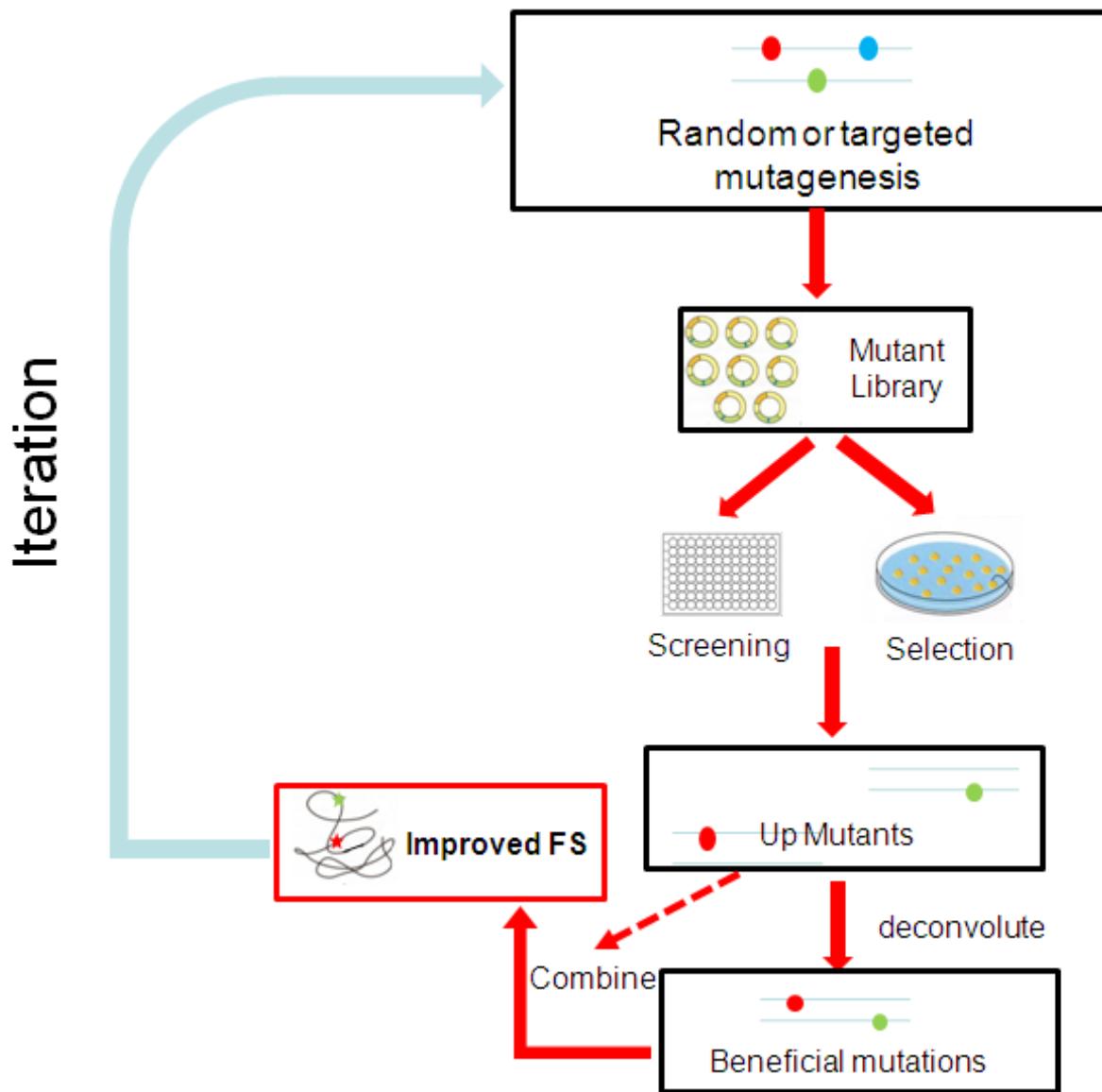
FS – how fast is it?



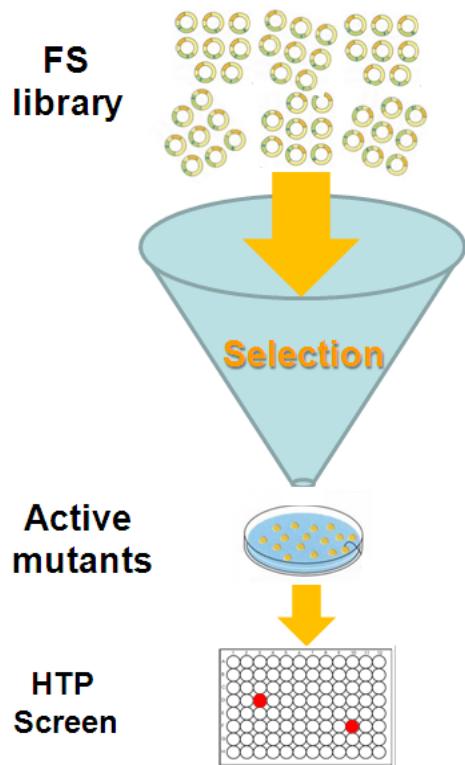
- Terpene synthase catalysis is limited by product release (e.g. Steady-state k_{cat} *in vitro*)
- If product removal is more efficient *in vivo*, then the *in vitro* k_{cat} may be an underestimate of the true *in vivo* k_{cat} .
- Ionization of FPP (rate = $\sim 4 \text{ sec}^{-1}$) is the rate limiting chemical step

We need a faster FS!

FS Engineering – Overview of FS Improvement Strategy



Filtering FS libraries by selection

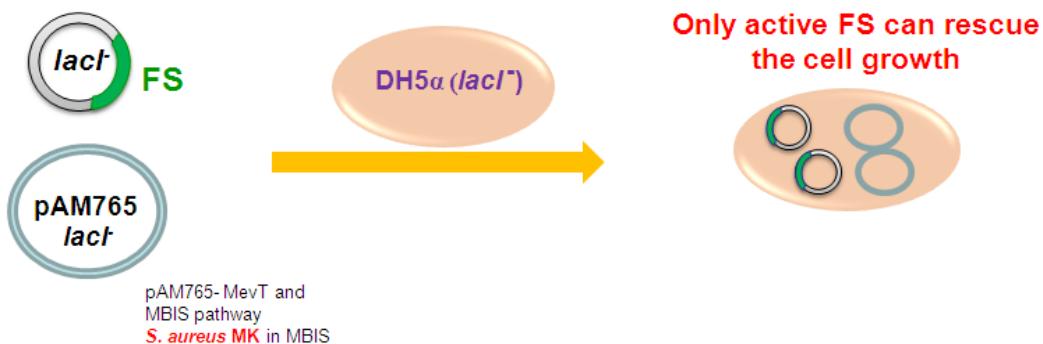


- High [FPP] is lethal to the cells
- Select for cells with active FS mutants
- Reduce the HTP burden
- Speed up the screening

Selection Method in *E. coli*

Selection basis

- FPP toxicity is lethal to the cell
- Active FS can rescue the cell from FPP toxicity



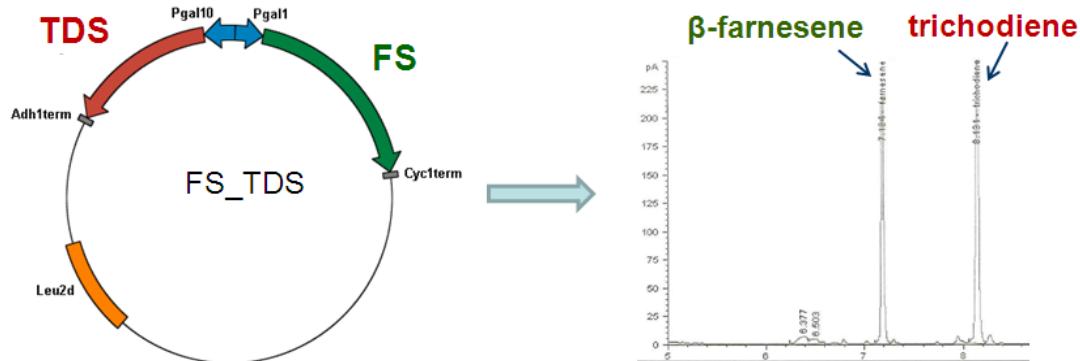
FS Selection in Yeast



"Classic" PCR stitching but with Error-Prone PCR for *AaFS* primary amplification

- **FPP-toxicity based selection in yeast**
- **Strain background: Y227, Cen.PK2**
- **Yeast 2.0 base strain**
- **Fully Galactose inducible**
- **Know “room-for-improvement” conditions**

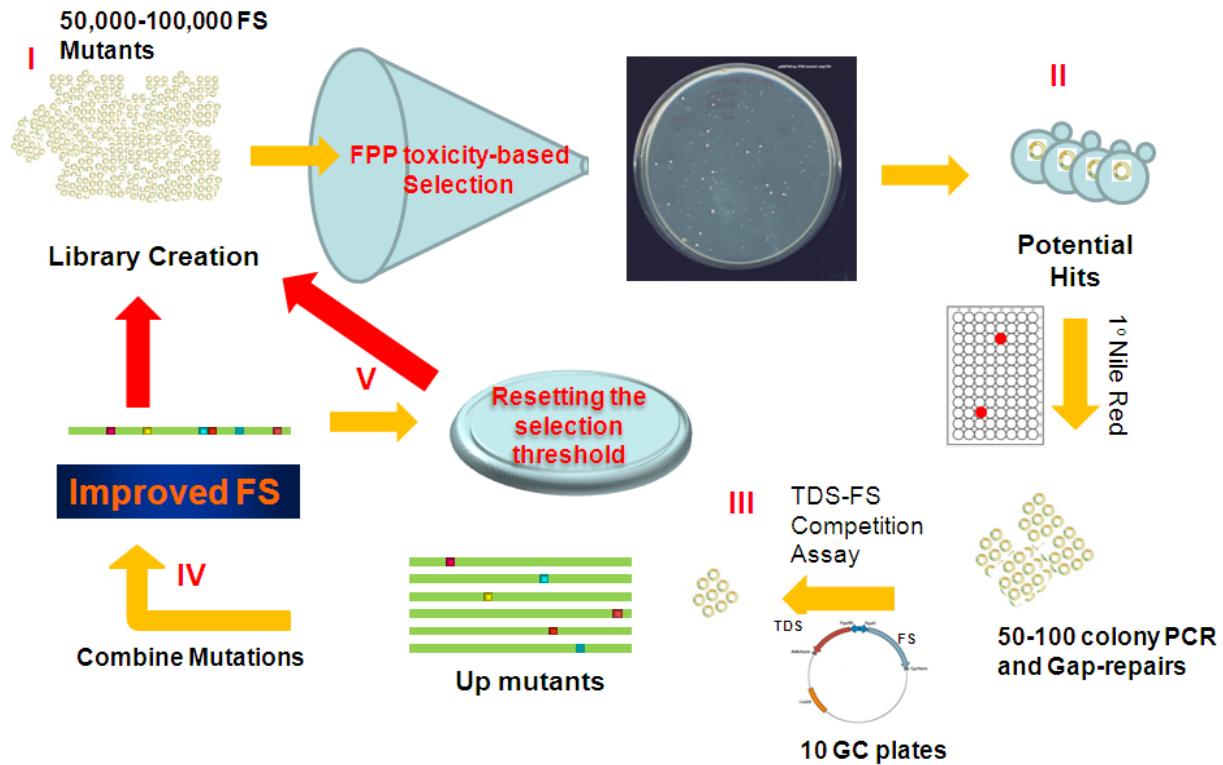
How to benchmark FS Mutants and validate improved enzymes



- Ratios of fene/trichodiene
- Single copy integration

FS	F-ene/T-ene
eFS S2D	0.82
Aa FS	1.32
Ad FS (kiwi)	1.66

Yeast Selection Workflow



Inter-Department Capabilities

4.5 Analytical Chemistry (Mike and Amy)

Analytical Chemistry (AC) is split into four subgroups: Methods Development, Operations, Proteomics/Metabolomics, and Strain Physiology.

Operations:

Rob Herman, Phil Norton, Richard Dutcher, Brian Van Deren, Felix Kha

Routine analyses are performed by our Operations group and data is accessed through Themis.
<http://themis.amyris.local/>

Themis screen shot

A screenshot of a Mozilla Firefox browser window showing the 'Analytical Chemistry Sample Running Database - Amy Shutkin - Mozilla Firefox' page. The URL in the address bar is http://themis.amyris.local/tray/ViewAll.php. The main content area is titled 'Index of Trays for Next Processing' and shows a table of trays. The table has columns for 'Tray Name', 'Status', and 'Last Update'. There are two entries: 'u Diesel, high resolution (titer filter V21.0)' and 'u HTP Diesel (49)'. A legend at the bottom left explains the status codes: 'Not Yet' (yellow), 'Ready to be Processed' (green), 'Processing' (orange), 'Hold/Block' (red), 'Sample Ready' (blue), and 'Rejected' (grey). A note at the bottom states: '© 2009 Amyris™. Contents of this website are confidential and proprietary. Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please click here.'

Analyses currently performed include:

- Farnesene (f-ene) titer for fermentation analysis and yield calculations
- HTP f-ene titer for strain screening
- Various assays to support enzyme engineering efforts (trichodiene, etc.)
- Organic acids and alcohols
- Squalene and ergosterol
- Sugars
- Sugars in cane syrup
- Trehalose and glycogen

Primarily, we use Agilent GCs and LCs. Additionally, we have two walk-up Agilent GC-MSs. Training is available.

Development:

Scott Fickes,; Veronica Rocha, Keerthini Manda, Don Diola

All methods performed by Operations are first developed by our Methods Development group typically as cross-departmental collaborations. Current and recent collaborative projects include High-Throughput Screening, Near InfraRed Development, and Microscale Assay Development. In addition, this team analyzes fuels impurities and degradation products.

Proteomics/Metabolomics:

Sara Gaucher, Peter Jackson, Marites Ayson, Nathan Moss

Proteomic assay methods include:

- Protein ID to determine presence of a protein and to mine data post-acquisition
- MRM (relative) to specifically target your favorite protein (YFP)
- MRM (absolute) to specifically target YFP and to measure absolute quantity (nmol/gDCW)
- iTRAQ is not currently available but we have the capability to run, if required

Metabolomic Methods:

- Relative quantification of pathway metabolites and co-factors (all or specific targets)
- Absolute quantification of pathway metabolites and co-factors (all or specific targets)

Sara Gaucher, Peter Jackson, Marites Ayson, Nathan Moss

Strain Physiology:

Lance Kizer, Derek Abbott, Bonny Lieu, Ivan Zamora

The primary aim of Strain Physiology is to develop screens for our strains and identify new engineering targets. Strain Physiology assays:

- Gene expression (Nanostring, microarray)
- Flux measurements
- Viability and growth assays

4.6 Introduction to Fermentation (Tim Leaf)

Key roles

- **Process development (“PD”)**
- **Strain testing (performance validation, “PV”)**
- **Material generation for purification & chemistry**
- **Process transfer (contractors, partners)**
- **Process scale-up (pilot plant, Brazil)**

Capabilities

- **26 x 2 L stirred tank fermenter vessels**
Much higher level of control than a flask culture
- **Control capabilities**
Temperature, pH, Oxygen, Feed delivery
- **Additional process monitoring**
Offgas analysis (mass spec)
- **Data logging and control software “MFCS”**
Log any process parameter continuously
Program process control in response to monitored variables or elapsed time
- **All fermentation data is available to Amyrisians in Hermes database**

Stirred Tank Fermentation

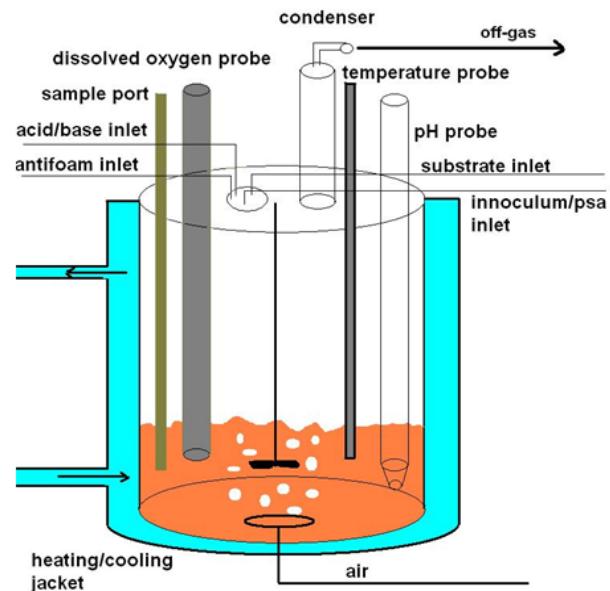
- pH
 - Monitored by pH probe
 - Controlled by acid/base delivery

- Dissolved oxygen
 - Monitored by dissolved oxygen probe
 - Controlled by agitation and gas inlet

- Temperature
 - Monitored by temperature probe
 - Controlled by heating/cooling jacket

- Off-gas compositions
 - Monitored by off-gas analyzer
 - Controlled by inlet gas and substrate consumption

- Cell growth
 - Measured by light absorption at 600 nm (OD600)
 - Can be monitored online with a probe
 - Controlled by just about everything

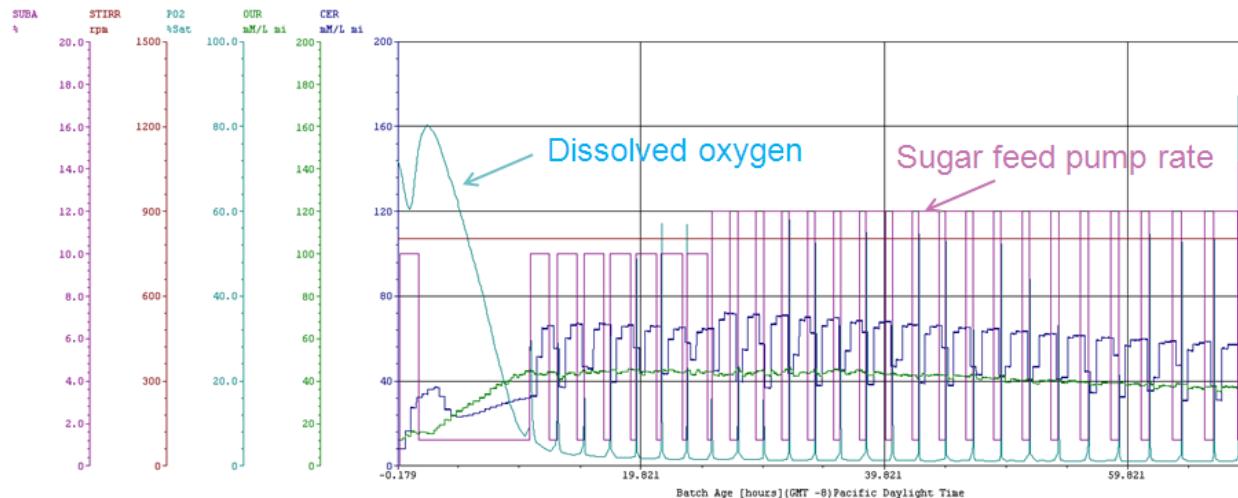


Process Conditions: Example

Protocol	<u>TMAP/syrup</u> <u>.30C, pH5,</u> <u>1000TR,</u> <u>72h MAPFX</u>	Run Conditions		Media Conditions	
		Process Length	3.00 (Days)	Tank Medium	TMAP FM YFL0082
		Vessel Size	2.00 (L)	% Inoculum	100.00 (%)
		Initial Volume	1.00 (L)	Initial Sucrose Concentration	22.90 (g/L)
		Temperature	30.00 (C)	Feed Type	Cane Syrup
		Initial pH	5.00	Feed Sucrose Concentration	763.60 (g/L)
		Base Used	NH4OH		
		Target OTR	100.00 (mmolO2/L/hr)		
		VVM	0.50		
		Agitation Rate	1050.00 (RPM)		
		Aerobicity	Micro-Aerobic		
		Feed Strategy	MAPFX		
		Antifoam Added	yes		
		Antifoam Type	Tergitol L-81		
		cell source	vial		
		Air Flow	0.50 (slpm)		

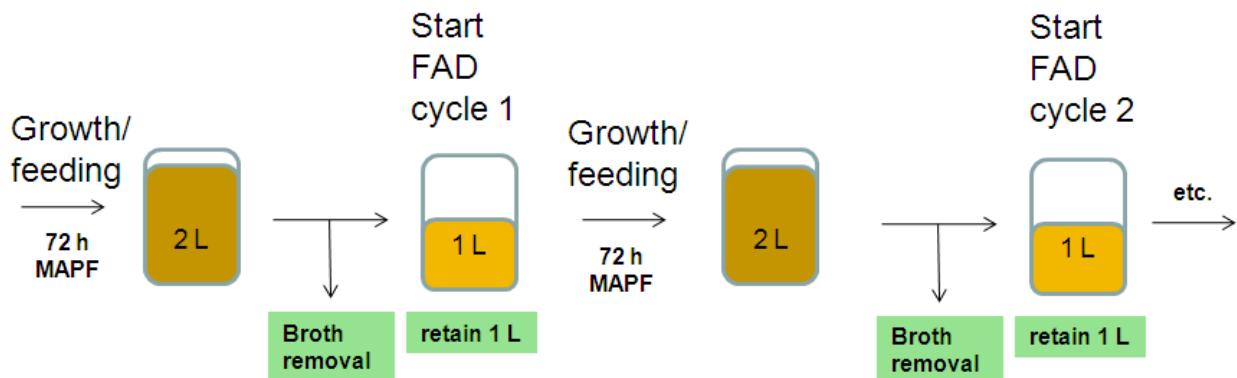
Micro-aerobic pulse feed process (“MAPF”)

- Fene strains also make ethanol when sugar is in excess
- Ethanol is consumed in the absence of sugar
- Pulse feeding prevents excess ethanol accumulation
- Feed is triggered by dissolved oxygen “spike” upon carbon exhaustion



Fill and Draw Operation

- Start with build-up of biomass
- Tank slowly fills with sugar feeding
- Draw broth and continue feeding
- Product yield improves by conserving biomass



Terminology

- DO: dissolved oxygen (%)
- CER: carbon dioxide evolution rate (mmol/L/hr)
- OUR: oxygen uptake rate (mmol/L/hr)
- OTR: oxygen transfer rate (mmol/L/hr)
- SOUR: specific oxygen uptake rate (mmol/gDCW/hr)
- DCW: dry cell weight (biomass)
- MAPF: micro-aerobic pulse feed, a sugar feed strategy
- FAD: fill-and-draw, an extended operation fed fermentation
- Fermentor = tank = vessel = bioreactor
- Titer (g product / L fermentor volume)
- Yield (g product / g substrate) (%)
- Productivity (g product / L fermentor volume / day)

5. Databases and Computational Tools

Help with Navigating Amyris' Databases:

Feel free to drop by our cubes or set up a time via outlook. Any of us would be happy to walk you through the process or answer any other questions you might have:

- Thumper (Victor) followed by short 15 minute session with Brian & Mike (Thumper developers)
- Argus (Lawrence and Matt)
- Spotfire (short overview & best of how one can best leverage the tool) Jack Schonbrun
- Request Systems & Superdog overview

KM will walk new hires through the request systems (overview to be given by Shawn)

KM will walk new hires through Superdog usage, tips & tricks & articles database (Marian)

5.1 Hermes (Chris D and Marian)

Hermes (<http://hermes.amyris.local>) serves 2 purposes. The first is as an index of activities and experiments carried on an Amyris, and the second is as a repository for fermentation data (primarily PV (Performance Validation) and PD (Process Development) data).

Recording the basics (hypothesis, rational, preliminary analysis) of what you are working on in Hermes, and then attaching associated notes and data (spreadsheets, presentations, or in some cases structured data) is the simplest way to give visibility to the rest of Amyris as to what you are working on, an easy way to point others to what you are working on (just give them a hermes experiment # or a URL), and a good way for you to explore and keep up to date on what other researchers are working on. It also serves as a repository for historical data (what has been worked on, what has been tried, etc). It is a product in continuous development, and the developers are very open to hearing your ideas or criticisms. At the bottom of every page in the application (and in all KM applications) there's a link to give feedback- that's the best place from which to send the developers your ideas or report if you've had any problems.

To use the application, just point your browser to <http://hermes.amyris.local>. Anyone with an Amyris Emeryville or Amyris Brazil login should be able to log in with their Amyris username and password. At the top of the page you'll see a number of tabs. The "experiments" and "runs" tabs are the most important. They'll take you to the index of experiments and the index of fermentation runs, respectively.

Both of these indexes are laid out in an excel like grid with input fields at the top of most columns which you can use to filter the results:

Experiment Index										
<input type="button" value="Clear"/> <input type="button" value="Columns"/> <input type="button" value="New Experiment"/>										
Experiments 1 - 10 of 132										
ID	Date	Hypothesis	Design	Property Sets	Project	Organism	Fermentat	Yield		
2222	2010-06-18	PERFORMANCE VALIDATION	Compare yield performance of 10 strains in I	All	01- Fermentation Services & Yeast	Bioreactor				
2215	2010-06-14	Measurements of fene flux, growth rate, r	Overall process for experimental strain: 1. Pr		22- CIA (Baseline Physiolog Yeast	96 Well Pls				
2212	2010-06-11	PERFORMANCE VALIDATION	Compare yield performance of 10 strains in I		01- Fermentation Services & Yeast	Bioreactor	15.23%			
2200	2010-06-04	PERFORMANCE VALIDATION	Compare yield performance of 10 strains in I		01- Fermentation Services & Yeast	Bioreactor	13.87%			
2193	2010-05-28	PERFORMANCE VALIDATION	Standard Temperate PV/high OTR new stan		01- Fermentation Services & Yeast	Bioreactor	14.31%			
2190	2010-05-25	Since we have observed a correlation be	Compare strain stability ranking from SY85 e		22- CIA (Baseline Physiolog Yeast	96 Well Pls				
2187	2010-05-24	PERFORMANCE VALIDATION	Standard Temperate PV/high OTR new stan		01- Fermentation Services & Yeast	Bioreactor	13.98%			
2176	2010-05-20	PERFORMANCE VALIDATION	Standard Temperate PV/high OTR new stan		01- Fermentation Services & Yeast	Bioreactor	13.44%			
2175	2010-05-20	FPP and farnesol levels of mutants comp	mutants and parents		22- CIA (Baseline Physiolog Yeast	96 Well Pls				
2164	2010-05-17	PERFORMANCE VALIDATION	Standard Temperate PV/high OTR new stan		01- Fermentation Services & Yeast	Bioreactor	12.38%			

Run Index									
<input type="button" value="Clear"/> <input type="button" value="Columns"/> <input type="button" value="CSV"/> <input type="button" value="Chart"/> <input type="button" value="Dashboards"/> <input type="button" value="Tag"/> <input type="button" value="Tag Scope"/> <input type="button" value="Open"/>									
Runs 1 - 10 of 150 (0 selected)									
□	Exp	Run	Description	Strain	Fermentation 1	Start Time	Titer	Yield	Tags
				All					MAPF2.0
<input type="checkbox"/>	1537	1	Y2622, mutagenized strain	Y2622	Bioreactor	2009-06-19 10:41:59	10.10	6.90%	MAPF2.0
<input type="checkbox"/>	1537	2	Y2624, mutagenized strain	Y2624	Bioreactor	2009-06-19 10:32:14	6.80	5.00%	MAPF2.0
<input type="checkbox"/>	1537	3	Y2666, a consolidated strain with ndt80	Y2666	Bioreactor	2009-06-19 10:58:48	9.70	6.30%	MAPF2.0
<input type="checkbox"/>	1537	4	Y2669, a consolidated strain (ura3 ^A of Y	Y2669	Bioreactor	2009-06-19 10:59:48	6.40	6.60%	MAPF2.0
<input type="checkbox"/>	1516	1	2nd run, yielded 8.2% in H1493	Y2445	Bioreactor	2009-06-05 10:27:28	12.10	8.40%	MAPF2.0
<input type="checkbox"/>	1516	2	2nd run, yielded 8.3% in H1493	Y2446	Bioreactor	2009-06-05 10:29:21	12.90	8.80%	MAPF2.0, meio
<input type="checkbox"/>	1516	5	industrial strain in Y728 background	Y2591	Bioreactor	2009-06-05 10:18:49	8.30	6.40%	MAPF2.0
<input type="checkbox"/>	1516	6	industrial strain in CAT-1 background	Y2594	Bioreactor	2009-06-05 10:29:57	2.60	3.40%	MAPF2.0
<input type="checkbox"/>	1500	2	control Fene strain	Y1893	Bioreactor	2009-06-02 11:06:31	8.40	6.10%	MAPF2.0
<input type="checkbox"/>	1500	4	mutant of Y2050, 1st run	Y2469	Bioreactor	2009-06-02 11:10:10	6.70	5.00%	MAPF2.0

These index “grids” are customizable per user (if you are signed in to the application). You can click the “columns” button at the top of each grid to choose the data columns that are interesting to you. You can shrink or expand the column width by dragging the sides of the header cells, drag and drop columns to change the order and stretch or shrink the entire grid as well as choose the number of results you like to see on each page. Any changes you make are sticky-the system will remember them until you change them to something else.

From the indexes you can double click on a row to see the more detailed view of that experiment or run (or right click to open that page in a new tab/window).

To create your own experiment, click on the “New Experiment” button at the top of the Experiment Index. You’ll get a form asking you to specify the hypothesis/rational for your experiment, and a few other details including the department and project associated with the activity, as well as the other staff

involved with the experiment (basically the other people who might need to edit/add data to the experiment). Once the form is submitted, you'll have a hermes experiment #. You can go back to that page, choose "edit" from the left hand navigation box to change/add details, or you can add notes and attachments at the bottom of the page.

If you are doing flask or bioreactor experiments, you can add runs to the experiment (one run per bioreactor or per set of replicates for flasks). If you are planning to have analytical chemistry assay your fermentations, you'll need to provide them with a hermes experiment number and run number for each sample, and the data will automatically get added to the experiment as soon as it's ready. Otherwise you can load data directly using the "import/export" link in the left navigation box of each experiment page. This will give you an excel spreadsheet that you can import any existing data into, add your new data and click a button (embedded in the spreadsheet) to have it uploaded to the experiment. If you need to do this and haven't been trained, it probably is best to get a walk through first, either from someone in your department who's done it before, or from Chris Dolan (dolan@amyris.com), Shawn Lobosco (lobosco@amyris.com) or Matt Ward (ward@amyris.com).

5.2 Strain (Kirsten and Candace)

Reasons to Enter Strains Correctly in Database

1. Don't waste time of other genetic engineers
 - A. People might use your strains as host strains for further modifications
 - B. They need to be able to find info in public database, rather than pestering you or looking in your notebook
2. Don't waste time of Jim, Katrin, and Chris Ring
 - A. Strains might be sent to Brasil, for which accurate customs forms describing genotype are needed
 - B. Strains might be submitted for GMM approval
 - C. Strains might be included in patent application
3. Permit powerful new pattern-finding data mining approaches
 - A. K2Y is developing approaches to pull data from Strain, Hermes, and Argus databases, which can be combined with data from your own spreadsheets
 - B. We'll be able to ask questions like "Is genetic element X correlated with strain behavior Y?" or to find unexpected correlations between any genetic element and any strain behavior feature

 Strain Database
AMYRIS

Advanced Search [\(?\)](#)

Welcome To Strain!

What's New:

- January 2010
 - New warning messages for improper parent strain relationships.
 - Easier to determine strain Banked/UnBanked status.
- December 2009
 - New 'Derived By' Options.
 - Required Fields changed.
 - New 'Add Daughter' option.
- Aug 2009
 - Bulk edit Strains, Integrations and Plasmids.
 - Bulk copy Strains, Integrations and Plasmids.
- July 23, 2009
 - Text filter Genetic Element list when creating/editing Integrations and Plasmids.
- July 17, 2009
 - Create a new host Ecoli Strain directly from an existing Plasmid.
 - Convert Strain Tree view into an image, or an editable drawing.
 - Improved full text search functionality.
 - New fields on list views (Parents and Children).
- Jan 29, 2009
 - Added Isoprene to available project list

Reminders:

- Did you know that you can automatically import your genetic elements for new integrations by uploading your CloneManager file in the GeneBank file.
- Can't find a particular item in a pull down list? Check your project filter under [Preferences](#).
- Something not working right? Let us know! Use the comment submission form at the bottom, or [email us](#).

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Fields in Strain- Top

Description--
key features of this strain?
Entry in this field should enable someone to find a strain they are seeking

Is Strain Banked-- now in more convenient location!

Y3385 View

Strain ID ⓘ	Y3385	Date Added	Sep 2, 2009 by Hanxiao Jiang
Description	Y3284 (ura3 ⁻ of Y3000) with PGAL7-zmPDC_PHXT3-ACS2@NDT80	Developers	Hanxiao Jiang Kirsten Benjamin
Strain Purpose	Production	Strain Background ⓘ	CEN.PK2
Project	Fuels - Diesel - yeast	Notebooks	336:152-155
Physical Status ⓘ	Banked View Locations	Who designed it? Who actually made it?	
Species	Saccharomyces cerevisiae	Isolates ⓘ	
Mating Type	@	Chromosome II Disomy	
Parents ⓘ View Tree	Y3284	Children ⓘ	Y3443 (Via Rational) Y3510 (Via Rational) Y3547 (Via Mutagenesis) Y3557 (Via Mutagenesis) Y3558 (Via Mutagenesis) Y3559 (Via Mutagenesis) Y3560 (Via Mutagenesis) Y3571 (Via Mutagenesis) Y3572 (Via Mutagenesis) Y3573 (Via Mutagenesis)
Source Well ⓘ		Derived Via ⓘ	Rational
			New Derived by box

Species and Background are linked!

PHENOTYPE:
Someone should be able to look at this field and know what plate to grow these cells on and whether the strain is Gal+ or Gal-

To make it easier to assess strain medium requirements at a glance, we decided not to include prototrophies (e.g., Leu+) and sensitivities (e.g., HygS), but only to include auxotrophies (e.g., Leu-) and resistances (e.g., HygR)

Plasmids pAM00404

Selection Markers ⓘ
Via Integrations:
HISMX
natA
kanA
hphA
URA3
Via Plasmids:
leu2-d
Amp

Mutations ⓘ
Verified By ⓘ

Amyris' Nomenclature ⓘ
Matalpha-hphA_Zpartial-TAF2partial: NDT80 upstream-URA3-PHXT3-ACS2-TPGK1-PGAL7-Z_mobilis_PDS_hphA_PTDH3-THL-Sce-tAHP1/HCCW1_HMG5-Bju-PYPD1/PTUB2THL-Cac_HXT3 US (^HXT3) : URA3 downstream (^URA3) ; GAL80 upstream_kenA_PGAL1-MK-Sce_GA80 downstream (^GAL80) ; ERG3 truncCDS_HISMX_PGAL1-PMD-Sce_PGAL10-PMK-Sce_Leu2 downstream; HIS3 upstream_HISMX_PGAL1-MK-Sce_PGAL10-PDK1-ERG3 upstream_PGAL1-HMGR-Sce_PGAL10-ID1-Sce_PADE1-ADE1 CDS; TRP1 upstream_PGAL1-HMGR-Sce_PGAL10-FPS-Sce_PT CYC1; (^GAL80 ^URA3 ^HXT3 ^NDT80)

Genotype ⓘ
[Expand/Collapse All](#)
+ Integration: i00476

Does integration include mutation!

Who designed it?
Who actually made it?

New Derived by box

Aliases: Names that this strain is known by outside of Amyris (e.g., "W303 wild-type" or "ATCC #5")

Aliases: Names that this strain is known by outside of Amyris (e.g., "W303 wild-type" or "ATCC #5")

New Information for Strains!

Primer on Yeast Community Conventions

- Genotype (DNA status)
 - Wild-type functional allele: *URA3* or *TRP1*
 - Mutant allele, general name: *ura3* or *trp1*
 - Mutant allele, specific name: *ura3-52* or *trp1-352* or *ura3Δ* (deletion, see below)
 - Deletion of gene: *ura3Δ* or *trp1Δ*
 - Dominant gain-of-function allele: *UPC2-1*
- Phenotype Examples (observations, behaviors)
 - Leucine prototroph: Leu-
 - Leucine auxotroph: Leu+
 - HygromycinB resistant: HygR
 - Temperature sensitive: Ts
 - Elongated bud
 - Petite
 - Peanut shmoo
 - Checkpoint defective
 - Crabtree positive

Primer on Yeast Community Conventions

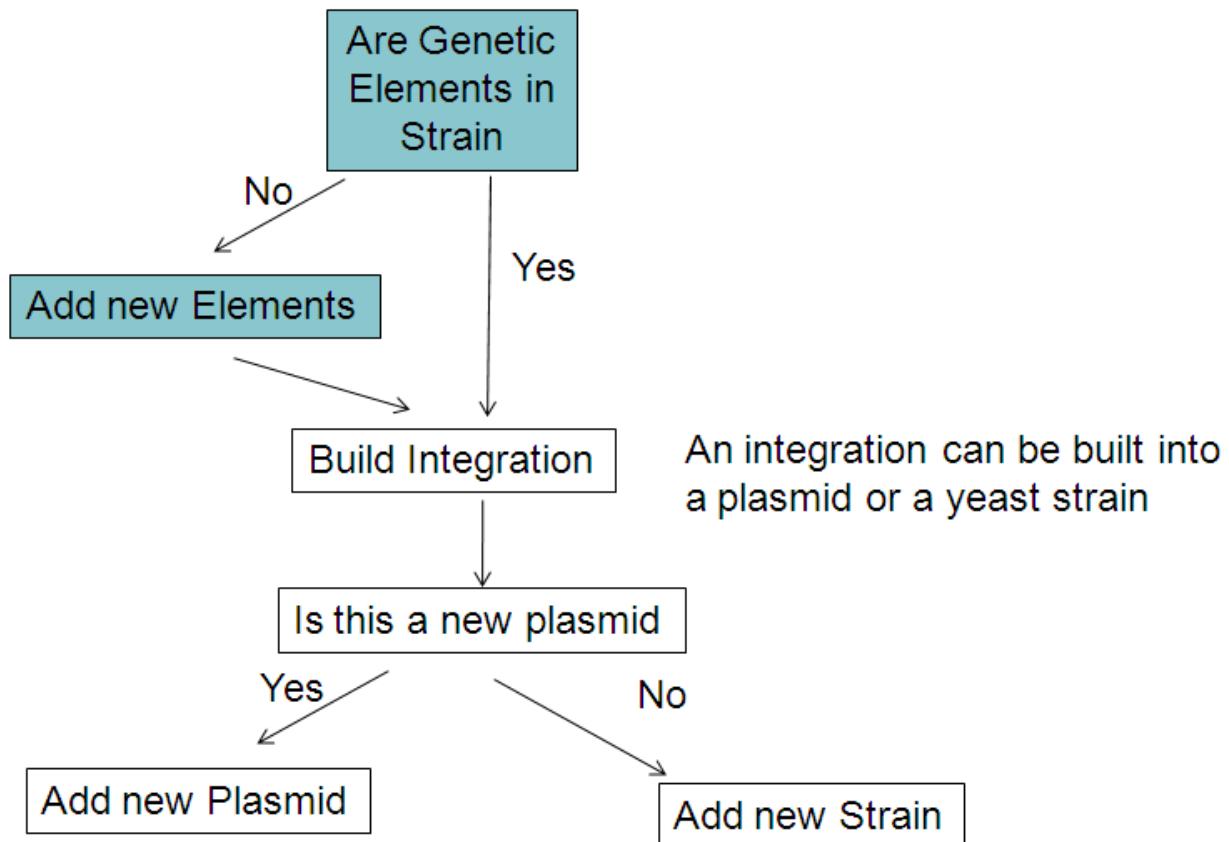
- In Phenotypes Box, we want to focus on phenotypes that describe cell growth or media requirement

Phenotype	What it means	Genotype
Leu+	Can grow in the absence of external Leucine in medium	LEU1 LEU2 etc.
Leu-	Cannot grow without added Leucine	leu1 or leu2 or...
His-	Cannot grow without added Histidine	his3 or his4 or his7 or...
Gal+	Can eat galactose	GAL1 GAL10 GAL7
Gal-	Cannot eat galactose	e.g., gal1Δ
Ts	Temperature sensitive (needs to grow at 25°)	Any one of hundreds of mutant alleles of genes
KanR	Resistant to G418	kanA or kanMX or kanB or...
HygR	Resistant to hygromycin B	hphA or hphB or...
NatR	Resistant to nourseothricin	natA or natB or...
Dser+	Can grow on D-serine as sole nitrogen source	dsdA or dsdB or...

Amyris Modification of Yeast Community Conventions

- The “Δ” symbol for deletion is replaced by ^ (shift-6)
- Strain entries show the final integration present not the path to arrive at the strain.
- A functional unit like PGAL3-CPR-TPGK1 contains dashes (-) between elements, and functional units are separated from each other with underscores (_)

Workflow-Adding a Yeast Strain



Genetic Element Index: Selection Markers

- Which gene in a plasmid or integration construct enables you to select cells that have taken up the construct?

Genetic Element Index

3' Primes	5' Primes	Genes	Promoters	Selection Markers	Tags	Terminators		
Name	Lit Name		Date Added	Added By				
300bp-URA3-300bp				06/09/08	Hanxiao Jiang			
ADE1	<i>S. cerevisiae</i> ADE1		05/07/08	Patrick Westfall				
Amp	<i>Bla</i>		04/16/08	Tom Treynor				
Broken_hyg_marker			07/06/09	Jed Dean				
can1	canavanine		08/21/08	Gordon Dang				
CHX	cyclohexamide		08/12/08	Gordon Dang				
Cml	<i>Cat</i>		04/17/08	Youngnyun Kim				
Crb	Carbenicillin		05/08/09	Tina Mahatdejkul				
dsdA	<i>K.lactis</i> PTEF-dsdA- <i>K.lactis</i> TTEF (D-serine utilization)		05/05/08	Kirsten Benjamin				
dsdB			05/20/08	Kirsten Benjamin				
frt-kan-frt			10/01/08	Tina Mahatdejkul				
HIS3	<i>S.cerevisiae</i> HIS3		05/07/08	Patrick Westfall				
HIS3_C. glabrata			07/02/08	Annie Tsong				
HISMX	PMX_5_kluyveri HIS3_TMX		01/15/09	Patrick Westfall				
hphA	<i>K.lactis</i> PTEF-hph- <i>K.lactis</i> TTEF (Hygromycin B resistance)		04/16/08	Patrick Westfall				
hphB			05/20/08	Kirsten Benjamin				
hphD			09/24/08	Victor Holmes				
...				

Genetic Element Index: Selection Markers

- Several Markers (genotypic cassettes) can confer resistance to G418:

kanA	K.lactisPTEF-kanR-K.lactisTTEF (G418 resistance)	12/08/08	Patrick Westfall
kanB		05/20/08	Kirsten Benjamin
kanD	kanD	10/23/08	Gordon Dang
kanMX	PMX-kanMX-TMX (G418 resistance)	04/16/08	Patrick Westfall

kanMX (at ERG9 locus in Y211) $P_{TEF1(Ashbya\ gossypii)}$ Tn903knar $T_{TEF1(Ashbya\ gossypii)}$

kanA (from pAM555) $P_{TEF1(K.\ lactis)}$ Tn903knar $T_{TEF1(K.\ lactis)}$

kanB $P_{TEF1(K.\ lactis)}$ Tn903knar (No T, best for simple deletions, use T in chromosomal destination from KO gene, to reduce integration of cassette at other markers in same strain)
Etc.

Jim's new markers (our favorite drug resistance markers with new Promoters and Terminators will be given names like kanE, kanF, etc.

Genetic Element Index: Genes

- Convention is to name genes in pathway with universal name (e.g., FPS) followed by species name (e.g., Eco or Sce). In “Lit Name” (Literature Name) will go the gene name according to literature and outside databases (e.g., ERG20)

Genetic Element Index

3' Primes	5' Primes	Genes	Promoters	Selection Markers	Tags	Terminators
		Name		Lit Name	Date Added	Added By
2094 (<i>C. kluyveri</i> ADA)					05/08/09	Annie Tsong
2095 (<i>E. coli</i> EutE)					05/08/09	Annie Tsong
2098 (<i>S. typhimurium</i> EutE)					05/08/09	Annie Tsong
AaADH1		ADH1			06/05/08	Patrick Westfall
AaALDH1					06/20/08	Andrew Main
AaCBR1		CBR1 from Aan			06/20/08	Ken Zahn
AaCYB5					06/20/08	Ken Zahn
AaDBR2					07/21/08	Anna Tai
ACAT1					03/09/09	Gale Wichmann
ACC1		Acetyl-CoA carboxylase			08/29/08	Victor Holmes
aceE		aceE			05/19/08	Annie Tsong
aceF		aceF			05/19/08	Annie Tsong
ACh1		acetyl-CoA hydrolase			08/08/08	Victor Holmes
ackA		ack			05/01/08	Tina Mahatdejkul
ACS (<i>E. coli</i>)					02/04/09	Youngnyun Kim
ACS (<i>S. enterica</i>)					02/18/09	Youngnyun Kim
ACS(L641P) <i>S. enterica</i>					02/04/09	Youngnyun Kim
ACS1					04/16/08	William Chit-Maung
ACS2		Sce Acetyl-Coa Synthetase			03/20/09	Victor Holmes
...

Genetic Element Index: Promoters

- “P” for promoter followed immediately by gene from which promoter was taken (e.g., PGAL1), and then any modifying description (e.g., oc or fixed)

Genetic Element Index

3'	3' Primes	5'	5' Primes	Genes	Promoters	Selection Markers	Tags	Terminators
Name			Lit Name		Date Added	Added By		
3prime truncated gal10 promoter					08/29/08	Raymond Lowe		
3prime truncatedPgal1					08/29/08	Raymond Lowe		
P(CTR3)*					04/30/09	Patrick Westfall		
P10%T7	P10%T7				05/20/08	Sunil Chandran		
pACS2					07/28/08	Annie Tsong		
pADH1					07/29/09	Jed Dean		
PADH2	ADH2 promoter				04/26/08	Patrick Westfall		
PCTR3	CTR3 promoter				04/16/08	Patrick Westfall		
PCTR3*					06/02/09	Patrick Westfall		
PCTR3m2	CTR3 promoter with second mac1 site				08/25/08	Victor Holmes		
PCTR3m3	CTR3 promoter with third mac1 site				09/03/08	Victor Holmes		
PCUP1	CUP1 promoter				04/29/08	Patrick Westfall		

Genetic Element Index: Terminators

- Put “T” for promoter followed immediately by gene from which promoter was taken (e.g., TADH1).
- When describing an Integration or cassette that contains a yeast gene followed by its own terminator, it is not necessary to explicitly add the terminator (e.g., sufficient to write ERG20, not necessary to write ERG20-TERG20)

Genetic Element Index

3'	3' Primes	5'	5' Primes	Genes	Promoters	Selection Markers	Tags	T	Terminators
Name				Lit Name		Date Added			Added By
CL1-degron						11/11/08			Andrew Main
F-Cph1 RE site						12/16/09			Gale Wichmann
FLAG						02/17/09			Gale Wichmann
FLAG-HIS						05/08/09			Yoseph Tsegaye
FLAG-myc-His						04/22/08			Rekha Nambudiri
GFP						04/23/08			Gordon Dang
His-6				His-6		07/07/08			Sunil Chandran
His-FLAG						04/22/08			Rekha Nambudiri
MTS (0)				MTS from Su9 - Original sequence		01/29/09			Gale Wichmann
MTS (1)				MTS from Su9 - DNA variant #1		01/29/09			Gale Wichmann
MTS (2)				MTS from Su9 - DNA Variant #2		01/29/09			Gale Wichmann
MTS (3)				MTS from Su9 - DNA variant #3		01/29/09			Gale Wichmann
mvs						04/30/08			Rekha Nambudiri
PEST				CLN2 C- terminus		04/29/08			Patrick Westfall
T7				T7 Gene 10 epitope		07/28/08			Arle Kruckeberg
URA3 Loopout Sequence 1						09/25/08			Victor Holmes
URA3 loopout sequence 3					→	09/25/08			Victor Holmes

Genetic Element Index: 5' Targeting Sequence (directing homologous recombination)

- First, indicate gene whose sequence or surrounding sequence is relevant
- Second, tell whether the sequence used was
 - “upstream” or “US” of the ATG of the gene
 - “CDS”=coding sequence of the gene
 - “truncCDS” (truncated version of CDS)
 - “T”=terminator of the gene

Genetic Element Index

3 Primes	5 Primes	Genes	Promoters	Selection Markers	Tags	Terminators
5' HO		HO 5' homology		03/03/09	Yandi Dharmadi	
5' IME2		Sc IME2		05/13/09	Kristy Hawkins	
ACC1 upstream	ACC1 upstream			08/29/08	Victor Holmes	
ACH1 Upstream				08/08/08	Victor Holmes	
ACS1US	ACS1 upstream			09/22/08	Annie Tsong	
ACS2 upstream				06/23/08	Gale Wichmann	
ADE1 upstream				05/07/08	Patrick Westfall	
ADE2 US				11/05/09	Hanxiao Jiang	
ADH1 upstream				05/28/08	Rekha Nambudiri	
ADH6 upstream				06/09/08	Hanxiao Jiang	
ALD4US	ALD4 upstream			10/14/08	Annie Tsong	
ALD4 F�4US	ALD4 F�4US			08/11/08	Annie Tsong	

Genetic Element Index: 3' Targeting Sequence (directing homologous recombination)

- **First, indicate gene whose sequence or surrounding sequence is relevant**
- **Second, tell whether the sequence used was**
 - “downstream” or “DS” of the TGA/TAA/TAG stop codon of the gene without regard for the functionality of the terminator
 - “CDS”=coding sequence of the gene
 - “truncCDS” (truncated version of CDS)
 - “P”=promoter of the gene

Genetic Element Index

3' 3 Primes	5' 5 Primes	Genes	Promoters	Selection Markers	Tags	Terminators
Name		Lit Name		Date Added		Added By
3' HO		HO 3' homology		03/03/09		Yandi Dharmadi
3' IME2		3' Sc IME2		05/13/09		Kristy Hawkins
ACH1 3_prime		Upstream of ACH1		01/28/09		Victor Holmes
ACS1DS		ACS1 downstream		09/22/08		Annie Tsong
ACS2 downstream				06/23/08		Gale Wichmann
ADE2 DS				11/05/09		Hanxiao Jiang
ADH1 downstream				05/28/08		Rekha Nambudiri
ADH5		ADH5		01/05/10		Yoseph Tsegaye
ADH6 3_prime				06/09/08		Hanxiao Jiang
ALD4DS		ALD4 downstream		10/14/08		Annie Tsong
ALD6 3_prime		ALD6 3 prime		08/11/08		Annie Tsong
AOX				08/27/09		Youngnyun Kim

Create Genetic Elements

AMYRIS

Main 0

- Strains
 - Yeast
 - E. coli
- Plasmids
- Integrations
- Genetic Elements
- Metabolism
- Storage (7)
- Strain Backgrounds
- Reports (7)
- Preferences (7)
- Help

Actions 0

[Add Element](#)

Recent Items 0

- V3883
- V3852
- V3854
- V3856
- V3851
- V2821
- V2339

Genetic Element Index

Name	Lit Name	Date Added	Added By
2094 (C. kluyveri ADA)		05/08/09	Annie Tsong
2095 (E. coli EutE)		05/08/09	Annie Tsong
2098 (S. typhimurium EutE)		05/08/09	Annie Tsong
AaADH1	ADH1	08/05/08	Patrick Westfall
AaALDH1		06/20/08	Andrew Main
AaCBR1	CBR1 from Aan	06/20/08	Ken Zahn
AaCYB5		06/20/08	Ken Zahn
AaCBR2		07/21/08	Anna Tai
ACAT1		03/09/09	Gale Wlichmann
ACCoA	Acetyl-CoA carboxylase	08/29/08	Victor Holmes
aceE	aceE	05/19/08	Annie Tsong
aceF	aceF	05/19/08	Annie Tsong
ACH1	acetyl-CoA hydrolase	08/08/08	Victor Holmes

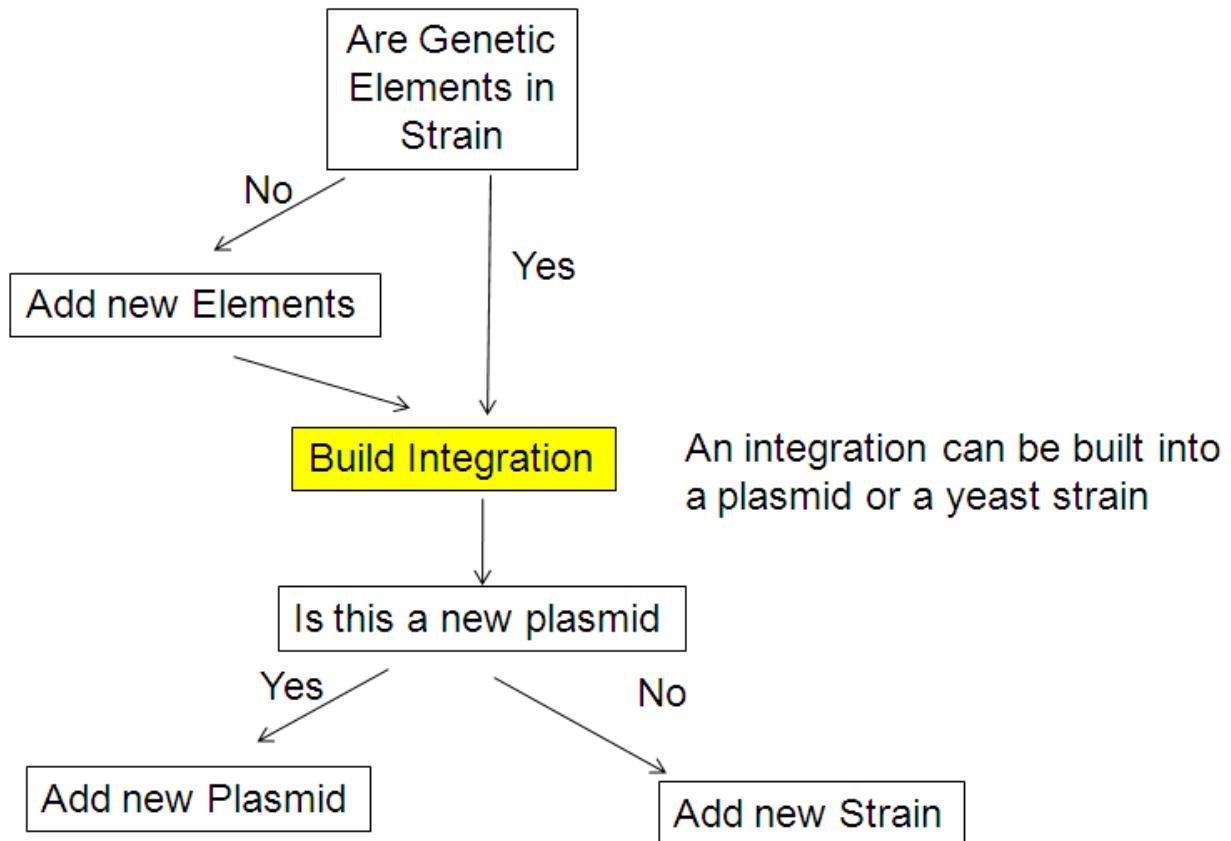
Genetic Elements with Mutations

New Genetic Element

<input type="button" value="Cancel"/>	<input type="button" value="Done"/>
Element Name	<input type="text" value="FS EG-6"/>
Literature Name	<input type="text"/>
Element Description	<input type="text"/>
Element Type	<input type="text" value="Gene"/> <input type="button" value="▼"/>
Clone Manager File	<input type="text"/> <input type="button" value="Browse..."/>
This is a mutation of	<input type="text" value="FS-Aan Farnesene synth"/> <input type="button" value="▼"/>
Associated Phenotypes	<input type="text" value="New:"/> <input type="button" value="▼"/>
Applicable To Yeast	<input checked="" type="radio"/> Yes <input type="radio"/> No
Applicable To EColi	<input checked="" type="radio"/> Yes <input type="radio"/> No

Enter in mutation-
will automatically
populate
Mutations tab of a
strain when used
in an integration

Workflow-Adding a Yeast Strain



Naming the Integration Cassette

Example of preferred naming scheme:

ndt80^::PHXT3-ACS2_URA3_PGAL7-ZmPDC

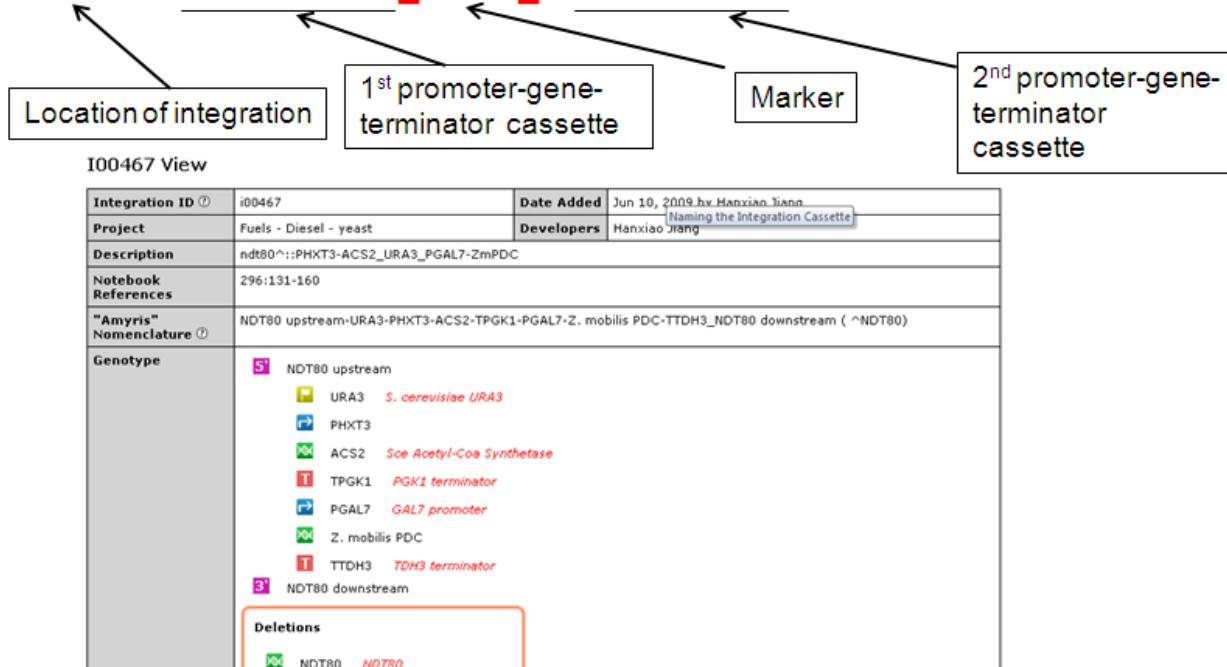


I00467 View

Integration ID	i00467	Date Added	Jun 10, 2009 by Hanxiao Jiang Naming the Integration Cassette
Project	Fuels - Diesel - yeast	Developers	Hanxiao Jiang
Description	ndt80^::PHXT3-ACS2_URA3_PGAL7-ZmPDC		
Notebook References	296:131-160		
"Amyris" Nomenclature	NDT80 upstream-URA3-PHXT3-ACS2-TPGK1-PGAL7-Z. mobilis PDC-TTDH3_NDT80 downstream (^NDT80)		
Genotype	<p>5' NDT80 upstream</p> <ul style="list-style-type: none">URA3 <i>S. cerevisiae URA3</i>PHXT3ACS2 <i>Sce Acetyl-Coa Synthetase</i>TPGK1 <i>PGK1 terminator</i>PGAL7 <i>GAL7 promoter</i>Z. mobilis PDCTTDH3 <i>TDH3 terminator</i> <p>3' NDT80 downstream</p> <p>Deletions</p> <ul style="list-style-type: none">NDT80 NDT80		

Example of preferred naming scheme:

ndt80^::PHXT3-ACS2_URA3_PGAL7-ZmPDC



Example of preferred naming scheme:

ndt80^::PHXT3-ACS2_URA3_PGAL7-ZmPDC



These descriptions are not as informative:

i00674 Plan XX Megastitch #3554. (Stitches 269, 54)

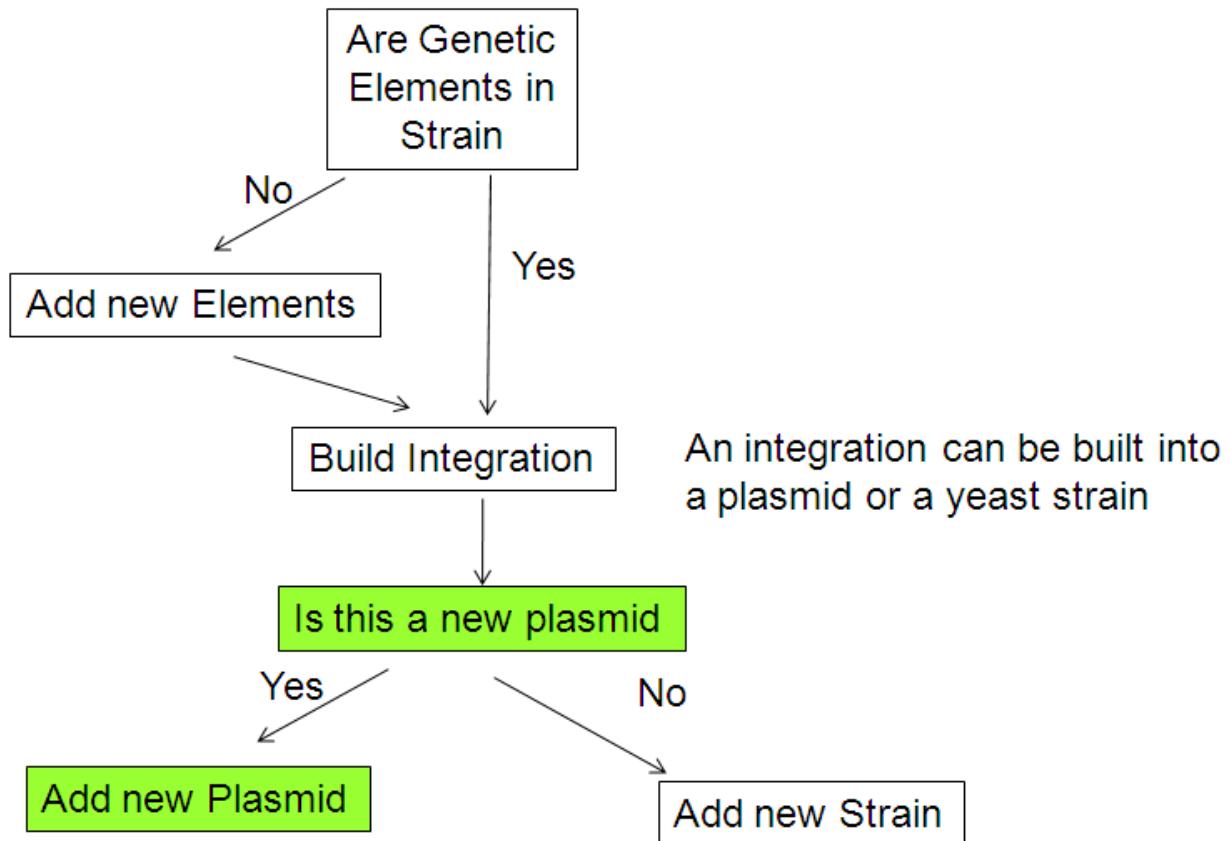
i00642 MevT AssA construct integrated into HXT3 with ura loop out



This would be more informative:

hxt3^::URA3 (loop out)_PGAL10-HMGR-Sau-TCYC1_PGAL1-ERG10-TADH1_PGAL2-ERG13-TPGK1_PGAL7-HMGR-Sau-TCYC1

Workflow-Adding a Yeast Strain

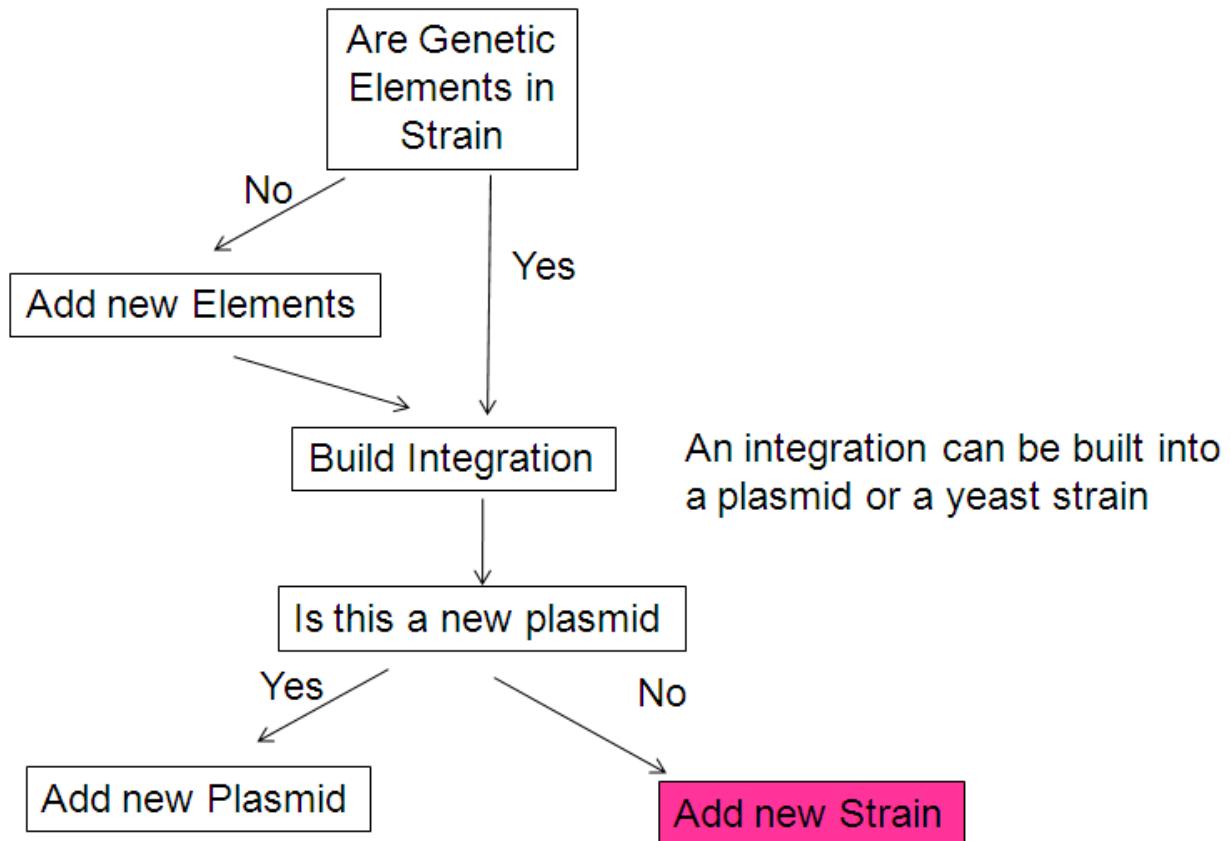


Adding a Plasmid

Add parent backbone adds correct markers

Plasmid Description			
Project	<input type="button" value="New"/> ▾		
Genetic Elements <small>?</small>	Element Tree <small>?</small> <input checked="" type="checkbox"/> Add to Root <small>?</small> TADH1 <small>ADH1 terminator</small> PGAL10 <small>GAL10 promoter</small> PGAL1 <small>GAL1 promoter</small> TCYC1 <small>CYC1 terminator</small> leu2-d <small>leu2-d</small> Trash <small>?</small>	Select Element <small>?</small> Filter: <input type="text"/> <input checked="" type="checkbox"/> 3 Prime (113) <input checked="" type="checkbox"/> 5 Prime (103) <input checked="" type="checkbox"/> Gene (306) <input checked="" type="checkbox"/> Promoter (59) <input checked="" type="checkbox"/> Selection Marker (36) <input checked="" type="checkbox"/> Tag (17) <input checked="" type="checkbox"/> Terminator (35)	For more information on how to use the drag and drop, watch this MoT video!
Plasmid Image <small>?</small>	<input type="button" value="Browse"/>	Clone Manager File <small>?</small> <input type="button" value="Browse"/> <small>(Allowed file types: *.gbk)</small>	
Developers	Begin typing to filter list Abhishek Murarka Adam Meadows Alison Russo Amrik Kar Ami Shukla	Included Integrations <small>?</small> Begin typing to filter list 672 /00671 - Plan XX Megastitch #6577 (Stitches /00670 - Plan XX Megastitch #6714 (Stitches /00689 - Plan XX Megastitch #6889 (Stitches /00688 - Plan XX Megastitch #6851 (Stitches	
Notebook References	New: Notebook = <input type="text"/> Page(s) <input type="text"/>	Parents <small>?</small> pAM00178 <small>delete</small> Begin typing to filter list pAM02012 - Lead Generation Stitch USBIC pAM02011 - PS Mutant Gen3-2 with pGai1 p pAM02010 - PS Mutant Gen3-1 with pGai1 p pAM02009 - PS Mutant EG-6 with pGai1 pro pAM02008 - PGAL1-PS(modified)-TCYC1 c	
How this was made <small>?</small>			

Workflow-Adding a Yeast Strain



Adding a Strain

- Add Yeast Strain- add a new strain de novo
- Copy Strain - start with one strain and make up to 50 exact copies- NEED TO EDIT
- Add Daughter- generates daughter strain from a specific parent- still need to add information
- Add any new genetic elements (not already in database)
- Add any new Integrations
- Add any new plasmids
- Add new yeast strain
 - Add a description
 - Add notebook/page number
 - Add Developers
 - Select Project
 - Select “Derived by”
 - Select Parent-
 - *Click on the Reload button of your browser. This will add the integrations, phenotype, selection markers, mating type etc from parent*
 - BE SURE and modify or delete incorrect information
 - Add new insertions, phenotype etc
- Hit “Save”

Add a New Strain from a Parent

The screenshot shows the 'New Strain' form with various fields and dropdown menus. Annotations are present:

- a**: Points to the 'Description' field.
- b**: Points to the 'Notebook References' section.
- c**: Points to the 'Developers' dropdown menu, which lists several names.
- d**: Points to the 'Project' dropdown menu.
- e**: Points to the 'Literature References' section.
- f**: Points to the 'Parents' dropdown menu, which lists various strain entries.
- Red arrows**: Point from the 'Parents' dropdown (f) to the 'Derived Via', 'Deletions', and 'Interractions' sections, indicating that these fields are derived from the selected parent strain.

Derived By Field

- Mutagenesis- *use for strains that were mutagenized*
- Rational- *use for strains that were derived through rational engineering*
- Spore Screen- *for screens of diversity (i.e industrial screens, screens for consolidation/deconvolution)*
- Mating- *for generation of strains that are diploid*
- Meiosis- *for strains derived from meiosis that generates a specific and known genotype at end*

- Random Libraries- *for screens from libraries of rational designs, combinatorial libraries, other libraries.*
- Evolved- *for generation of strains through evolution (selection or archived “broken” strains).*
- None- (*leave bank*) no derivation- *for example parental strains or strains acquired through ATCC*
- Undetermined parentage- *for strains where a specific parent is unknown*

Alternate Route: Add a Daughter

- 1. Go to parent entry and click “Add daughter”**
- 2. Add a description**
- 3. Add notebook/page number**
- 4. Add Developers**
- 5. Select Project**
- 6. Select “Derived by”**
- 7. Update any changes to integrations, phenotype, plasmids etc.**
- 8. Hit Save**

Add a Daughter

New Strain

Required fields: Project, Description, Developer

1	Description	
2	Notebook References	New: Notebook # <input type="text"/> Page(s) <input type="text"/>
3	Aliases	<input type="text"/>
4	Strain Purpose	<input type="text"/>
5	Developers	Begin typing to filter list Abhishek Murarka Adam Meadows Alison Russo Amar Klar Amy Shutkin Lindsey Munn
6	Verified By	Begin typing to filter list Abhishek Murarka Adam Meadows Alison Russo Amar Klar Amy Shutkin Lindsey Munn
	Literature References	<input type="text"/>
	Other Info	<input type="text"/>
	Plasmids	pAM00940 delete Begin typing to filter list pAM02012 - Lead Gene, Van Stich USBIO4-UF pAM02011 - FS Mutant Gen3-2 with pGal1 prom. pAM02010 - FS Mutant Gen3-1 with pGal1 prom. pAM02009 - FS Mutant EG-6 with pGal1 promoter pAM02008 - PGAL1-P(promised)-TCY1 cloned pAM02007 - EG-6 with pGal1 promoter
	Deletions	Begin typing to filter list 2094 (C. kluwey ADA) 2095 (E. coli EutE) 2098 (S. typhimurium EutE) AaCBR1 - CBR1 from Aan CC1 - Acetyl-CoA carboxylase MCA1 - Malic aciduria
	Phenotype	KanR (G418, yeast) delete Gal+ (eats galactose) delete Leu- delete New: <input type="text"/>
	Integrations	i00211 delete i00094 delete i00008 delete i00006 delete

Copy Strain

- This creates an exact replicate of existing strain
- There is NO step to double check-Strain is automatically in database and any editing is automatically saved (beware!)
- Need to edit and correct differences in the copies.

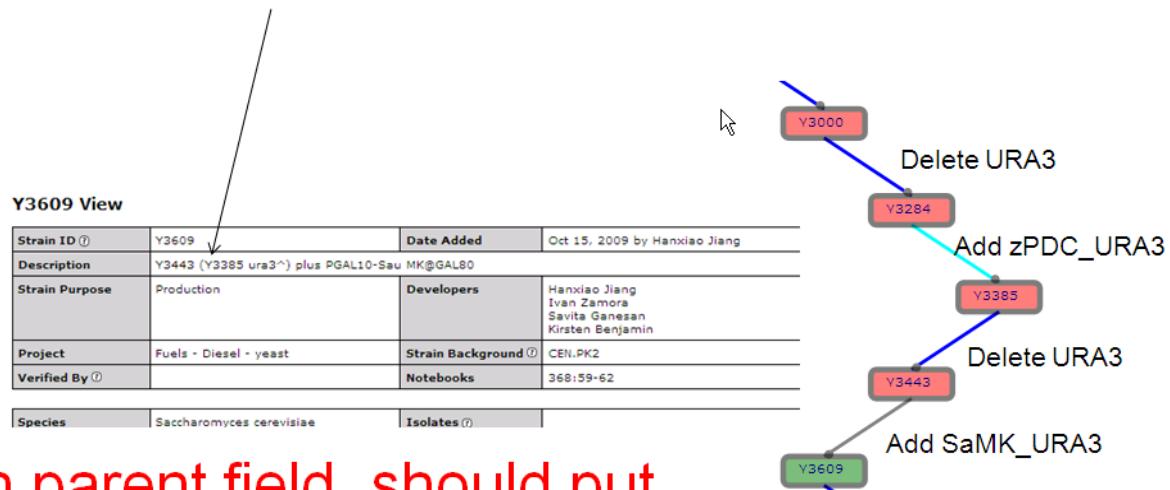
Alerts

- Organism is different from parent
- Strain background is different from parent
- Number of Parents and Mating Type Alerts:

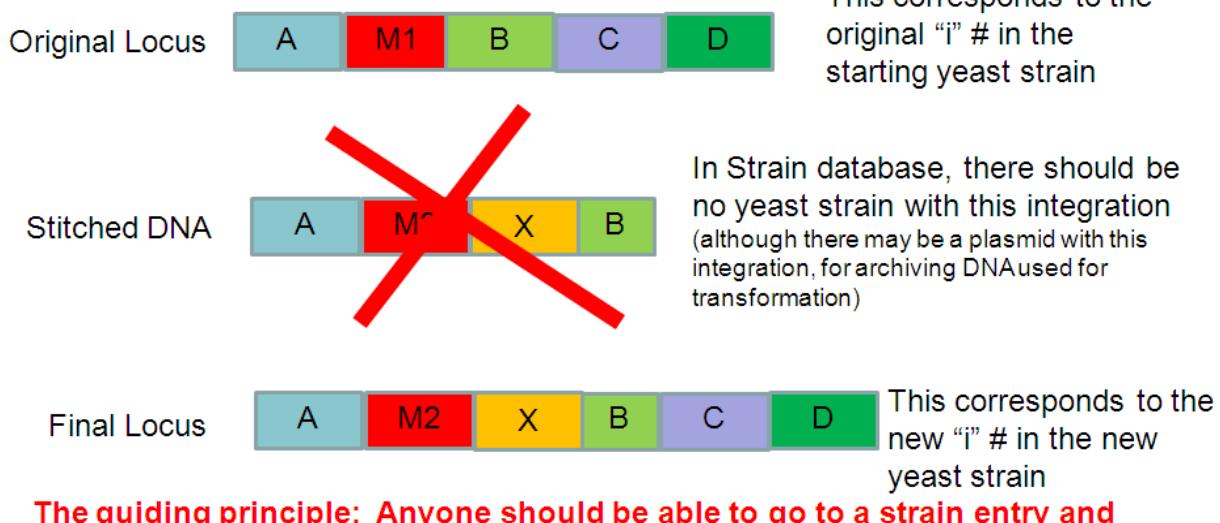
Derived By Type	Usual # parents	Alert if:
Any		3 or more
Rational	1	2 or more
Mutagenesis	1	2 or more
Spore Screen	1 parent but a/α	1 parent but NOT a/α
Spore Screen	2 parents- one a and one α	2 parents- both a or both α
Mating	2 parents one a and one α	2 parents- both a or both α OR 1 parent
Random Libraries	1 parent	2 or more
Meiosis	2 parents one a and one α	2 parents- both a or both α OR 1 parent
Evolved	1 parent	2 or more

Using Strain Correctly: Who is my parent

- Example: Y3609 description includes the parent and grandparent in description

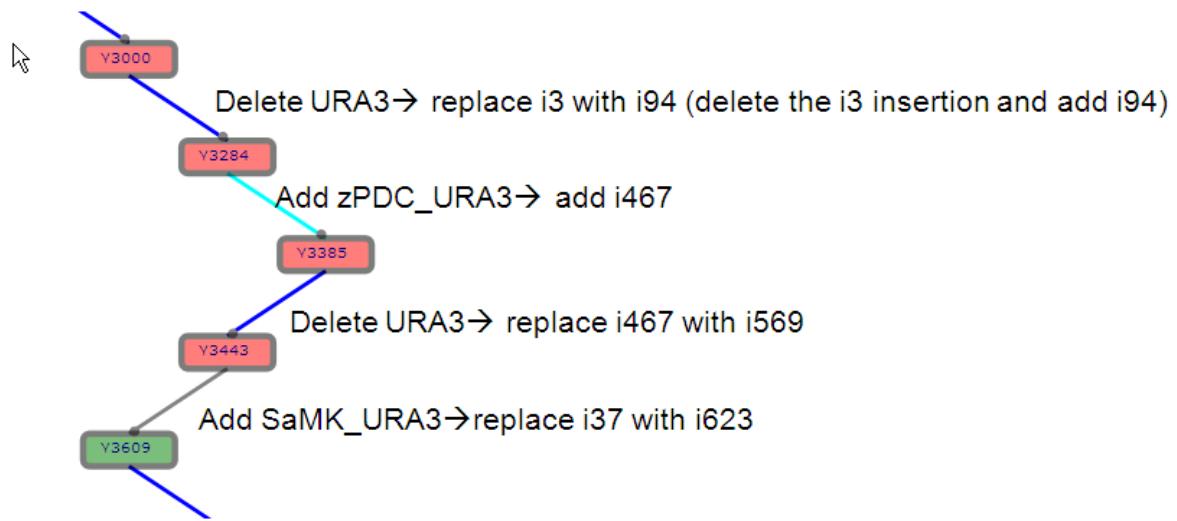


Using Strain Correctly: Integration into integration



The guiding principle: Anyone should be able to go to a strain entry and obtain the DNA sequence of each engineered locus by looking at a single integration (should not have to examine all integrations to see if 2 of them apply to the same locus, and then do CloneManager work to reconstruct what the locus looks like in the given strain).

Using Strain Correctly: Integration into integration



5.3 Thumper (Victor)

Overview

- Thumper is a set of tools for construction of RYSE-based integrations as well as a database for tracking all RYSE-associated parts.
- For all users
 - Helps search existing RABits, stitches, megastitches etc.
 - Helps create new DNA constructs including designing primers, ordering primers, generating combinatorial libraries, etc.
- Specifically for ASE,
 - Manages cycles of 1000 strains with subsets, called tricks, corresponding to individual scientists' requests.
 - Processes molecular biology data and generates robotic worklists.

Searching for Parts?

- Thumper keeps Megastitches, Stitches, and RABits.

welcome to thumper - Mozilla Firefox

File Edit View History Bookmarks Tools Help

http://thumper/ welcome to thumper ASE Issue Tracker Saccharomyces Geno... Inbox - Outlook Web ... Elm Pandora Radio Liste... welcome to thumper Automated Strain Engineering - Biology... Jump to blt: go

Active User: Victor Holmes

genotypes megastitches stitches rabbits cycles trix admin sign out

Welcome to Thumper 2.1, home of Automated Strain Engineering

Version 2.1 (Feb 10):

- Stitches and MegaStitches are now grouped into Tricks and Cycles.

Version 2.0 (Nov 09):

- Bulk upload rabbits and mules.
- Bulk upload stitches and megastitches.
- Generate Robot worklists for stitches and megastitches.
- Trac Status, Location and Volume details for rabbits, stitches and megastitches.

Version 1.1:

- Explore the Hutch
- Request a RABit or set of RABIT's
- Create a MULE which can later be transformed into a rabbit.
- Easily choose Gene's and Primers from CENPK2 & CAT1 (with more coming soon)
- Autogenerate a clone manager file for a RABIT
- Use the amazing eStitcher

If you're a member of the Gene Trix team, you can also:

- Approve a rabit for construction
- Modify/Update requests and RABIT's

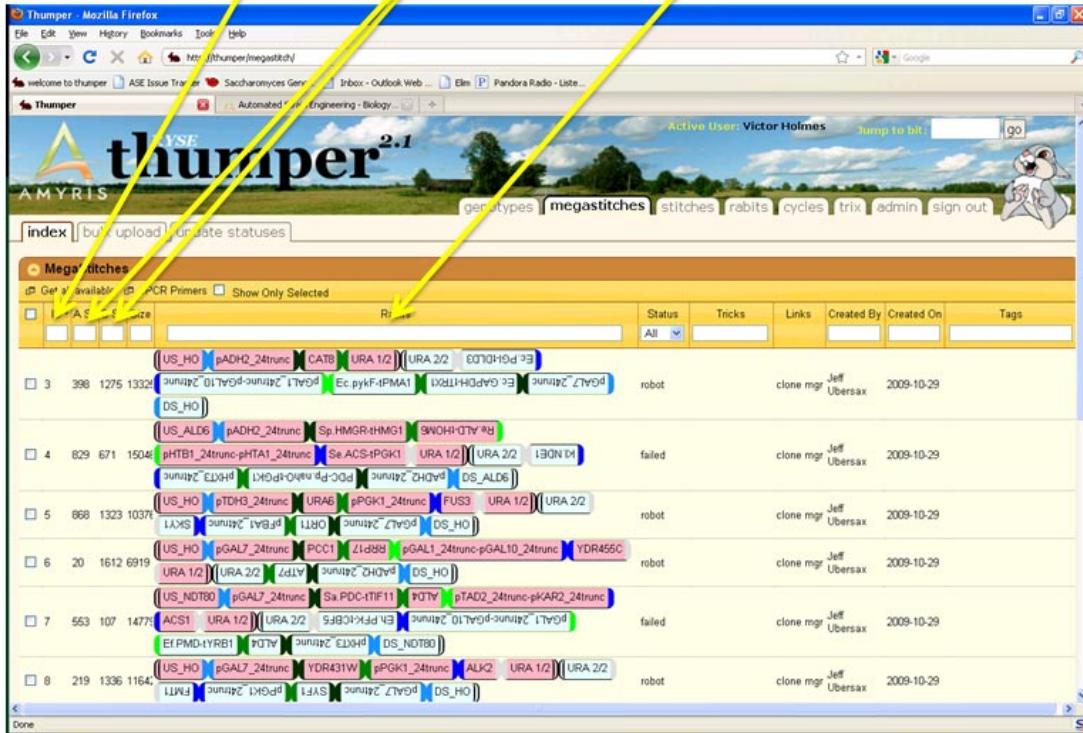
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Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please [click here](#).

Done

How can I find stitches / megastitches?

- Search by unique ID, Stitch, key word / RABit name, etc



How can I create megastitches / stitches?

- Populate a list in excel, then cut and paste into thumper.

The image contains two side-by-side screenshots of the Thumper web application interface, both titled "Bulk Upload".

Bulk Upload MegaStitches: This screenshot shows a table for inputting data for megastitches. The columns are labeled "Mega Stitch ID", "Stitch A ID", and "Stitch B ID". The data entered is:

Mega Stitch ID	Stitch A ID	Stitch B ID
1	2	
1	4	

A note below the table says: "Leave ID blank to get an ID signed, or provide it if you are changing an existing megastitch (you will only be able to change mega stitches you added)."

Bulk Upload Stitches: This screenshot shows a table for inputting data for stitches. The columns are labeled "Stitch ID", "Slot 1", "Slot 2", "Slot 3", "Slot 4", "Slot 5", "Slot 6", "Slot 7", "Slot 8", "Slot 9", and "Tags". The data entered is:

Stitch ID	Slot 1	Slot 2	Slot 3	Slot 4	Slot 5	Slot 6	Slot 7	Slot 8	Slot 9	Tags
1234	3423	2001								Lead Consolidation Round 6
234	343	2001	204							Funky Stuff, Libraries

A note below the table says: "You can provide a stitch id (and any new tags) if you want to re-use an existing stitch in your trick. If you provide a set of rabbits that already exists as a stitch, the existing stitch will be added to your trick. You can decide whether or not to restitch existing stitches when you generate the stitch robot worklist for your trick."

Below the tables, there are several footer links and notices:

- Feedback: "Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please [click here](#).
- Copyright: "© 2008 Amyris Biotechnologies™. Contents of this website are confidential and proprietary."
- Trick Selection: "Trick: ASE Cycle 004 (For Lance)"
- Report Information: "A ticket has been created for this report, you can view it [here](#). An email with information about this ticket has been sent to your email address."

..or create stitches virtually with eStitcher

- eStitcher allows plug and play assembly of library bits to make new stitches.
 - Search by keywords in “Insert Name” and build up your stitch from 0 linker to 9 linker.
 - Can add mutliple RABits for any slot to make a combinatorial library!

The screenshot shows a Mozilla Firefox browser window displaying the eStitcher interface on the Thumper website. The title bar reads "eStitcher - Thumper - Mozilla Firefox". The address bar shows the URL "http://thumper/stitch/eStitcher". The main content area is titled "eStitcher™ 2.0". At the top, there is a navigation menu with links like "genotypes", "megastitches", "stitches", "rabits", "cycles", "trix", "admin", and "sign out". Below the menu, a sub-menu for "stitches" is open, showing options like "index", "bulk upload", "combinatorial sampler", "eStitcher", "update statuses", and "bulk tag". A yellow arrow points down to the "Insert Name" search bar in the "eStitcher" sub-menu. The main search form has a checkbox "Include MULE's" checked, and a section titled "The Hutch" with various dropdown filters for "ID", "Label", "Kind", "Linker", "Direction", "Breed", "Insert Name", "Source", "Size", "Created By", "Created C", "Status", and "Votes". Below the search form, a message says "No records to view". At the bottom of the page, there is a copyright notice: "© 2008 Amyris Biotechnologies™. Contents of this website are confidential and proprietary. Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please [click here](#). Thank you very much for your feedback! A ticket has been created for this report, you can view it [here](#). An email with information about this ticket has been sent to your email address."

How can I find RABits?

- “Status” tells you where a bit is between created virtually (Mule needs similarity check) and QC’d in the freezer (RABit).
- Search by ID, Linkers, Type (breed), keyword, or creator

The screenshot shows a Mozilla Firefox browser window displaying the Thumper web application. The title bar reads "Hutch - Thumper - Mozilla Firefox". The address bar shows the URL "http://thumper.rabit/". The page header includes the Thumper logo, a user icon for "Victor Holmes", and navigation links for "genotypes", "megasatches", "stitches", "rabits" (which is highlighted in blue), "cycles", "trix", "admin", and "sign out". A yellow arrow points from the text "Right-click on a row to go to the rabbit page." to the first row of the table. Another yellow arrow points from the text "The Hutch" to the top-left of the table.

ID	Kind	Linker	Direction	Breed	Insert	Source	Size	Created By	Status	Votes	Tags
55	rabit	45	forward	Promoter	pTDH3_truncated	S. cerevisiae sp. CENPK2	559	Rekha Nambudiri	Rabit	x0 x0	
1043	mule	23	forward	Gene	tri1	S. cerevisiae sp. CENPK2	1181	Raymond Lowe	MULE Not Ordered	x0 x0	
57	rabit	56	forward	Promoter	pTDH3_truncated	S. cerevisiae sp. CENPK2	559	Rekha Nambudiri	Rabit	x0 x0	
1045	mule	23	forward	Gene	ARP9	S. cerevisiae sp. CENPK2	1990	Raymond Lowe	MULE Needs Similarity Check	x0 x0	
59	rabit	23	forward	Promoter	pENO1_full	S. cerevisiae sp. CENPK2	500	Rekha Nambudiri	Rabit	x0 x0	
1047	mule	01	reverse	3' loci (downstream)	hd3	S. cerevisiae sp. CENPK2	500	Kirsten Benjamin	MULE Not Ordered	x0 x0	
61	rabit	34	forward	Gene w/ stop	HMGR	S. aureus	1329	Rekha Nambudiri	Rabit	x0 x0	
1049	mule	45	forward	Marker	NATA	Hybrid Sc I	1456	Rekha Nambudiri	MULE Not Ordered	x0 x0	
63	rabit	23	reverse	Terminator	ITDH3	S. cerevisiae sp. CENPK2	263	Rekha Nambudiri	Rabit	x0 x0	
1051	mule	29	forward	3' loci (downstream)	3'NQMI	S. cerevisiae sp. CENPK2	500	Timothy Gardner	MULE In House	x0 x0	
65	rabit	67	forward	Terminator	ITDH3	S. cerevisiae sp. CENPK2	263	Rekha Nambudiri	Rabit	x0 x0	

Create Tags for easy searching

- Put any tag on any stitches or RABits for easy searching or to guide future users

The screenshot shows two windows of the Thumper web application:

- Bulk Upload Stitch Tags:** A form where users can input 'Stitch ID' and 'Tag Label 1', 'Tag Label 2', etc. A yellow arrow points from the explanatory text to the 'Tags' input field.
- The Hatch:** A list of RABits with columns for ID, Kind, Linker, Direction, Breed, Insert, Source, Size, Created By, Status, Votes, and Tags. A yellow arrow points from the explanatory text to the 'Tags' column.

Both windows include a 'Tags' column where users can enter tags like 'ase 006'.

Generate Shopping Lists of Parts

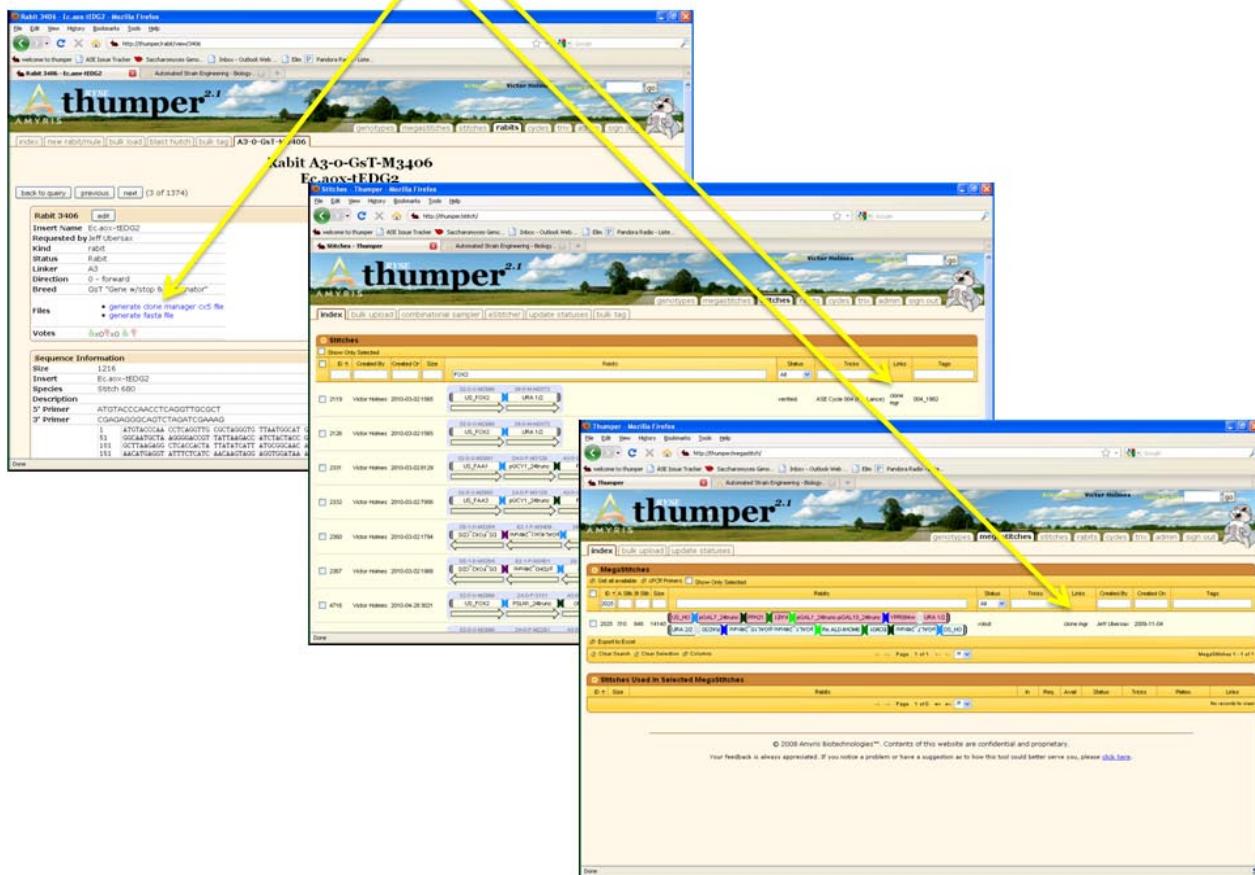
After you search for and select any number of megastitches or stitches,

The screenshot shows a Mozilla Firefox browser window displaying the Thumper web application. The URL is <http://thumper/megastitch>. The page title is "Thumper". The navigation bar includes links for "index", "bulk upload", and "update statuses". The main content area has tabs for "genotypes", "megastitches", "stitches", "rabits", "cycles", "trix", "admin", and "sign out". The "megastitches" tab is active, showing a table titled "Megastitches (4 selected)". The table lists four rows of data, each representing a megastitch with various components and status information. Below this table is a link to "Export to Excel". The "stitches" tab is also visible, showing a table titled "Stitches Used In Selected Megastitches". This table lists six rows of data, each representing a stitch used in the selected megastitches, with columns for ID, Part, Quantity (In), Required Quantity (Req), Availability (Avail), Status, Tricks, Plates, and Links. A yellow arrow points from the top table to the bottom table.

the bottom of the screen shows all the parts you'll need and how much of each.

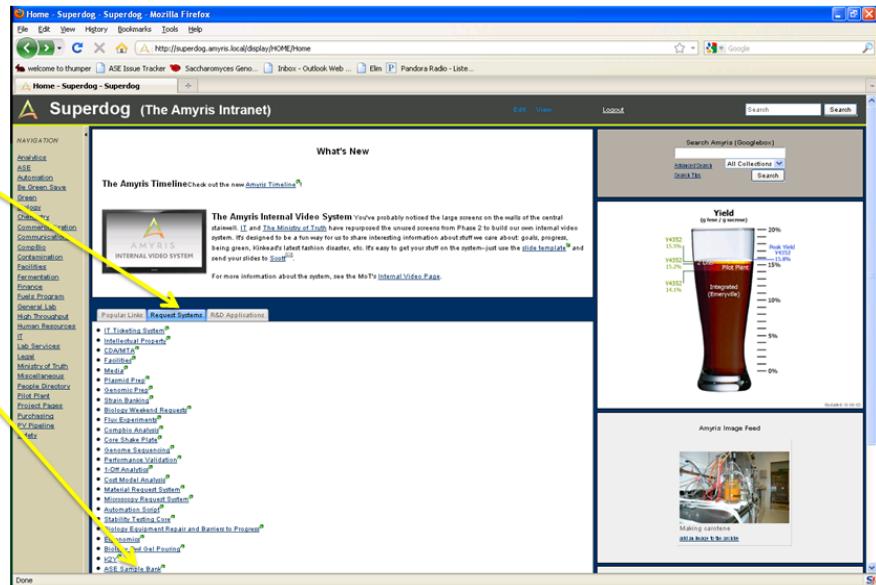
One-click Clone Manager Files

- Clone Manager files are downloadable for all DNAs



Request Parts on ASE page

- You can submit a request for cloned RABits or Stitches under Request Systems / ASE Sample Bank request page



More Questions?

- Just TRY IT!
- Ask Victor Holmes,
Brian Hawthorne,
Mike Bissell



5.4 Argus (Lawrence and Matt Ward)

What is ARGUS?

- ARGUS is a database for 96-well shake plates and shake plate experiments. Store, search, and retrieve shake plate data of any user.
- It is also the interface for the CORE request system.
- For technical help, ask Matt Ward. For CORE ask Jericho Pantaleon. For all else, ask Lawrence Chao.

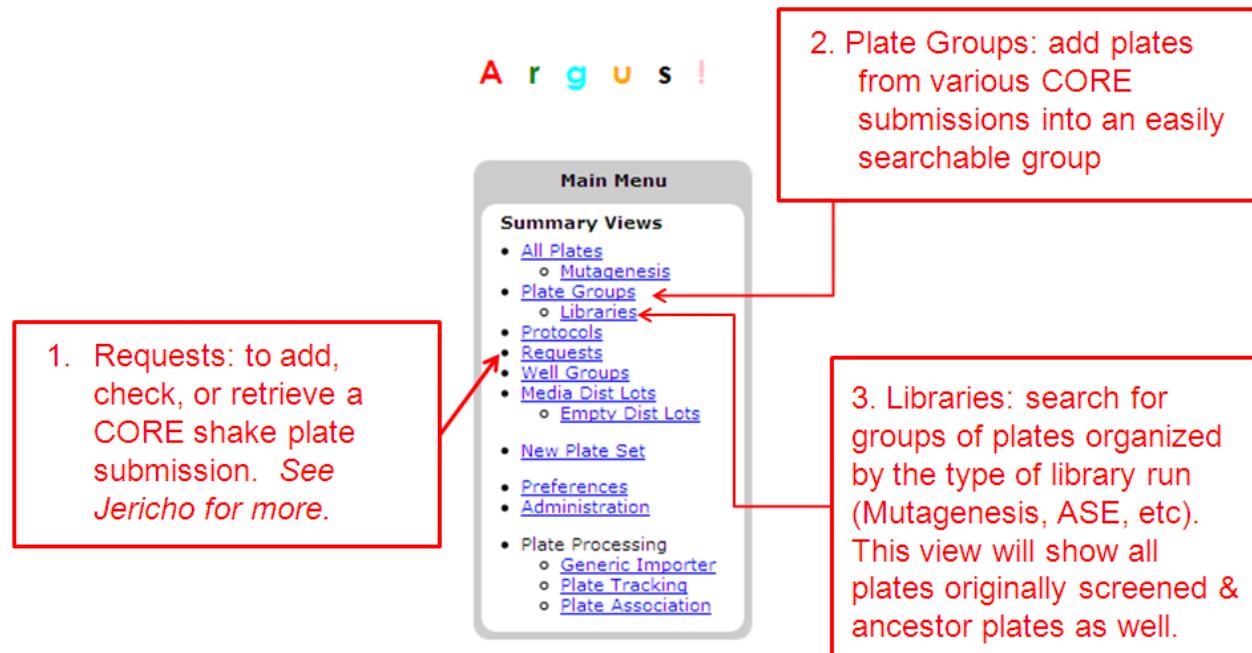
ARGUS Home Page (<http://argus.amyris.local/index.php>)

The screenshot shows the Argus home page with several UI elements highlighted:

- Main Menu:** A red box highlights the main menu bar at the top left.
- Search bar:** A red box highlights the search bar at the top right, labeled "Search bar: enter UID to look up specific plates".
- User Information:** A red box highlights the user information area at the top right, showing "User: Lawrence Chao (logout)" and search buttons.
- Advanced Search:** A red box highlights the "Advanced Search" button and dropdown menu at the top right, labeled "Advanced search: use more stringent parameters".
- Welcome To Argus!**: The main content area is labeled "Welcome To Argus!".
- Main Menu Tree:** The left sidebar contains a tree view of the main menu:
 - Summary Views
 - All Plates
 - Mutagenesis
 - Plate Groups
 - Libraries
 - Protocols
 - Requests
 - Well Groups
 - Media Dist Lots
 - Empty Dist Lots
 - New Plate Set
 - Preferences
 - Administration
 - Plate Processing
 - Generic Importer
 - Plate Tracking
 - Plate Association
- What's New:** A list of recent changes:
 - May 2010
 - Multi plate editing (editing individual properties of several plates on the same page).
 - Better Library support.
 - Empty Plate Distribution Lot tracking system.
 - April 2010
 - Media Distribution Lot tracking system.
 - March 2010
 - New Plate Ids.
 - Feb 2010
 - New Plate Ids.
 - Search plate groups.
 - New Fields: Temperature.
 - December 2009
 - New quicker excluding of wells - just click on the well!
 - New "Matrix CSV" export format for Assay Plates. It's accessible from the main plate list page.
 - November 2009
 - Improved Searching Speed.
 - New Tie Ins with Strain Database.
 - Auto Submission of Trichodiene Plates to Themis.
 - October 2009
 - New Core Request Submission Form.
- © 2009 Amyris Biotechnologies. Contents of this website are confidential and proprietary. Your feedback is always appreciated. If you notice a problem or have a suggestion as to how this tool could better serve you, please click [here](#).

Main Menu: 3 Things to Know

All other options are for the use of by the HTS group.



Searching: 3 Critical Criteria

- **Unique ID (UID):** every piece of plastic has a barcode that distinguish it from other plates.
- **Plate Groups:** clusters of plates grouped by users.
- **Owner:** simplest method of finding YOUR submissions to CORE and grouping them into plate groups

5.5 Fonzie (Darren)

- Fonzie is a set of computational tools to help accelerate progress in Biology. Check them out: <http://limonium.amyris.local/>

6. Program Management (Tim Gardner)

Flexibility, Clarity and Transparency on R&D Goals

Mission of Program Management:

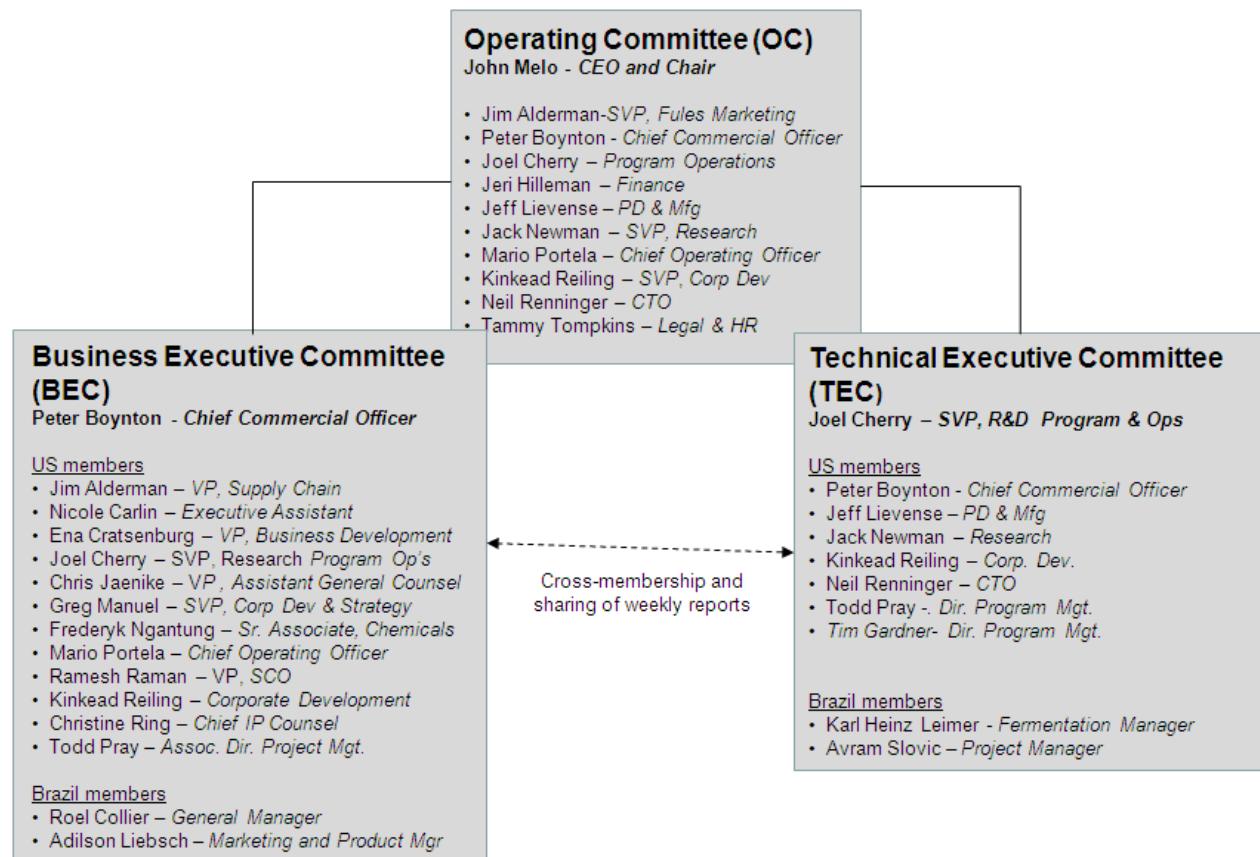
Goal setting: break corporate goals into projects and project objectives

Flexibility: Rapid response of resources to address challenges

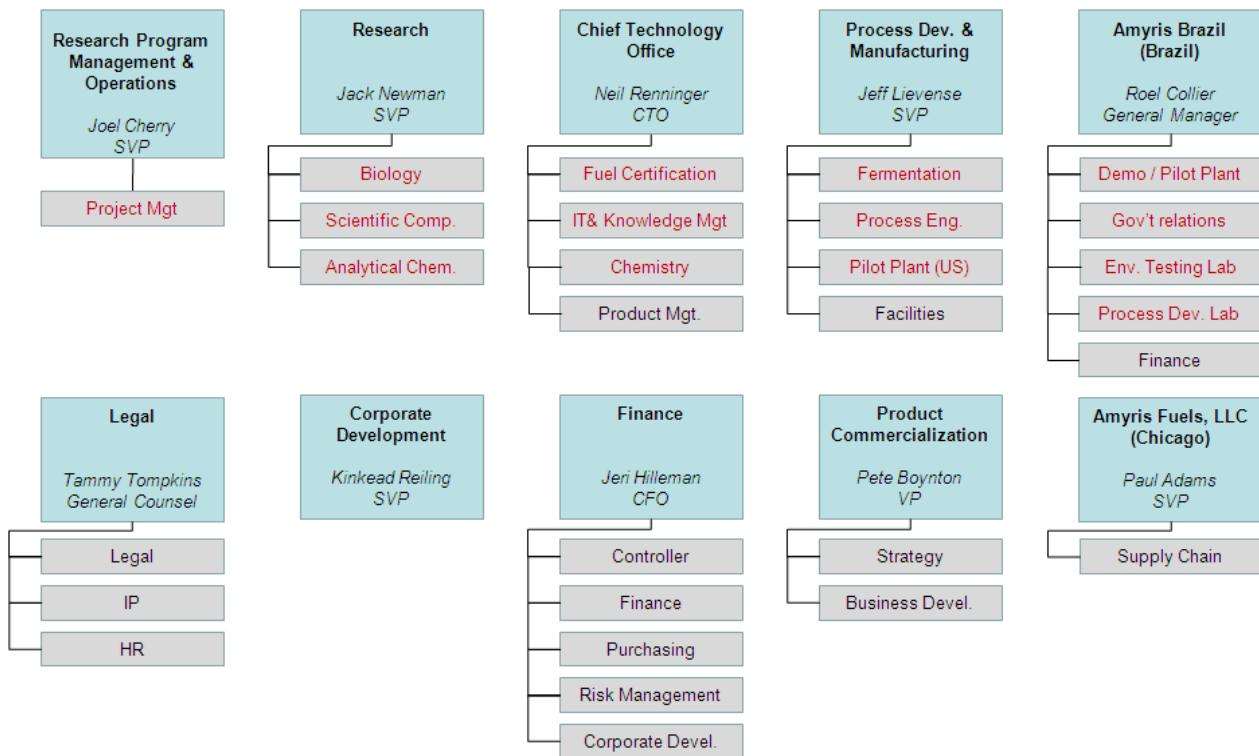
Transparency : Who is working on what. Reporting progress toward objectives

Clarity: Where decision-making lies

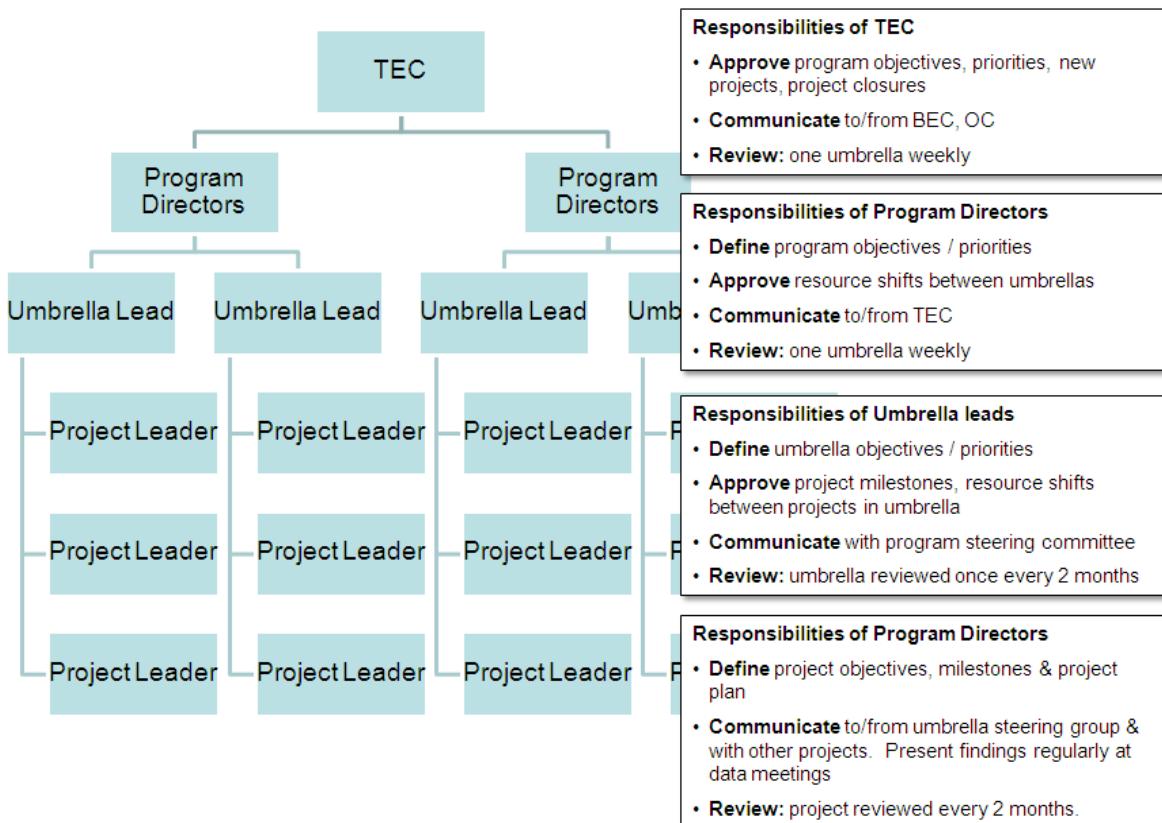
R&D Program Operations: Executive Governance



Corporate Line Management

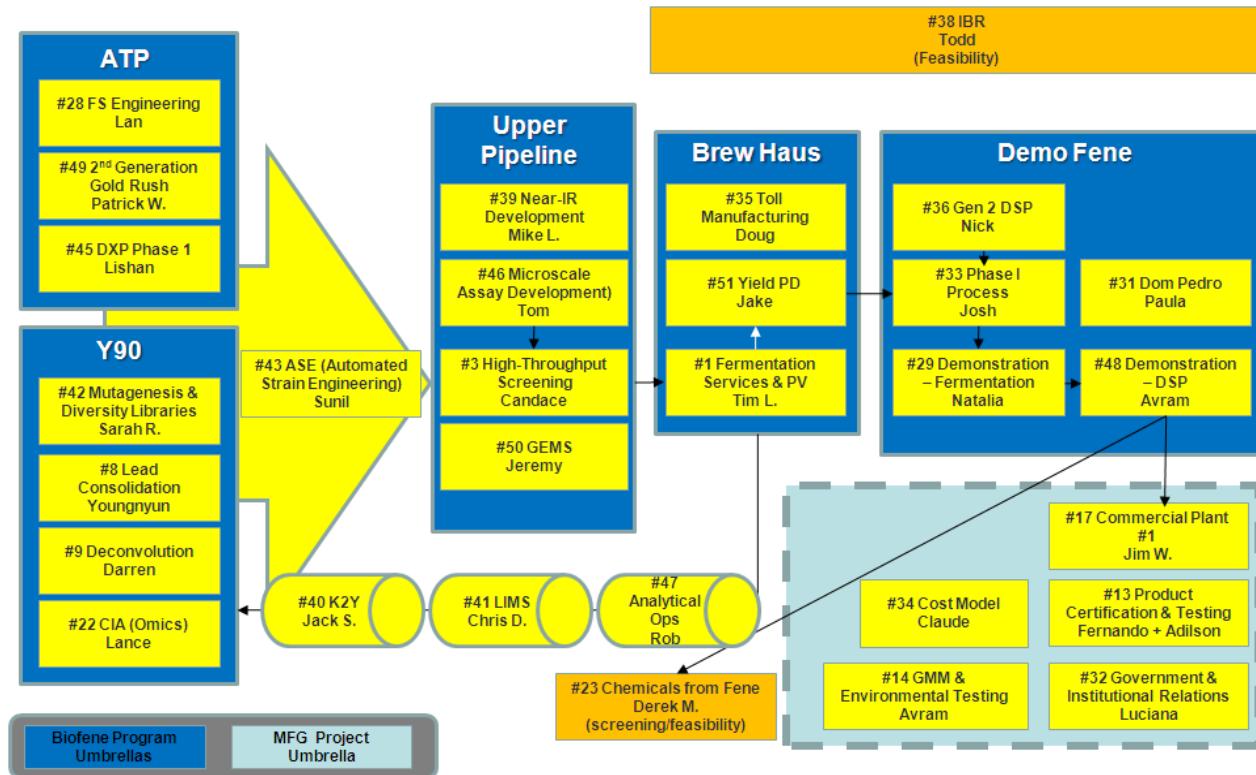


R&D Program Structure & Governance



June 2010 R&D Projects, Umbrellas and Workflow

(note that projects are dynamic—this is a snapshot as of 7/10)



Role of Project Leader (ProLer)

Project Leaders are facilitators- they communicate the overall goals to the project members and do everything in their power to help those members achieve the goals. They lead by interaction, inspiration, persuasion, and enthusiasm, not by dictation, hesitation, or confusion.

Their MO:

Establishing a framework of interaction between team members defined by:

- » Defining responsibilities, timeframes, and deadlines
- » Assigning tasks with attention to expertise and talent of members in such a way that members have a reasonable chance of success, are challenged, can grow personally and professionally, and are rewarded for their success.

Communicating the rationale behind the framework (the big picture) to project members and seeing that tasks stay aligned with project/program objectives

» This requires constant monitoring through reading of project member reports, project meetings, and most importantly, casual regular contact.

Taking corrective action to reassign tasks, shift priorities, and resolve disputes

» This requires familiarity with the tasks and goals of the project and constant communication of the "big picture" and how the smaller tasks fit into it.

» Most important is decisiveness to maintain a sense of project momentum while avoiding project inertia. By this I mean that an area of research that does not lead to the endpoint goal should be stopped without delay, and interesting new areas that will benefit the overall goal should receive additional resources immediately, often by making hard decisions about where those resources come from. Although a dialog is critical, the leader is responsible for the ultimate success or failure of a project, and must take responsibility by acting as the mediator, consensus gatherer-builder, and ultimate decision maker.

Using time efficiently to communicate

» Meetings should be directed towards problem solving and information sharing, not simply to inform the project leader of what is going on, but to inform all participating. Redundancy between information shared at different meetings should be minimized.

Project Description (Example)

The screenshot shows a web-based project management system. At the top, there's a header with a logo, the title "Superdog (The Amyris Intranet)", and navigation links for "Edit", "View", and "Login".

The main content area has a left sidebar titled "NAVIGATION" containing a list of departmental links such as Analytics, Be Green Save Green, Biology, Chemistry, Commercialization, Communications, CompBio, Contamination, Facilities, Fermentation, Finance, Fuels Program, General Lab, High Throughput, Human Resources, IT, Lab Services, Legal, LOKI, Ministry of Truth, Miscellaneous, People Directory, Pilot Plant, Project Pages, Purchasing, PV Pipeline, and Safety.

The main content area displays a project titled "Brazilian Process Development" with a project number "#21". Below the title is a link to "back to top of project space".

Below the title, there are tabs for "Overview" (which is selected), "Monthly Reports", "Presentations", and "Meeting Minutes".

The "Overview" section contains the following details:

- Project leader:** Paula Delgado
- Relevant corporate goal(s):** Robust pilot process in Campinas using partner cane juice
- 2009 Q4 deliverables:**
 - 1) PRIORITY-Support Campinas Pilot Plant.
 - 2) PRIORITY-Successfully run four bioreactors per week in the research lab (3 x 7.5L and 2 x 15L TECNAL)
 - 3) PRIORITY-Successfully execute process and strain transfers from Emeryville to Campinas. Implement strain transfer protocol coordinated with PV.
 - 4) AS TIME PERMITS-Continue the fene production screening effort on cane juice, molasses and syrup with the more recent strain (using flask and bioreactor processes).
 - 5) AS TIME PERMITS-Continue the microbial contamination profiling on cane juice, molasses and syrup.
 - 6) AS TIME PERMITS-Investigate the supplementation needs for fene production using raw materials and the more recent strain.
 - 7) PRIORITY-Continue to develop the Analytical Chemistry lab.
- Tracking:** Current yield and productivity of strains in fermentation will be tracked and experiments will be inputted to Hermes. All raw material test results will be inputted into Perseus.. tanks per week, tank failures, lead strains yield on a weekly basis (adopt Performance Validation metrics for use in Campinas)

At the bottom of the "Overview" section, there's a note about the experimental path or dependencies for a non-experimental project:

PRIORITY-Deliverable #1: Laboratory staff will be the primary support for the effort to identify contaminants in 5kL BC and root cause of contamination in 5kL BC. This will include Laboratory staff training PP and/or Technical Team staff for topics such as sterile technique, microscope use, and plating technique. Promote new strains to PP as quickly as possible. When identification and root cause analysis of 5kL BC contamination is complete further investigation of how to control contaminations will be performed on a as time allows basis.

PRIORITY-Deliverable #2: We are planning to have 4 bioreactors running per week. Two bioreactors will run process develop experiments and two bioreactors for raw material screening with continual communication with Emeryville for experimental planning and rapid strain promotion. The fifth bioreactor will be used on an as needed basis.

Overall Project Matrix Allocation (Example)

ID	Project Title	Project Leader	Sponsor	Line Mgr	Direct staffing to projects from each department										Support FTE	Total	
					Bio	CompBio	Ferm	DSP	Chem	PE	KM	AnChem	Pilot	Campinas	Subtotal	AnChem	
1	Performance validation	Tim Leaf	Jeff	Jorge	0.25	4.84						0.10	5.19	0.50	5.69		
3	Random winners & HTP development	Zach Serber	Jack	Jim	11.40	1.50	0.10			0.90	3.10			17.00	1.25	18.25	
8	Lead consolidation	Kirsten Benjamin	Jack	Candace	3.23		0.10				1.88			5.21	0.20	5.41	
9	Deconvolution	Darren Platt	Jack	Tim Gardner	1.00	1.50									2.50		
10	Fermentation development & scaleup	Paul Hill & Jake Lenihan	Jeff	Jorge	0.05	8.15	0.25			0.25	5.00			13.70	0.40	14.10	
12	DSP development & scale-up	Glenn Dorin	Jeff	Derek		3.00		1.25	0.10	0.41	4.05			8.81		8.81	
13	Fuel certification and testing	Fernando Garcia	Neil	Neil	0.53	0.50				0.42		0.50	1.95		0.15	2.10	
14	GMM approval	Avram Slovic	Neil	Roel	0.15							5.50	5.65			5.65	
15	Campinas pilot plant tech transfer	Jonas Nolasco & Josh Leng	Jeff	Patelli		0.25	0.25			0.20	0.70	16.50	17.90		0.10	18.00	
17	Commercial plant design & construction	Glenn Dyson	Jeff	Jeff	0.25	0.50			2.50			3.50	6.75			6.75	
21	Brazilian Process Development	Paula Delgado	Jeff	Patelli		0.33						7.90	8.23			8.23	
22	Baseline Physiology	Lance Kizer	Jack	Mike L	1.25					1.00	7.38			9.63		9.63	
23	New Product Opportunities	Derek McPhee	Neil	Jeff				4.00	0.25		0.17	0.25		4.67		4.67	
25	Sanofi	Chris Paddon	Jack	Jack	3.00		1.20				0.50			4.70		4.70	
26	Lead generation	Tim Gardner	Jack	Jack	6.33	1.50								7.83	0.20	8.03	
27	Plan X2	Jeff Ubersax	Jack	Candace	11.09					1.10	1.45			13.64	1.25	14.89	
28	FS engineering	Lishan Zhao	Jack	Jim K	5.25							5.25	0.20		5.45		
	Core services				2.25		5.00			2.90				10.15		10.15	
	Direct department total				44.00	6.00	21.00	4.00	4.00	4.00	6.00	15.75	10.00	34.00	148.75	4.25	
	Indirect allocation (e.g. AC support)											4.25					
	- Department overall staff				44.00	6.00	21.00	4.00	4.00	4.00	6.00	20.00	10.00	34.00	153.00		
	= Over (under) allocation				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		

Project-Specific Allocation Detail (Example)

Fermentation development & scaleup (Multiple Items)		<input checked="" type="checkbox"/>
Dept	Sum of Fermentation development & scaleup	
⊖ AnChem	0.25	
Amy Shutkin	0.15	
Robert Herman	0.1	
⊖ Bio	0.05	
Kirsten Benjamin	0.05	
⊖ DSP	0.25	
Ronald Henry	0.25	
⊖ Ferm	8.15	
David Melis	0.8	
Diana Eng	0.9	
Douglas Pitera	0.8	
Eleonora Bellissimi	1	
Elisa Rodriguez Porcel	0.5	
Hiroko Tsuruta	1	
Jacob Lenihan	1	
Jorge Galazzo	0.25	
Paul Hill	0.9	
Tizita Horning	1	
⊖ Pilot	5	
Jeremiah Kim	0.6	
Jeremy Weissbar	1	
Joshua Leng	0.5	
Michael Young	0.7	
Patrick Poon	0.3	
Stanley Louie	0.5	
Michael Waskowiak	0.4	
Hannah Carlos	1	
Direct allocation	13.7	

Project tracking / Review

Dashboard:

Purpose: Approve/Resolve

- Δ milestones/goals
- Δ resources
- Δ objectives
- Issues

Dashboard – Project leads fill in dashboard and discuss w/ Umbrella lead. **Umbrella lead approves milestone changes**. Requests for changes in resources and objectives are handled as quickly as possible after discussion with the appropriate groups (other projects, other umbrellas or the TEC as needed).

Weekly Highlights

Purpose: Inform

- Results
- Impact
- Status

Weekly Highlights – Project lead prepares short (bullet form) weekly highlight of achievements. Umbrella lead prepares a short summary of project highlights. Program director prepares a short summary of the program. Report is consolidated and distributed to R&D.

2 Month Review

Purpose: Review / Revise

- At Showtime
- One umbrella per Showtime
- Report = Showtime slides + text summary
- Reviewed after Showtime by review panel

2 Month Review – Every 2 months all projects in an umbrella present their results at Showtime, the weekly R&D meeting. The objectives, experimental path, risks and next steps of the project are reviewed immediately following Showtime by a panel composed of a TEC member, the Program Director, the Umbrella Lead, the Project lead, the Line Sponsor, and ad hoc reviewers.

Weekly Dashboard (Example)

	D	E	F	G	AD
1	Project 45				
2	DXP Phase 1				
3					
4	Project Leader: Lishan Zhao				
5	2010 Project Milestones				
6	File managed by Tammy Mellon; email comments to mellon@amyris.com				
7	Milestone	Date Due	Status	Hide	7/14/2010
18	Yeast strains with isc genes evaluated by metabolomics and genetic screen	25-Jun-10	Approved		Completed
19	Fe-55 assay for Fe-S cluster developed	25-Jun-10	Approved		Completed
20	Determine the LOD of HDMAPP by performing spike and recovery in yeast metabolite extraction of Y4470	2-Jul-10	Approved		Completed
21	Measure the stability of HDMAPP spiked into yeast cell lysate (to confirm the absence of endogenous activity that might convert HDMAPP to some other compound)	21-Jul-10	Approved		On Schedule
22	Establish in house anaerobic capability for in vitro activity assay	9-Aug-10	Approved		On Schedule
23	Test all soluble IspG and IspH homologues for in vitro activity using artificial electron donors after expression and purification from yeast	31-Aug-10	Approved		On Schedule
24	Construct yeast strains with enhanced Fe-S machinery and test for increased [Fe-S] incorporation capacity (readout: Fe-S content or IspG/IspH activity)	27-Aug-10	Cancellation Approved		Not Started
25	Test various flavodoxin/ferredoxin - Flavodoxin reductase as redox partners for in vitro activity	30-Sep-10	Approved		Not Started
26					
27					
28					
29					
30					
31					
32					
33					
34					
35					
36					
37					
38					
39					

Milestones / General Issues / Staffing Requests / Ready |

Showtime! Presentations and Project Review

Showtime! Is a weekly R&D-wide meeting to present recent results

- Immediately followed by review
- Pre-read slides provided 3 days in advance
- Rotating 5-7 week schedule
- Organized by project umbrellas

Review: immediately follows Showtime:

Output: Modified project plans with 3 month objectives

Review Panel

Provide feedback to ProLer, Program Coordinator, Program Director on direction, approach and impact of project.

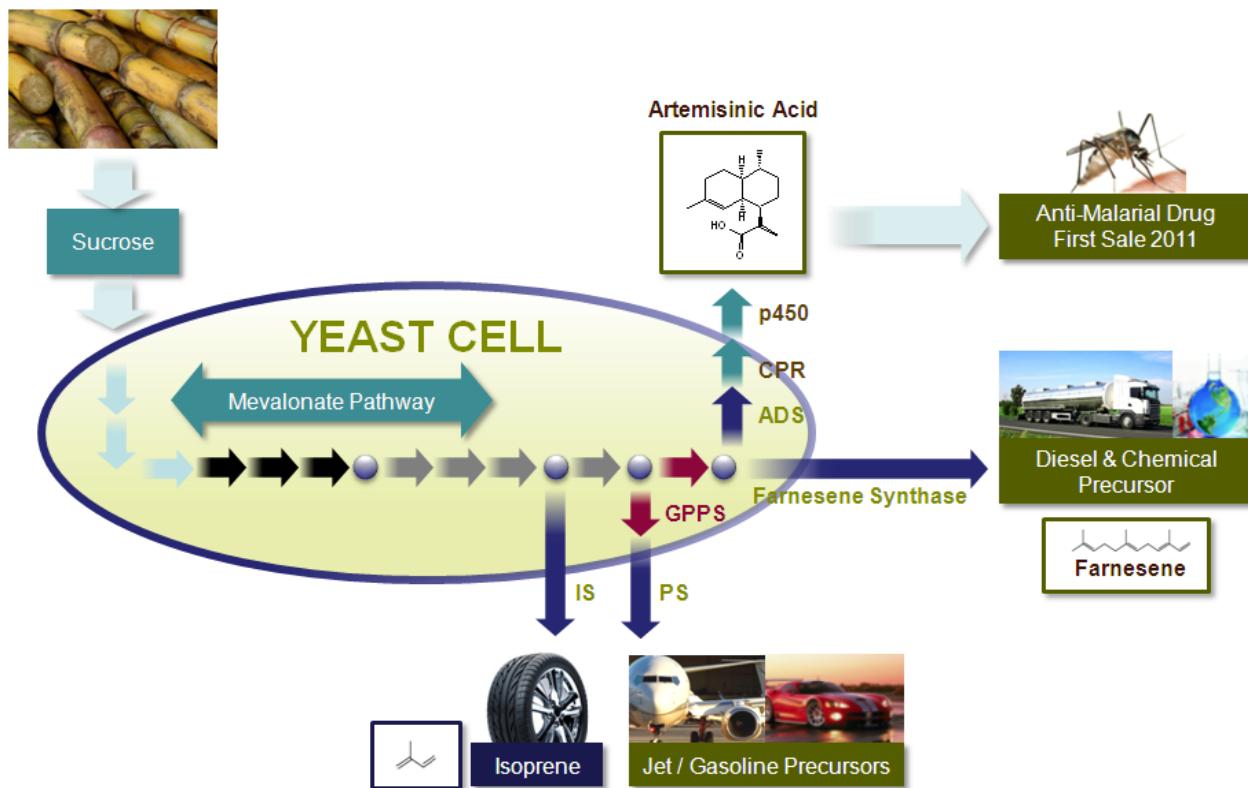
Reviewer	Focus area
Program Coordinator	Alignment with umbrella objectives
Program Director	Alignment with program objectives
TEC sponsor	Alignment with corporate objectives
Line manager of ProLer	Staffing, Infrastructure

Meet directly after Showtime for 1.5 hours.

Weekly Milestone Summary (Example)

Count of 11/5/2009	Column Labels	Not reported	Behind Schedule	Blocked	Cancelled	Completed	On Schedule	Grand Total
Row Labels								
Baseline Physiology		1			2	7	10	
Brazilian Process Development						7	7	
Campinas Pilot Plant Transfer		3		1		23	27	
Commercial Plant Design & Construction		1			1	7	9	
DSP development & scale-up		1				8	9	
Fermentation Development & Scale-Up		12					12	
FS Engineering			1		2	3	6	
Fuel Certification				1	1	8	10	
GMM Approval		12					12	
High-Throughput Screening					6	12	18	
Hit Deconvolution					5	2	7	
Lead Consolidation			1		4	14	19	
Lead Generation					8	5	13	
New Product Opportunities		6					6	
Performance Validation					2	5	7	
Plan X2		10					10	
Sanofi						7	7	
Grand Total		40	8	1	1	31	108	189

Platform Delivers Multiple Products



For more information

<http://superdog.amyris.local/display/PROJ/Research+and+Development+Projects>

7. Logistics

7.1 Amyris Meetings

Meeting	Purpose	Occurrence	Day/Time	Attendees
Showtime!	High level project update	Weekly	Tues/9:30-11	Amyris
Joint project meeting	Data level research update	Weekly	Thur/12-1:30	Research
Research House Keeping (RHK)	State of Research/Technology downloads	Biweekly	Fri/9-10	Research
Project Meetings	Planning and execution of project goals	As determined by project leader	As determined by project leader	Project team
Town Hall	Executive Team communication to Amyris	As needed	TBD	Amryis

7.2 Conference and Travel (Ann Guiney)

Preparations:

- AC Travel tab on Analytics wiki
- Download, complete, & sign the **Conference Reimbursement and Travel Authorization** forms
 1. Approvals from your manager & VP required
Work with your manager to estimate the projected cost of attending the conference
 2. After your manager has signed off, give forms to Ann Guiney for Jack to sign
 3. Retain copies of your signed forms for your reimbursement requests



AC  Ann



Traversa Self-Registration:



- Log-in & book your domestic travel

<https://traversa.travelport.com/amyris>

- It's easy, just like Expedia.com
- Air, rental cars, lodging
- DON'T make travel reservations outside of Traversa or your reimbursement will be at risk! See Ann for international travel plans.
 - Note: Amyris pays for airfare up front, but the employee is responsible for hotel, rental car, & meals expenses which are reimbursed through filling out an expense report form

Conference Rate Lodging

- If your conference has reserved a block of rooms for discounted local lodging:
 - Book your own room at the conference rate
 - This is an exception to the Traversa travel booking guidelines but



it's ok (wink, wink)

- Submit lodging receipts with expense report for reimbursement following conference
- See Ann G. if your up front out-of pocket expenditure creates undue hardship as advance payment can be arranged

Conference Registration

- If your conference offers early-bird registration discounts:
 - Pre-register with your own credit card to take advantage of the discount
- Submit your registration receipt for reimbursement following the conference
- If you can register with a check, download & submit a signed check request
 - Finance cuts checks on Thursdays

- **Timing is everything**

Reimbursement Policy

- **Following the conference**
 - Download, complete & sign an expense report
 - Attach copies of your signed Conference Reimbursement & Travel Authorization forms to your signed expense report
 - Have your manager sign the expense report
 - Submit your completed expense report to Fereen in Finance
 - Reimbursement is deposited in your next pay check



8. Glossary of Terms and Definition of Acronyms

ASE: Automated Strain Engineering

Bistitches (Also known as megastitch): Two stitches usually combined during yeast transformation and usually with a split marker to provide overlap. Using bistitches both allows 10 RABits per integration and allows more combinations of genetic elements to be tested.

CER: carbon dioxide evolution rate (mmol/L/hr)

DCW: dry cell weight (biomass)

DO: dissolved oxygen (%)

FAD: fill-and-draw, an extended operation fed fermentation

Fermentor = tank = vessel = bioreactor

HTS: High throughput screening. Sometimes referred to as “HTP”.

Linker: A standard DNA sequence which, like Velcro or couplers between railroad cars, is used to connect the different RABits in a stitch. Linkers 0-9 are used to connect bits where no read-through is expected. Linkers A-G are used in positions where polymerase will read through.

MAPF: micro-aerobic pulse feed, a sugar feed strategy

Megastitch: see Bistitch

Mule: PCR product that may contain a RYSE linker, but is not plasmid borne (not capable of autonomously replicating)

OTR: oxygen transfer rate (mmol/L/hr)

OUR: oxygen uptake rate (mmol/L/hr)

Productivity (g product / L fermentor volume / day)

RABit: Ryse Associated Bit. Any piece of DNA, usually a promoter, gene, terminator etc cloned into a vector and flanked by two linkers which allow interconnection. The linker-flanked DNA piece in a RABit can always be cleanly excised from the vector using a Sap1 restriction enzyme.

RYSE: Rapid Yeast Strain Engineering. A system that converts DNA into modular, interchangeable, connectable parts. Individual parts (or bits) are called RABits which are connected through Linkers into biologically functional stitches and bistitches (also known as megastitches).

SOUR: specific oxygen uptake rate (mmol/gDCW/hr)

Stitches: An assembly of RABits (PCR product) that usually contains a selectable marker, genes and promoter to be inserted, and the chromosomal locus to either delete or alter. Stitches are usually made from two to ten RABits, though ASE limits itself to 5 RABits per stitch.

Titer (g product / L fermentor volume)

Yield (g product / g substrate) (%)

Additional acronyms can be found on the analytical chemistry superdog page:

<http://superdog/display/ANAL/AC>

9. Appendix: Relevant Literature (Kirsten and Chris P)

Use the google box to search for journal articles

To order journal articles:

<http://superdog/display/MOT/Access+to+Scientific+Literature>



Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ympben

Selection and optimization of microbial hosts for biofuels production

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Stress tolerance
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ABSTRACT

Currently, the predominant microbially produced biofuel is starch- or sugar-derived ethanol. However, ethanol is not an ideal fuel molecule, and lignocellulosic feedstocks are considerably more abundant than both starch and sugar. Thus, many improvements in both the feedstock and the fuel have been proposed. In this paper, we examine the prospects for bioproduction of four second-generation biofuels (*n*-butanol, 2-butanol, terpenoids, or higher lipids) from four feedstocks (sugars and starches, lignocellulosics, syngas, and atmospheric carbon dioxide). The principal obstacle to commercial production of these fuels is that microbial catalysts of robust yields, productivities, and titers have yet to be developed. Suitable microbial hosts for biofuel production must tolerate process stresses such as end-product toxicity and tolerance to fermentation inhibitors in order to achieve high yields and titers. We tested seven fast-growing host organisms for tolerance to production stresses, and discuss several metabolic engineering strategies for the improvement of biofuels production.

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1. Metabolic engineering challenges in biofuels production

The creation of liquid transportation fuels from renewable biomass has been a long-standing research goal (Czyzewski and Wilke, 1976). Biomass is renewable, and is abundant in places where other liquid fuels, chiefly petroleum and its byproducts, are not readily available (Perlach et al., 2005).

Biomass resources are widely distributed, but often with densities of 0.4 kg/L or lower. The challenge of fuels production from biomass is to liquefy and increase the bulk density of the resource, all while preserving its energy content. The final fuel product should (i) have a high energy density on a mass as well as a volume basis, (ii) be produced at yields near the stoichiometric maximum from a given biomass feed, and (iii) enjoy compatibility with existing fuel distribution infrastructure. No single fuel currently satisfies all of these criteria (Table 1).

Ethanol as made from corn or sugar cane has an acceptable energy density and can be produced from a variety of biomasses at excellent yields. However, ethanol largely fails the requirement for compatibility with existing fuel infrastructure. Gasoline, the predominant liquid fuel in the US, enjoys over 95,000 miles of dedicated pipeline for its distribution (<http://www.pipeline101.com/overview/products-pl.html>), over 160,000 filling stations (Yergin et al., 2006), usually with underground gasoline storage tanks, and over 240,000,000 heavy- and light-duty passenger

vehicles already in use (<http://www.pipeline101.com/overview/products-pl.html>). Together, this infrastructure is worth hundreds of billions of dollars. Ethanol can cause materials corrosion and draw water into the fuel mixture, properties which render it unsuitable for use in the existing fuel distribution infrastructure. Increased use of ethanol in large quantities may thus necessitate large infrastructure investments.

Other proposed fuels, in particular, biodiesel, butanol, and various terpenoid compounds may be compatible with existing fuels infrastructure, but efficient, high-yielding processes for their production are not yet commercially feasible. The principal barrier to the production of these advanced biofuels is the development of robust, high-yielding microbes and processes for their production.

In this paper, we survey the feedstocks that may be useful for biofuels production, and similarly survey the molecules that have been proposed as second-generation liquid biofuels, and consider how various feedstocks and products influence the choice and optimization of suitable host organisms for biofuels production. Although substantial opportunities for metabolic engineering of higher plants exist (Ragauskas et al., 2006), our focus is limited to examining microbial conversions of biomass to liquid fuels.

2. Biofuels feedstocks and products

Metabolic pathways for the synthesis of most proposed biofuels proceed through common metabolic intermediates such as acetyl-CoA or pyruvate. The pathways can thus be effectively divided into "feed" pathways (Fig. 1a), which convert biomass to

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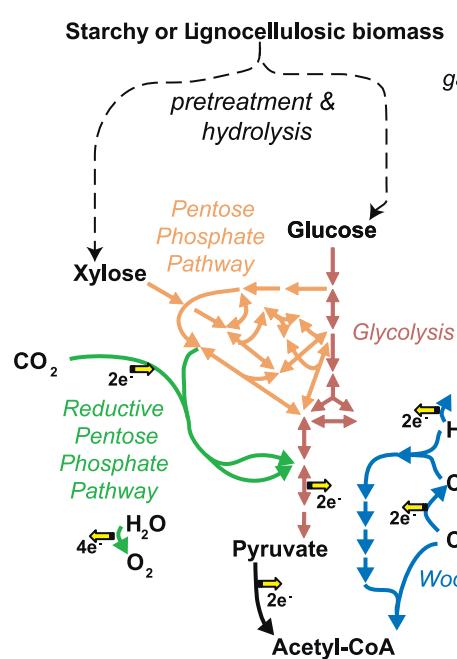
E-mail address: gregstep@mit.edu (G. Stephanopoulos).

Table 1Energy densities, maximum yields, and compatibility with existing fuel distribution infrastructure for various types of biomass and liquid fuels^a

	Molar energy density (kJ/mol)	Formula mass	Volumetric energy density (MJ/L)	Mass energy density (MJ/kg)	Bulk density (kg/L)	Maximum stoichiometric yield from glucose (kg/kg)	Infrastructure compatibility?
Petrofuels							
Gasoline	100–105	34.7	42.3	0.72–0.78	—	—	Yes
No. 2 diesel	~200	38.3	45.3	0.80–0.89	—	—	Yes
Example biofuels feedstocks							
Hybrid poplar wood	—	—	6–7.1	19.38 (dry basis)	0.310–0.370	—	No
Glucose	2803	180.155	24.3	15.6	1.562	—	No
Syngas ($\text{CO}+\text{H}_2$)	569	30.026	—	18.95	—	—	No
Candidate biofuels							
Ethanol	1367	46.07	23.6	29.9	0.792	0.511	No
<i>n</i> -Butanol	2676	74.121	29.2	36.1	0.81	0.411	Probable
Vegetable oil (canola)	35390	~887	36.2–36.7	39.8–40.0	0.910–0.917	~0.353	No
Biodiesel (methyl-esterified virgin canola)	12080	~298	35.7	40.6	~0.88	—	Partial and increasing
α -Pinene (representative terpenoid)	6205	136.234	38.9	45.5	0.8539	0.324	Unknown

^a Data compiled from Balatinecz and Kretschmann (2001), Brown (2003), Davis and Diegel (2007), Henstra et al. (2007), Lide (2006–2007), Shahidi (2005), Thomas (2000), Transportation Energy Data Book: Edition 26, (2007).

a



b

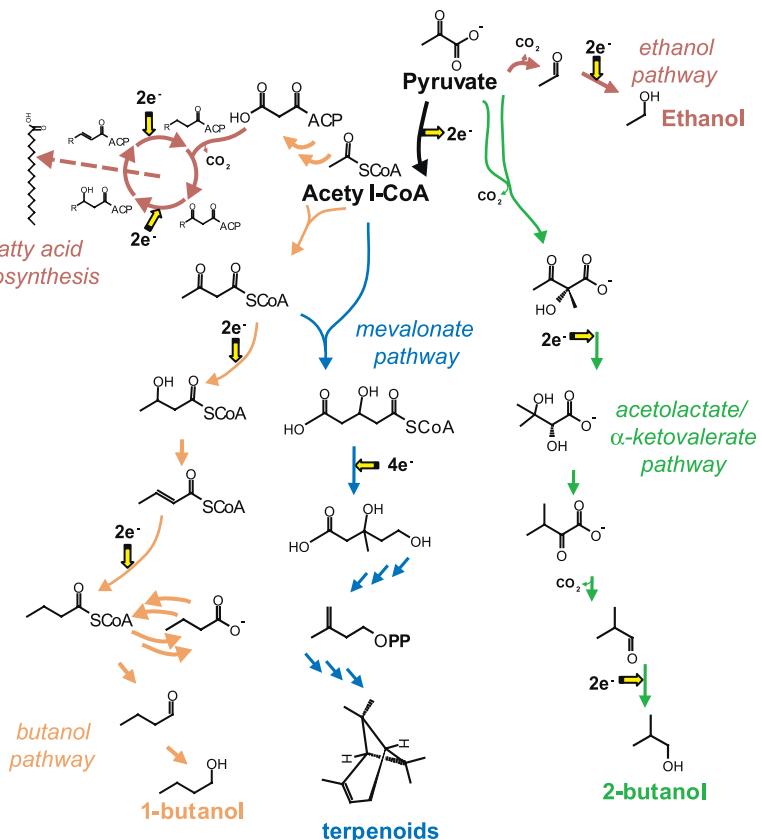


Fig. 1. Pathways for the metabolic conversion of biomass into biofuels can be loosely divided into (a) feed pathways, which convert carbohydrate biomass into the central metabolic intermediates pyruvate and acetyl-CoA; and (b) product pathways, which convert these central intermediates into fuels. Reducing equivalents, shown with yellow arrows, are generated in (a) and consumed in (b). For reasons of clarity and familiarity, molecular structures are not drawn in (a).

the common metabolic intermediates, and “production” pathways, which convert the intermediate to the chosen fuel (Fig. 1b). Generally speaking, the feed pathways create reducing equiva-

lents which are required by production pathways for the synthesis of the biofuel. Consumption of reducing equivalents by other pathways, for example aerobic respiration, is thus undesirable.

2.1. Feedstocks

2.1.1. Starches and simple sugars

Ethanol, today's predominant biofuel, is presently manufactured using feedstocks such as cane-derived sucrose and corn-derived starch. In Brazil, sugar cane juice and sugar cane molasses are used as sources of sucrose. Using *Saccharomyces cerevisiae* and closely related yeast strains as the host, industrial ethanol yields on sucrose are up to 93% of the stoichiometric maximum. Both continuous and batch production is used, with residence times in the fermentors being 6–10 h (da Silva et al., 2005). Production is more than 15 billion gallons (57 billion liters) per year.

In the US, the predominant feedstock is maize. Studies have reported industrial yields in the range of 2.65–2.71 gal per bushel (0.388–0.397 L/kg) of maize, which amounts to approximately 93.6–95.8% of the stoichiometric maximum (Johnson, 2006; McAloon et al., 2000). Presently, industrially attained ethanol titers post-fermentation are around 9% by weight (McAloon et al., 2000), although this figure may increase with improvements in strain ethanol tolerance (see Section 3.2).

The process for production of ethanol from these sources is highly efficient, with feedstock cost generally amounting to 60–80% of the cost of production, although this figure is subject to the usual vagaries of the agricultural commodities markets. The metabolic pathway for starch or sucrose conversion into fuel begins with enzymatic hydrolysis, which yields glucose. The glucose is converted to pyruvate via the familiar pathway of glycolysis (Fig. 1a). Because processes for starch- or sugar-derived ethanol are so efficient, they are a convenient benchmark by which to evaluate other proposed biofuels processes.

2.1.2. Cellulosics

Cellulosic biomass enjoys a much more massive resource base than what is available from maize or sugarcane (Perlach et al., 2005). It is expected to play a dominant role in biofuels production in the near future.

However, cellulosic biomass is more difficult to convert into fermentable sugars than is corn or sugar cane, because (i) five-carbon sugars, mainly xylose, account for 10–25% of the total carbohydrates; (ii) of the presence of lignin, a highly recalcitrant network polymer of aromatic alcohols that accounts for 17–25% of common cellulosic biomasses (van Maris et al., 2006); and (iii) cellulose is much more resistant to hydrolysis than starches and simple oligosaccharides. The first obstacle can be overcome through the selection and/or engineering of microbes capable of the anaerobic fermentation of xylose and other five-carbon sugars to ethanol (see below). These five-carbon sugars are metabolized via the pentose phosphate pathway (Fig. 1a). Enzymatic degradation of lignin is too slow to be practical industrially, but lignin can be productively burned for power production, or gasified for thermochemical conversion.

The third obstacle is the most important. Cellulosic biomass recalcitrance necessitates chemical "pretreatment" of cellulosic biomass to a partially hydrolyzed product that cellulase enzymes can more easily digest. A number of pretreatment variations have been proposed, but many generate fermentation inhibitors (see Section 3.2).

Two main metabolic engineering approaches have long been studied for provision of cellulase and sugar fermentation to ethanol. The first is aimed at genetically modifying *S. cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, or other organisms so that pathways for utilization of five-carbon sugars and for ethanol synthesis exist in the same organism (Ho et al., 1998; Ingram et al., 1999; Mohagheghi et al., 1998). Recently, promising results with *S. cerevisiae* have been reviewed (van Maris et al., 2006). The advantages of *Saccharomyces* are high ethanol tolerance, and the

ability to ferment at low pH and forgo medium sterilization. The disadvantage is that *Saccharomyces* (like *E. coli* and *Z. mobilis*) cannot utilize cellulose or derived oligosaccharides directly. This necessitates that a biorefinery install its own dedicated on-site cellulase production system, usually involving the aerobic cultivation of organisms such as *Trichoderma reesei* (Zhang et al., 2006).

The diversion of feedstock to cellulase production, and the expenses incurred by aerobic cell growth (e.g. electricity and capital for air compression and culture agitation) add to the cost of fuel production. A 2000 study estimated that cellulase production accounted for about ~\$0.073/kg (\$0.22/gal) of ethanol, or about 14% of the production cost (McAloon et al., 2000) in a *Zymomonas*-based process, in rough agreement with past estimates (Lynd, 1996). The assumed biomass cost in these studies was \$35–\$42 per dry ton.

Another approach, called consolidated bioprocessing (CBP) and formerly referred to as direct microbial conversion (DMC), uses highly cellulolytic organisms like the thermophilic *Clostridium thermocellum* either exclusively or in co-culture with other thermophilic, higher-producing sugar fermenters (Lynd et al., 2002; Ng et al., 1981). These organisms permit cellulase production, cellulose hydrolysis, and fermentation to occur anaerobically in the same process vessel. Advantages are (i) that the thermophilic nature of these organisms may also obviate requirements for medium sterilization by allowing operation at 60 °C, (ii) most clostridia can efficiently use xylose without recombinant modification, and (iii) the cellulase production and cellulose digestion can occur in the same vessel. Disadvantages of CBP are (i) the lower solvent resistance of clostridia (Demain et al., 2005), (ii) comparative difficulty in genetic modification of clostridia, even despite recent developments (Tyurin et al., 2004), and (iii) increased energetic demands for cellulase production in anaerobic environments (Lynd et al., 2002).

Thus, the effect of consolidated processing on effective cellulose conversion costs is unclear. Anaerobic, single-vessel cellulase production may appear to eliminate the tradeoff between resources to dedicated cellulase production and ultimate biomass-to-fuel conversion, but, "an analogous tradeoff exists for single organisms that both produce cellulase and ferment the resulting hydrolysis products" (Lynd, 1996). CBP may have the potential to lower cellulose conversion costs, but to be realized, the development of strains and processes which dramatically increase rates of anaerobic cellulose conversion must be developed (Lynd et al., 2002).

2.1.3. Syngas

Syngas, sometimes called producer gas, is a mixture of carbon monoxide, hydrogen, and carbon dioxide. It can be prepared easily from biomass (or fossil fuels) by treatments at high temperature in the absence of oxygen (Sutton et al., 2001). Syngas can serve as the sole carbon and energy source for a variety of microorganisms, including ethanol and butanol producers. Carbon monoxide is oxidized to carbon dioxide by water via carbon monoxide dehydrogenase (CODHs). This reaction provides reducing power for anabolic CO metabolism via the Wood-Ljungdahl pathway (Ragsdale, 2004), where CO is converted to acetyl-CoA via acetyl-CoA synthetase (ACS) enzyme (Fig. 1a).

Hydrogen can be metabolized by many of the same organisms which metabolize carbon monoxide. In some cases, however, hydrogen metabolism may be inhibited by carbon monoxide itself, or by other trace syngas components such as nitric oxide (Ahmed et al., 2006; Ragsdale, 2004). Increasing the tolerance of microbes to syngas-associated stresses is an intriguing possibility, but approaches are just beginning to be studied.

Nonetheless, a variety of clostridia such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, and *Clostridium carboxidivorans* can simultaneously utilize both of these substrates and overproduce ethanol, butanol, and/or acetate (Rajagopalan et al., 2002; Sipma et al., 2006). For example, Lewis and his co-workers (Datar et al., 2004; Rajagopalan et al., 2002) have reported ethanol yields on CO of 45% the theoretical, with productivities in the neighborhood of 1 g/L/day and titers of up to 3 g/L.

Relative to synthesis of fuels from syngas via heterogeneous catalysis (Phillips et al., 2007), fermentation-based processes may (i) be operated at lower pressures, (ii) enjoy superior specificity for the desired products, and (iii) are likely to be more tolerant of sulfur impurities in syngas than traditional heterogeneous catalysts.

2.1.4. Light and carbon dioxide

Photosynthetic organisms tap the energy in light to generate reducing equivalents from water, thus allowing fixation and use of carbon dioxide as a growth substrate. The pathway for incorporation of CO₂ is through the reductive pentose phosphate pathway, also known as the Calvin cycle. The enzyme Rubisco catalyzes the conversion of CO₂ and ribulose-5-phosphate to two trioses, which can subsequently be converted to pyruvate (Fig. 1a).

Biomass from microbial photosynthesis is being explored for biofuels production. Compared to higher plants, microbial photosynthesizers can be genetically modified more easily, and also enjoy faster life cycles and rapid growth.

Maximum attainable cell densities and productivities are important determinants of the cost of algal biomass. Generally, cell densities in outdoor algae ponds reach no more than 0.5–1.0 g/L of dry cell weight, even when supplying highly enriched sources of carbon dioxide (such as the flue gas of a coal or natural gas-fired power plant). This necessitates mobilization of considerable land and water resources for algal culture. Cost estimates made in the 1980s by a Department of Energy program for algal biomass grown in large-scale ponds was \$0.19–\$0.41/kg of biomass (unadjusted for inflation) (Sheehan et al., 1998).

An alternative is to use photobioreactors for growth. Algal photobioreactors can obtain cell densities near 20 g/L (Lee, 2001), but estimates of the cost of algal biomass grown in closed bioreactor systems have been up to \$12–\$32/kg dry weight (Grima et al., 2003; Lee, 2001), indicating that these systems are likely far more costly than open ponds. Various elaborate modulations of the intensity and the spatial and temporal distribution of the incident light flux have been proposed (Gordon and Polle, 2007; Ono and Cuello, 2004) as methods for increasing productivities.

A US Department of Energy review of its extensive research program on algal biomass production in the 1970s and 1980s details the isolation of several promising algal strains, as well as methods for genetic transformation of algae, and improvement of lipid yields (Sheehan et al., 1998). Representative productivities in outdoor pond systems were 15–20 g/m²/d. Lipid contents of ~40% (w/w) and specific growth rates of ~2 d⁻¹ were representative. More recently, robust genetic methods have been reported for *Chlamydomonas reinhardtii*, *Chlorella* subspecies, and other algae (Coll, 2006; Stevens and Purton, 1997).

Algal biomass may become an attractive option for fuels production if metabolic engineering can boost productivities, photosynthetic efficiencies, or lipid content dramatically over historical levels, or if carbon taxes or emissions caps necessitate CO₂ capture from flue gas emissions from fossil fuel power plants.

2.2. Products

2.2.1. Ethanol

Ethanol is produced from pyruvate in two steps: pyruvate decarboxylase converts pyruvate to acetaldehyde, and alcohol dehydrogenase reduces the acetaldehyde to ethanol (Fig. 1b). This pathway is commonly exploited in *Saccharomyces* yeast hosts, or in the Gram-negative gamma-proteobacteria *Z. mobilis* or recombinant *E. coli*.

The maximum attainable ethanol concentration depends on the host and medium composition, but is usually between 4 and 16 wt%, depending on the host. Processes for the distillation of ethanol from dilute fermentation broth were known even in ancient Egypt (Stichlmair, 2000), but distillative separation of ethanol from the fermentation broth requires considerable energy input. To provide a basis for comparison with other fuels without specifying separation process specifics, we estimated the reversible work of separation for ethanol and several other fuels from an aqueous mixture as a function of concentration (Fig. 2; full description of calculation methods are in the supplemental materials available online). For reference, energy input required to separate ~9 wt% ethanol from water is about 5 MJ/kg for corn ethanol in the US (Johnson, 2006). (This implies that distillation operates at about 5% of the thermodynamically maximal efficiency.)

Distillation represents a higher percentage of the cost of production for corn ethanol than for cellulosic ethanol, because feedstock pretreatment and cellulase production still dominate projected costs for cellulosic ethanol (see Section 2.1.2 and references therein).

2.2.2. Butanol

n-Butanol is a fermentation product of *Clostridium acetobutylicum* and *Clostridium bjerinkci* (Ezeji et al., 2007b). It is produced from acetyl-CoA through the dimerization of two acetyl-CoAs into acetoacetyl-CoA. Subsequent enzymes catalyze the four-electron reduction and dehydration of acetoacetyl-CoA to butyric acid. The butyrate can then be re-metabolized by some clostridial species via a further four-electron reduction to n-butanol. In clostridia, acetone (not shown in Fig. 1b) is often produced concomitantly with butanol by decarboxylation of acetoacetyl-CoA.

The total pathway from glucose to butanol is, in principle, redox balanced. However, in some organisms, including *C. acetobutylicum* and *E. coli*, hydrogen is produced via formate-hydrogen lyase (Liu et al., 2006) and represents a competing outlet for reducing equivalents. In principle, hydrogenase activity or alternate formate dehydrogenases (Sanchez et al., 2005) could recover these reducing equivalents. The clostridial pathway has been functionally expressed in recombinant *E. coli* hosts, although

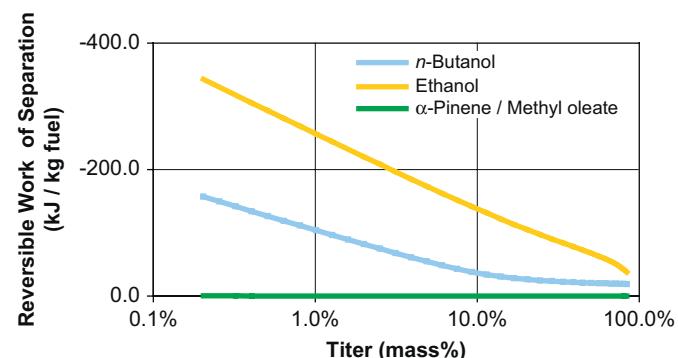


Fig. 2. The reversible work of separation of ethanol, butanol, alpha-pinene, or methyl oleate (biodiesel) from aqueous solution as a function of titer.

the observed productivity fell short of what was observed for clostridia (Atsumi et al., 2008).

An alternative approach to butanol production that starts from pyruvate instead of acetyl-CoA has been proposed in the patent literature (Donaldson et al., 2007). Decarboxylative dimerization of pyruvate to acetolactate is followed by reduction to α -ketovalerate, decarboxylation, and further reduction to butanol. The enzymes composing this pathway are not usually found in the same organism. Notably, this pathway results in production of the 2-butanol isomer. Relative to 1-butanol, the 2-butanol isomer may be preferred as a fuel.

Butanol has a considerably lower reversible work of separation from water than ethanol, which implies that its separation from fermentation broth will be considerably more energy efficient (Fig. 2). However, it is far more toxic to most microorganisms. In clostridia, 13 g/L has been reported as a usual *n*-butanol titer (Ezeji et al., 2004). Butanol yields on glucose of 0.76 mol/mol and productivities of 0.76 g/L/h have been reported for clostridial cultures sparged with hydrogen (Ezeji et al., 2004).

2.2.3. Higher lipids

Acetyl-CoA is the ultimate source of carbon for fatty acid biosynthesis. In many organisms, long-chain fatty acids biosynthesized through the ATP-requiring carboxylation to malonyl-CoA, followed by cycles of decarboxylative addition of malonyl-CoA to acyl units and β -reduction (Fig. 1b). Long-chain fatty acids, as methyl esters (Zhang et al., 2003) or decarboxylated derivatives (Maki-Arvela et al., 2007) are attractive as renewable substitutes for diesel fuel.

Oils from higher plants are also synthesized in this way. Easily extracted oils from palm trees and soybeans can be converted to biodiesel and are widely used for biofuels production. The obstacle that has stopped wider adoption of this technology is the insufficient availability of low-cost feedstocks (Hill et al., 2006). Nonetheless, about 0.225 billion gallons (0.805 billion liters) were produced in the United States in 2006. Like corn ethanol, government subsidies for biodiesel production are currently required to sustain commercial interest in these technologies.

Lipid production by algae has the potential to get around current biodiesel feedstock limitations (Chisti, 2007; Hankamer et al., 2007). Improving lipid production by algae has been an important metabolic engineering goal for many years, and remains so today (see Section 2.1.4). More recently, interest has developed in the generation of biofuels via lipogenic (or oleaginous) yeasts and other microbes (Voelker and Davies, 1994). This technology would allow microbial conversion of cellulosic materials to lipids.

Lipids do not mix with water, and as such, the reversible work of separation from aqueous solution is near zero. One drawback may be that lipids are often accumulated intracellularly, which could require extraction of the lipids from crude cell pastes (Grima et al., 2003). Separation of the lipids from undesirable biomass components, perhaps by solvent extraction or other means, will require its own energy inputs. An alternative metabolic engineering strategy is to engineer secretion of the lipid products.

Many details of fatty acid secretion even in model organisms like *E. coli* and *S. cerevisiae* are poorly understood. These areas may be attractive targets for metabolic engineering.

2.2.4. Terpenoids

Isoprenoids are a broad class of metabolites synthesized from isoprenyl pyrophosphate (IPPP), the pyrophosphate ester of 3-methylbut-2-en-1-ol, or its isomer dimethylallyl pyrophosphate

(DMAP), the pyrophosphate ester of 3-methylbut-2-en-1-ol. These molecules are synthesized either from glyceraldehyde-3-phosphate and pyruvate via the methylerythritol pathway (omitted from Fig. 1b), or from acetyl-CoA via the mevalonate pathway (Fig. 1b).

IPPP and DMAP can be dimerized or polymerized to an astonishing array of olefinic hydrocarbons or their alcohol derivatives. For fuel applications, water-insoluble liquid products such as the hemiterpenoid alcohol 3-methylbut-2-en-1-ol (prenol) (Withers et al., 2007), or perhaps monoterpene and sesquiterpene hydrocarbons such as limonene, pinene, or cadinenes, may be preferred targets.

Strains and processes capable of converting sugars to terpenoids at yields similar to the ethanol process have not yet been reported in the scientific literature. For example, a representative titer for the sesquiterpene hydrocarbon amorphadiene has been reported as about 0.5 g/L (Ro et al., 2006).

Terpenoid production has usually been examined only under aerobic culture conditions, but for large-scale, high-yield production, anaerobic processes are desired. Obviation of the oxygen requirement for terpenoid overproduction is one key metabolic engineering objective for biofuels applications of this pathway.

2.3. Requirements for yield, titer, and productivity

Ultimately, any host organism for biofuels production must perform at high yield, because of the significant cost for feedstock. Years of research has revealed the enzymological and genetic details of metabolic pathways for ethanol and butanol fermentation, and allowed their functional pathway expression in convenient host organisms (Atsumi et al., 2008; Ingram et al., 1999). Carbon monoxide dehydrogenase has been functionally expressed in recombinant *E. coli* (Loke et al., 2000), suggesting that research to develop recombinant syngas fermenting organisms may also be attractive.

Equally important, comprehensive stoichiometric models of metabolism permit the design of efficient genetic backgrounds for fuel production (Alper et al., 2005b; Fong et al., 2003; Ibarra et al., 2002). These models can also be used to design genetic backgrounds in which biomass yield is positively correlated with the desired product yield, allowing simple evolutionary strategies to be used to optimize the yield of the desired product. We conjecture, therefore, that of yield, productivity, and titer, the yield of a given pathway for fuel production may be the most amenable to traditional metabolic engineering strategies for strain optimization.

However, a similarly diverse and effective array of techniques is not available to optimize productivities, or, in particular, titers. Mechanisms for dealing with the stresses associated with biofuels production, such as osmotic stress, membrane disruption, and the presence of various toxins appear to be host-specific and often poorly understood (Cakar et al., 2005; Taherzadeh et al., 1997a). The selection of a fitting host organism is thus essential for the development of biofuels production processes.

3. Biofuels hosts

3.1. Desirable properties

Finding or constructing an optimal host for biofuel production is the most obvious requisite of any metabolic engineering effort, but is far from trivial. In this context, the ideal host would degrade lignocellulosic components, ferment the resulting sugars (both hexoses and pentoses) at high rates and with high yields,

and tolerate high titers of the end-product at high temperatures and extreme pH (to avoid cooling and sterilization costs). The fact that biodiversity is remarkable, especially for microorganisms, may suggest that an ideal host for biofuel production already exists, so that the real challenge is finding it. Since there is no clear path for solving this needle-in-a-haystack problem, most researchers have opted to tackle the design problem of combining desirable characteristics into an engineered host using recombinant DNA technology. Any such effort usually entails the alteration of phenotypes dictated by multiple genes. The systematic implementation of this approach requires substantial knowledge of the host to be modified, which has favored the use of "laboratory strains" that are research-friendly, but not necessarily robust enough for industrial applications.

The characteristics that have made laboratory strains like *E. coli*, *S. cerevisiae*, and *Bacillus subtilis* so popular are also to a large extent those that will be needed for the development of industrially relevant production strains. The first requisite is genetic competence, the ability of a strain to accept foreign DNA in a controllable fashion. High transformation efficiencies are desirable, especially for the successful use of combinatorial libraries for screening genes or gene variants that confer a particular phenotype of interest (see discussion in following section). Even though transformation protocols have been used for a long time, they tend to be host-specific and based on empirical observations rather than on fundamental principles. Furthermore, their success seems to depend on a variety of factors such as the activity of host restriction and DNA-modification systems (Alegre et al., 2004; Matsushima et al., 1989), genetic background (Umemoto et al., 1996), origin of replication and marker of the vector (Aukrust et al., 1995), to name a few. A second trait that makes laboratory strains attractive is the availability of well-characterized metabolic engineering "modules" that allow manipulation of the genotype in different ways. Examples include promoters of various strengths (Alper et al., 2005a), termination sequences, repressor-inducer systems, plasmids (with known copy number, replication mechanism, compatibility with other plasmids, etc.), chromosome integration cassettes for building knockouts or stable replication of genes, etc. A third feature is the availability of "omics" platforms and algorithms for genome-wide characterization of cellular responses to different manipulations and environments (microarrays, metabolic network models, etc.). A fourth, and most understated feature of all, is the great amount of accumulated knowledge on the physiology of laboratory strains provided by generations of researchers.

The need for host strains that are "domesticable" and predictable arises also from overall process considerations. Understanding the relationship between growth phase and product formation, as well as the kinetics of both is essential for process design. For example, solventogenesis in *C. acetobutylicum* is a stationary-phase phenotype controlled by global transcriptional regulators that respond to different cues (Ravagnani et al., 2000; Wilkinson et al., 1995), knowledge of which should be considered in the implementation of butanol fermentations. Another important aspect associated with any large-scale fermentation is the cost of maintaining sterile conditions and the possibility of contamination. The use of antibiotics is undesirable not only for economic reasons, but also for the development of resistance. Running fermentations at low pH has been an effective measure in this regard, but requires acid-tolerant hosts. High temperature has also been used when the biotransformation is carried by a thermophilic microorganism (Sakai and Ezaki, 2006). However, the microorganism that produces the compound of interest most efficiently (i.e. with the highest productivity, titer, yield from cellulose, etc.), is rarely also

highly tolerant to acid, heat, or similar restrictive environments. Because environmental tolerance is generally a phenotype dictated by the expression of many genes, those traits are hard to engineer or introduce into a desired host. For this reason, strains that are easy to manipulate and are well characterized are good starting points for the development of production platforms.

3.2. Strain improvement

Improving environmental tolerance has been an active area of study because maximum productivity and titer are key determinants for the profitable fermentative production of commodity chemicals. Creative reactor or process design strategies have been successfully implemented to ameliorate product toxicity problems (Ezeji et al., 2007b), but such approaches are mostly complemented by strain engineering. In general, genetic changes for strain improvement may be of two types: rational (or directed) and random (or combinatorial). The first is straightforward when a genetic sequence that is likely to impact a trait of interest is known. The second is preferred when this knowledge is lacking, and relies in our ability to construct genetically diverse populations and screen them.

Random approaches have gained special attention, because detailed physiological mechanisms of tolerance remain widely uncharacterized. Traditionally, these efforts have been focused on long-term adaptation or serial rounds of mutagenesis and selection, usually referred to as "classical strain improvement" (CSI). The mutagenesis step is accomplished with chemical or physical mutagens (e.g. nitrosoguanidine and UV, respectively), producing libraries of variants that can be then screened for improvement in tolerance. A clear limitation of this approach is the non-transferability and intractability of the resulting genotypic changes. More recently, a variety of random-search (i.e. library-based) methods for strain improvement have emerged that are based on phenotypic alteration using tractable elements. These include artificial transcription factors (Park et al., 2003), global transcription machinery engineering (Alper et al., 2006; Alper and Stephanopoulos, 2007), libraries of siRNAs (Berns et al., 2004), randomized ribozymes (Miyagishi et al., 2005), knockout and overexpression libraries (Jin and Stephanopoulos, 2007), and others.

Although obvious, the fact that any genetic manipulation results in changes to an existing, functional genome is sometimes ignored when pursuing a strain improvement effort. This fact implies that the type of changes resulting in improvement depend on the genetic background of the host strain. As such, there is a limit for improvement defined by the maximum number of changes that can be implemented experimentally. Let us take the engineering of thermostolerance as an example: even if chaperones and proteases are overexpressed, thermophilic versions of key enzymes are introduced, membrane properties are modified, etc., the makeup of an organism that makes it tolerant to heat is a property of the system in its entirety, and resists simplification. This does not imply that tolerance cannot be improved with respect to that of the wild-type, but that the practical relevance of the limit for improvement depends on the phenotype we choose to engineer. Therefore, the prospects of finding an ideal strain for biofuel production reside on a balance between selecting the right host as a starting point and choosing which properties to change.

This fact is underscored by the data in Fig. 3, which shows that the tolerance of commonly used mesophilic, facultatively anaerobic biofuels production hosts to two stresses. *B. subtilis* beats the other six hosts by a large margin in the case of tolerance to 1-butanol, but is very sensitive to the fermentation inhibitors in dilute-acid pretreated (Schell et al., 2003), quicklime-neutralized corn stover hydrolysate (CSH). (Details of the CSH material are

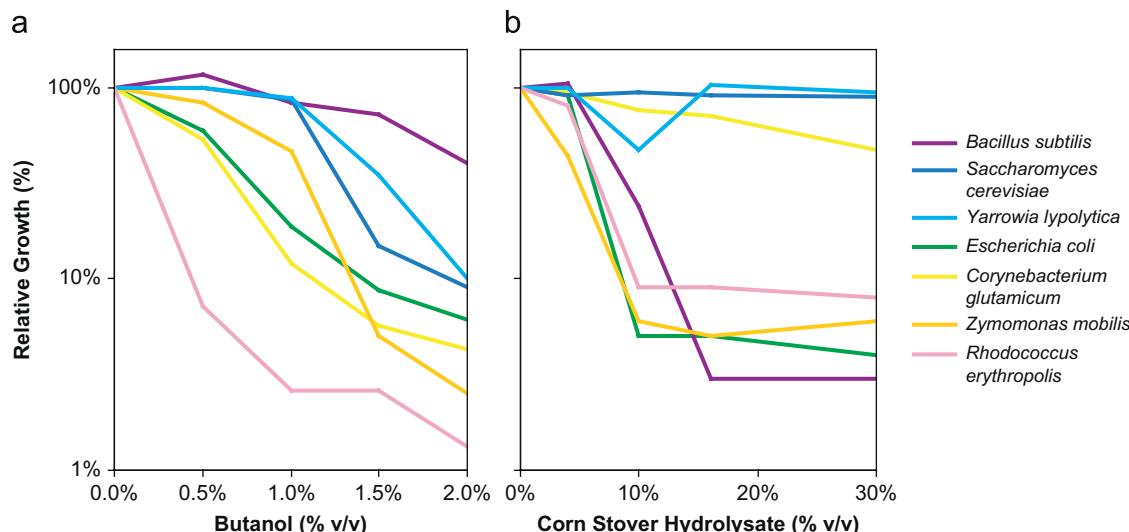


Fig. 3. The tolerance of seven common mesophilic, facultatively anaerobic host organisms to the stresses of (a) butanol tolerance, or (b) tolerance to neutralized corn stover hydrolysate. See supplemental material for full experimental details.

available in the supplemental materials.) Conversely, *Corynebacterium glutamicum* is comparatively much more tolerant to the CSH than to butanol. The yeasts *S. cerevisiae* and *Yarrowia lipolytica* are reasonably tolerant to both of these stresses. Data like that in Fig. 3 are useful for qualitatively comparing the different hosts. But, without tailoring temperatures, growth media, and, in the case of CSH, pretreatment and neutralization conditions to each particular host, it is difficult to extrapolate such data to process conditions.

3.3. Engineering environmental tolerance to common process stresses

Environmental stresses come in many disparate forms, only some of which have been reasonably well-studied. Heat tolerance has received significant attention, as running fermentations at higher temperatures curbs cooling costs and reduces the risk of contamination. Heat increases the fluidity of the membrane (Balogh et al., 2005; Mansilla et al., 2004; Shigapova et al., 2005), which in turn interferes with energy transduction and pH maintenance (Sikkema et al., 1995). In addition, it causes protein denaturation and aggregation, directly affecting most cellular functions (Riezman, 2004). Thermotolerance has been enhanced both by rational and random schemes. Overexpression of heat shock protein hsp22.4 (from *Chaetomium globosum*) in *S. cerevisiae* increased cell viability after a 4-h shift to 51°C (Liu et al., 2007). This approach has been extended to other organisms, from bacteria (Fiocco et al., 2007) to very complex multicellular systems (Feder et al., 1996; Welte et al., 1993). Thermotolerance has also been improved by preconditioning (Shigapova et al., 2005), overproduction of metabolites such as trehalose (Purvis et al., 2005), and supplementation with protectants such as betaine and choline (Holtmann and Bremer, 2004). These three and other compounds act similarly by altering the osmolarity of the cytoplasm, changing the water–protein interactions, stabilizing them and preventing aggregation (Cayley and Record, 2003; Conlin and Nelson, 2007; Ignatova and Giersch, 2006).

Combinatorial approaches have also been successful. For example, subjecting a mutagenized *Saccharomyces* population to serial freeze–thaw cycles followed by selection has delivered mutants with increased tolerance to multiple stresses, including heat (Cakar et al., 2005). Screening of artificial transcription factor

libraries has been used to engineer thermotolerance in *S. cerevisiae* (Park et al., 2003) and to determine a gene responsible for this phenotypic alteration in *E. coli* (Park et al., 2005).

Osmotolerance has also been studied as a target for strain improvement, as high substrate, product or salt concentrations that increase osmotic pressure are commonly encountered in industrial fermentations (Thatipamala et al., 1992; Varela et al., 2004). Some of the same strategies outlined for enhancing thermotolerance can be or have been applied. For example, trehalose and betaine have been reported to increase osmotolerance in *E. coli* (Miller and Ingram, 2007), and the protective role of trehalose in yeast has been reported (Hounsa et al., 1998). Transcriptional engineering has delivered yeast strains improved for growth in high salt (Park et al., 2003) and glucose (Alper et al., 2006). Similarly, this method has improved the tolerance of *E. coli* to a variety of stresses, as well as improved metabolite over-production (Alper and Stephanopoulos, 2007).

3.4. Biofuels-specific stresses

The problem of environmental tolerance is not only a challenge for fermentation technology in general, but is particularly relevant to the production of biofuels for two reasons. First, when the production strain cannot degrade lignocellulosic material directly, the substrate for fermentation may contain toxic compounds. This has been widely discussed for feedstocks derived from lignocellulosic hydrolysates, as pretreatment by acid hydrolysis produces a mixture of oligosaccharides, organic acids, phenolic derivatives, and furans (Sakai et al., 2007), all but the first of which are inhibitors of growth for many microorganisms. Second, the product of fermentation may itself be toxic, in which case the titer could be inherently limited. A classic example of this is ethanol production in *Saccharomyces* (Alper et al., 2006).

Lignocellulosic derivatives that have received most attention are furfural, hydroxymethyl furfural (HMF), acetic acid, and phenolic compounds. The amount and identity of the inhibitors after detoxification of hydrolysates depend on the method used (overliming, laccase treatment, charcoal, etc.) (Klinke et al., 2004). Although toxicity and detoxification issues have been mostly explored for ethanol, some studies for other fermentations such as butanol exist (Ezeji et al., 2007a). Because different compounds exert toxicity through different mechanisms and their effects

appear to be coupled (Ezeji et al., 2007a; Klinke et al., 2004; Taherzadeh et al., 1997a), solving the problem of environmental tolerance to the substrate cocktail by rational approaches and simple process modifications seems unlikely. However, partial successes from this front have been reported. Overexpression of ADH6 in *S. cerevisiae*, an 5-HMF-reducing enzyme, has enhanced conversion of the inhibitor which could be probably used in detoxification, but no increase in ethanol productivity was reported (Pettersson et al., 2006). A similar effort with ZWF1, encoding a glucose-6-phosphate dehydrogenase, resulted in higher furfural tolerance (Gorsich et al., 2006). Simultaneous overexpression of the genes would probably be synergistic, as the reduction of the inhibitor by ADH6 is NADPH-dependent, and ZWF1 is hypothesized to help this step by committing its substrate to the pentose-phosphate pathway, which produces the reduced cofactor. Overexpression of the enzyme phenylacrylic acid decarboxylase (from PAD1) resulted in *Saccharomyces* strains improved in ethanol productivity in the presence of ferulic and cinnamic acids (Larsson et al., 2001).

Manipulation of the fermentation pH has been used for alleviating tolerance to acetic acid, as toxicity is mainly effected by the protonated species (Lawford and Rousseau, 1998; Taherzadeh et al., 1997b). However, pH control is undesirable because of the additional cost associated with it, and because low pH reduces the risk of contamination as discussed above. Transferring a gene of acid-resistant *Oenococcus oeni* that responds to different stresses resulted in an *E. coli* strain with improved low pH tolerance (Morel et al., 2001). These or similarly constructed hosts may be better suited for fermentations at low pH.

Improvements from random approaches have also been reported. Genome shuffling of ethanologenic *Candida krusei* has delivered acetic acid-resistant mutants that perform better than the parent in ethanol fermentations in the presence of the inhibitor (Wei et al., 2008). The usefulness of CSI methods for improving tolerance of yeasts to lignocellulosic hydrolysate components has also been reported (Liu et al., 2005; Sonderegger et al., 2004). Similarly, studies describe that *Pichia stipitis* long-term adapted to increasing concentrations of hardwood hydrolysate partially neutralized or alkalinized with lime had higher ethanol productivity and titer (Nigam, 2001a,b).

Product inhibition has also been addressed, mainly for yeast ethanol fermentations. Most fuels have solvent-like properties, and are thought to impact the cell physiology similarly. In general, solvents and other lipophilic hydrocarbons partition into the membrane and disrupt its fluidity, which in turn results in ion leakage (Graca da Silveira et al., 2002; Ingram and Vreeland, 1980). Similar to the case of heat, this increase in membrane fluidity causes dissipation of the transmembrane pH and leads to an energy shortage, which ultimately results in cessation of growth. The harmful effect of membrane fluidity is therefore accentuated at high temperature and also in the presence of feedstock-derived inhibitors (Demain et al., 2005; Klinke et al., 2004), which results in an especially challenging problem.

Many ethanol-tolerant yeast strains have been independently isolated in the context of alcoholic beverage production, through hundreds of years of artificial trait selection. These are usually poorly characterized, but application of modern techniques has helped in the elucidation of possible ethanol-tolerance mechanisms in these strains (Shobayashi et al., 2007; Watanabe et al., 2007). Following a comparison of sake-brewing and laboratory strains using microarrays, overexpression of tryptophan-biosynthesis genes (TRP1-5) resulted in enhanced ethanol tolerance (Hirasawa et al., 2007). The mechanism behind this phenotype seems to be similar to that of strains with higher levels of trehalose (Kim et al., 1996), and for the same reasons outlined for thermotolerance.

The complexity of fuel/solvent tolerant phenotypes has invited the use of random approaches. A mutant of the TATA-binding protein, coded by SPT15, increased yeast tolerance to high-glucose, high-ethanol conditions through the alteration in expression of hundreds of genes (Alper et al., 2006). The improved phenotype could not be recovered from localized changes in gene expression, which is in tune with the complex nature of the stresses and underlines the need for *global* cellular engineering for strain improvement. A similar effort in *E. coli* delivered a mutant sigma factor with improved ethanol tolerance (Alper and Stephanopoulos, 2007). Overexpression of two genes in *C. acetobutylicum* that were found by screening a genomic DNA library resulted in increased butanol tolerance (Borden and Papoutsakis, 2007). Other localized genetic manipulations have been explored, like null mutations *ura7* and *gal6* that were selected by challenging a knockout library of *Saccharomyces* in ethanol (Yazawa et al., 2007). The mutant strains grew and consumed glucose faster in the presence of 8% ethanol compared to the wild-type, through changes in membrane lipid composition and other mechanisms that were not fully understood. CSI programs have also delivered ethanol-tolerant *E. coli* (Yomano et al., 1998) and butanol-tolerant *C. beijerinckii* mutants (Annous and Blaschek, 1991; Qureshi and Blaschek, 2001).

Even with the substantial research interest that this problem has attracted, environmental tolerance for biofuel fermentations continues to be a challenge. A main reason for *Saccharomyces* fermentations of ethanol being traditionally preferred is that this microorganism has been bred to precisely this end for hundreds of generations. Artificial evolution has already solved many of the limitations, but it has not had time to solve others (like tolerance to lignocellulosic hydrolysates) that are of practical significance today. Rational genetic manipulations based on "omics" data will likely be part of the answer, but random methods will also play a central role. Given that the genotypic space is practically infinitely large, better and more efficient ways of searching the phenotypic space are needed. Such research will contribute to the improvement of industrial strains rapidly enough to face the challenges of liquid fuel shortages.

4. Conclusions

Today's predominant microbially produced biofuel is starch-derived ethanol. However, further expansion of production capacity will require use of lignocellulosic feedstocks. The two best-developed technologies for conversion of lignocellulosics to fuel are (i) pretreatment and enzymatic hydrolysis to fermentable sugars, or (ii) gasification to syngas. Microbial photosynthetic processes may also render carbon dioxide as an attractive biomass feedstock.

An additional and orthogonal problem with starch-derived ethanol is the chemical properties of ethanol itself. Other fuel molecules, such as butanol, or liquid hydrocarbons of lipid or terpenoid origin, have higher energy densities, mix less readily with water, can be easier to separate from fermentation broth, and are compatible with the existing fuel distribution infrastructure. A primary barrier to the introduction of these technologies is insufficient microbe performance. That is, metabolic engineering research to improve the yield, titer, and productivity of microbial strains will be essential to the development of later-generation biofuels. We conjecture that yield may be the easiest of the trifecta to engineer, because the experimental tools for pathway manipulation, and the metabolic models required for evolutionary strain optimization are already in place. However, a similar toolset for improvement in productivity and titer is not available; in many cases, regulatory pathways governing are incompletely

understood. Further elucidation of these pathways is a much-needed avenue for future research.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.06.009.

References

- Ahmed, A., et al., 2006. Effects of biomass-generated producer gas constituents on cell growth, product distribution and hydrogenase activity of *Clostridium carboxidiورans* P7(T). *Biomass Bioenergy* 30, 665–672.
- Alegre, M.T., et al., 2004. Transformation of *Lactobacillus plantarum* by electroporation with in vitro modified plasmid DNA. *FEMS Microbiol. Lett.* 241, 73–77.
- Alper, H., Stephanopoulos, G., 2007. Global transcription machinery engineering: a new approach for improving cellular phenotype. *Metab. Eng.* 9, 258–267.
- Alper, H., et al., 2005a. Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. USA* 102, 12678–12683.
- Alper, H., et al., 2005b. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* 7, 155–164.
- Alper, H., et al., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science* 314, 1565–1568.
- Anous, B.A., Blaschek, H.P., 1991. Isolation and characterization of *Clostridium acetobutylicum* mutants with enhanced amylolytic activity. *Appl. Environ. Microbiol.* 57, 2544–2548.
- Atsumi, S., et al., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.*, doi:10.1016/j.ymben.2007.08.003.
- Aukrust, T.W., et al., 1995. Transformation of *Lactobacillus* by electroporation. *Methods Mol. Biol.* 47, 201–208.
- Balatinecz, J.J., Kretschmann, D.E., 2001. In: Dickmann, D.I., et al. (Eds.), *Properties and Utilization of Poplar Wood*. NRC Research Press, National Research Council of Canada, Ottawa (Chapter 9).
- Balogh, G., et al., 2005. The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. *FEBS J.* 272, 6077–6086.
- Berns, K., et al., 2004. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 428, 431–437.
- Borden, J.R., Papoutsakis, E.T., 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* 73, 3061–3068.
- Brown, R.C., 2003. *Biorenewable Resources: Engineering New Products from Agriculture*. Iowa State Press, Ames, IA.
- Cakar, Z.P., et al., 2005. Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 5, 569–578.
- Cayley, S., Record Jr., M.T., 2003. Roles of cytoplasmic osmolytes, water, and crowding in the response of *Escherichia coli* to osmotic stress: biophysical basis of osmoprotection by glycine betaine. *Biochemistry* 42, 12596–12609.
- Chisti, Y., 2007. Biodiesel from microalgae. *Biotechnol. Adv.* 25, 294–306.
- Coll, J.M., 2006. Methodologies for transferring DNA into eukaryotic microalgae. *Spanish J. Agric. Res.* 4, 316–330.
- Conlin, L.K., Nelson, H.C., 2007. The natural osmolyte trehalose is a positive regulator of the heat-induced activity of yeast heat shock transcription factor. *Mol. Cell. Biol.* 27, 1505–1515.
- Cysewski, G.R., Wilke, C.R., 1976. Utilization of cellulosic materials through enzymatic-hydrolysis. 1. Fermentation of hydrolysates to ethanol and single-cell protein. *Biotechnol. Bioeng.* 18, 1297–1313.
- da Silva, E.A., et al., 2005. Isolation by genetic and physiological characteristics of a fuel-ethanol fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation. *J. Ind. Microbiol. Biotechnol.* 32, 481–486.
- Datar, R.P., et al., 2004. Fermentation of biomass-generated producer gas to ethanol. *Biotechnol. Bioeng.* 86, 587–594.
- Davis, Stacy C., Diegel, Susan W., 2007. *Transportation Energy Data Book*, Edition 26. Office of Energy Efficiency and Renewable Energy, US Department of Energy, Report no. ORNL-6978.
- Demain, A.L., et al., 2005. Cellulase, clostridia, and ethanol. *Microbiol. Mol. Biol. Rev.* 69, 124–154.
- Donaldson, G.K., et al., 2007. Fermentative production of four-carbon alcohols. US patent application 20070092957 A1, Filed 26 April 2007.
- Ezeji, T.C., et al., 2004. Acetone butanol ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. *Appl. Microbiol. Biotechnol.* 63, 653–658.
- Ezeji, T., et al., 2007a. Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnol. Bioeng.* 97, 1460–1469.
- Ezeji, T.C., et al., 2007b. Bioproduction of butanol from biomass: from genes to bioreactors. *Curr. Opin. Biotechnol.* 18, 220–227.
- Feder, M.E., et al., 1996. Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. *J. Exp. Biol.* 199, 1837–1844.
- Fiocco, D., et al., 2007. Improved adaptation to heat, cold, and solvent tolerance in *Lactobacillus plantarum*. *Appl. Microbiol. Biotechnol.* 77, 909–915.
- Fong, S.S., et al., 2003. Description and interpretation of adaptive evolution of *Escherichia coli* K-12 MG1655 by using a genome-scale in silico metabolic model. *J. Bacteriol.* 185, 6400–6408.
- Gordon, J.M., Polle, J.E., 2007. Ultrahigh bioproduction from algae. *Appl. Microbiol. Biotechnol.* 76, 969–975.
- Gorsich, S.W., et al., 2006. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 71, 339–349.
- Graca da Silveira, M., et al., 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Appl. Environ. Microbiol.* 68, 6087–6093.
- Grima, E.M., et al., 2003. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnol. Adv.* 20, 491–515.
- Hankamer, B., et al., 2007. Photosynthetic biomass and H-2 production by green algae: from bioengineering to bioreactor scale-up. *Physiol. Plantarum* 131, 10–21.
- Henstra, A.M., et al., 2007. Microbiology of synthesis gas fermentation for biofuel production. *Curr. Opin. Biotechnol.* 18, 200–206.
- Hill, J., et al., 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl. Acad. Sci. USA* 103, 11206–11210.
- Hirasawa, T., et al., 2007. Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. *J. Biotechnol.* 131, 34–44.
- Ho, N.W.Y., et al., 1998. Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* 64, 1852–1859.
- Holtmann, G., Bremer, E., 2004. Thermoprotection of *Bacillus subtilis* by exogenously provided glycine betaine and structurally related compatible solutes: involvement of Opu transporters. *J. Bacteriol.* 186, 1683–1693.
- Hounsa, C.G., et al., 1998. Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology* 144 (Pt 3), 671–680.
- <http://www.pipeline101.com/overview/products-pl.html>. Accessed 4 December 2007.
- Ibarra, R.U., et al., 2002. *Escherichia coli* K-12 undergoes adaptive evolution to achieve in silico predicted optimal growth. *Nature* 420, 186–189.
- Ignatova, Z., Giersch, L.M., 2006. Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant. *Proc. Natl. Acad. Sci. USA* 103, 13357–13361.
- Ingram, L.O., Vreeland, N.S., 1980. Differential effects of ethanol and hexanol on the *Escherichia coli* cell envelope. *J. Bacteriol.* 144, 481–488.
- Ingram, L.O., et al., 1999. Enteric bacterial catalysts for fuel ethanol production. *Biotechnol. Prog.* 15, 855–866.
- Jin, Y.S., Stephanopoulos, G., 2007. Multi-dimensional gene target search for improving lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* 9, 337–347.
- Johnson, J.C., 2006. Technology assessment of biomass ethanol: a multi-objective, life cycle approach under uncertainty. In: Johnson, J.C. (Ed.), *Chemical Engineering*. MIT, Cambridge, MA, p. 280.
- Kim, J., et al., 1996. Disruption of the yeast ATH1 gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications. *Appl. Environ. Microbiol.* 62, 1563–1569.
- Klinke, H.B., et al., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotechnol.* 66, 10–26.
- Larsson, S., et al., 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. *Appl. Microbiol. Biotechnol.* 57, 167–174.
- Lawford, H.G., Rousseau, J.D., 1998. Improving fermentation performance of recombinant *Zymomonas* in acetic acid-containing media. *Appl. Biochem. Biotechnol.* 70–72, 161–172.
- Lee, Y.K., 2001. Microalgal mass culture systems and methods: their limitation and potential. *J. Appl. Phycol.* 13, 307–315.
- Lide, D.R. (Ed.), 2006–2007. *CRC Handbook of Chemistry and Physics*. Chapman & Hall/CRC Press, Boca Raton, FL.
- Liu, Z.L., et al., 2005. Enhanced biotransformation of furfural and hydroxymethyl-furfural by newly developed ethanologenic yeast strains. *Appl. Biochem. Biotechnol.* 121–124, 451–460.
- Liu, X., et al., 2006. Construction and characterization of ack deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. *Biotechnol. Prog.* 22, 1265–1275.
- Liu, Z.H., et al., 2007. A heat shock protein gene (hsp22.4) from *Chaetomium globosum* confers heat and Na(2)CO₃ (3) tolerance to yeast. *Appl. Microbiol. Biotechnol.* 77, 901–908.

- Loke, H.K., et al., 2000. Active acetyl-CoA synthase from *Clostridium thermoaceticum* obtained by cloning and heterologous expression of *acsAB* in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 97, 12530–12535.
- Lynd, L.R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. Annu. Rev. Energy Environ. 21, 403–465.
- Lynd, L.R., et al., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–577 (table of contents).
- Maki-Arvela, P., et al., 2007. Catalytic deoxygenation of fatty acids and their derivatives. Energy Fuels 21, 30–41.
- Mansilla, M.C., et al., 2004. Control of membrane lipid fluidity by molecular thermosensors. J. Bacteriol. 186, 6681–6688.
- Matsushima, P., et al., 1989. Transduction and transformation of plasmid DNA in *Streptomyces fradiae* strains that express different levels of restriction. J. Bacteriol. 171, 3080–3084.
- McAloon, A., et al., 2000. In: Service, N.A.R. (Ed.), Determining the Cost of Producing Ethanol from Corn Starch and Lignocellulosic Feedstocks. NREL, Golden, CO.
- Miller, E.N., Ingram, L.O., 2007. Combined effect of betaine and trehalose on osmotic tolerance of *Escherichia coli* in mineral salts medium. Biotechnol. Lett. 29, 213–217.
- Miyagishi, M., et al., 2005. Chemistry-based RNA technologies: demonstration of usefulness of libraries of ribozymes and short hairpin RNAs (shRNAs). Nucleic Acids Symp. Ser. (Oxf.), 91–92.
- Mohagheghi, A., et al., 1998. Cofermentation of glucose, xylose, and arabinose by mixed cultures of two genetically engineered *Zymomonas mobilis* strains. Appl. Biochem. Biotechnol. 70–72, 285–299.
- Morel, F., et al., 2001. Improved acid tolerance of a recombinant strain of *Escherichia coli* expressing genes from the acidophilic bacterium *Oenococcus oeni*. Lett. Appl. Microbiol. 33, 126–130.
- Ng, T.K., et al., 1981. Ethanol-production by thermophilic bacteria-fermentation of cellulosic substrates by cocultures of clostridium-thermocellum and clostridium-thermohydrosulfuricum. Appl. Environ. Microbiol. 41, 1337–1343.
- Nigam, J.N., 2001a. Development of xylose-fermenting yeast *Pichia stipitis* for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate. J. Appl. Microbiol. 90, 208–215.
- Nigam, J.N., 2001b. Ethanol production from hardwood spent sulfite liquor using an adapted strain of *Pichia stipitis*. J. Ind. Microbiol. Biotechnol. 26, 145–150.
- Ono, E., Cuello, J.L., 2004. Design parameters of solar concentrating systems for CO₂-mitigating algal photobioreactors. Energy 29, 1651–1657.
- Park, K.S., et al., 2003. Phenotypic alteration of eukaryotic cells using randomized libraries of artificial transcription factors. Nat. Biotechnol. 21, 1208–1214.
- Park, K.S., et al., 2005. Phenotypic alteration and target gene identification using combinatorial libraries of zinc finger proteins in prokaryotic cell. J. Bacteriol. 187, 5496–5499.
- Perlach, R.D., et al., 2005. Biomass as a Feedstock for a Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion-Ton Annual Supply. U.S. Department of Energy/Department of Agriculture. Report nos. DOE/GO-102995-2135 and ORNL/TM-2005/66.
- Petersson, A., et al., 2006. A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. Yeast 23, 455–464.
- Phillips, S., et al., 2007. Thermochemical Ethanol via Indirect Gasification and Mixed Alcohol Synthesis of Lignocellulosic Biomass. National Renewable Energy Laboratory, U.S. Department of Energy. Report no. NREL/TP-510-41168.
- Purvis, J.E., et al., 2005. Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress. Appl. Environ. Microbiol. 71, 3761–3769.
- Qureshi, N., Blaschek, H.P., 2001. Recent advances in ABE fermentation: hyperbutanol producing *Clostridium beijerinckii* BA101. J. Ind. Microbiol. Biotechnol. 27, 287–291.
- Ragauskas, A.J., et al., 2006. The path forward for biofuels and biomaterials. Science 311, 484–489.
- Ragsdale, S.W., 2004. Life with carbon monoxide. Crit. Rev. Biochem. Mol. Biol. 39, 165–195.
- Rajagopalan, S., et al., 2002. Formation of ethanol from carbon monoxide via a new microbial catalyst. Biomass Bioenergy 23, 487–493.
- Ravagnani, A., et al., 2000. Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. Mol. Microbiol. 37, 1172–1185.
- Riezman, H., 2004. Why do cells require heat shock proteins to survive heat stress? Cell Cycle 3, 61–63.
- Ro, D.K., et al., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.
- Sakai, K., Ezaki, Y., 2006. Open l-lactic acid fermentation of food refuse using thermophilic *Bacillus coagulans* and fluorescence in situ hybridization analysis of microflora. J. Biosci. Bioeng. 101, 457–463.
- Sakai, S., et al., 2007. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested *Corynebacterium glutamicum* R. Appl. Environ. Microbiol. 73, 2349–2353.
- Sanchez, A.M., et al., 2005. Effect of different levels of NADH availability on metabolic fluxes of *Escherichia coli* chemostat cultures in defined medium. J. Biotechnol. 117, 395–405.
- Schell, D.J., et al., 2003. Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor: investigation of yields, kinetics, and enzymatic digestibilities of solids. Appl. Biochem. Biotechnol. 105–108, 69–85.
- Shahidi, F., 2005. Bailey's Industrial Oil and Fat Products. Wiley.
- Sheehan, J., et al., 1998. A Look Back at the US Department of Energy's Aquatic Species Program—Biodiesel from Algae. National Renewable Energy Laboratory, Golden, CO.
- Shigapova, N., et al., 2005. Membrane fluidization triggers membrane remodeling which affects the thermotolerance in *Escherichia coli*. Biochem. Biophys. Res. Commun. 328, 1216–1223.
- Shobayashi, M., et al., 2007. Genome-wide expression profile of sake brewing yeast under shaking and static conditions. Biosci. Biotechnol. Biochem. 71, 323–335.
- Sikkema, J., et al., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59, 201–222.
- Sipma, J., et al., 2006. Microbial CO conversions with applications in synthesis gas purification and bio-desulfurization. Crit. Rev. Biotechnol. 26, 41–65.
- Sonderegger, M., et al., 2004. Fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains. Biotechnol. Bioeng. 87, 90–98.
- Stevens, D.R., Purton, S., 1997. Genetic engineering of eukaryotic algae: progress and prospects. J. Phycol. 33, 713–722.
- Stichlmair, J., 2000. Distillation and Rectification. Ullmann's Encyclopedia of Industrial Chemistry. Wiley-VCH Verlag GmbH.
- Sutton, D., et al., 2001. Review of literature on catalysts for biomass gasification. Fuel Process. Technol. 73, 155–173.
- Taherzadeh, M.J., et al., 1997a. Characterization and fermentation of dilute-acid hydrolysates from wood. Ind. Eng. Chem. Res. 36, 4659–4665.
- Taherzadeh, M.J., et al., 1997b. Acetic acid-friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*? Chem. Eng. Sci. 52, 2653–2659.
- Thatipamala, R., et al., 1992. Effects of high product and substrate inhibitions on the kinetics and biomass and product yields during ethanol batch fermentation. Biotechnol. Bioeng. 40, 289–297.
- Thomas, A., 2000. Fats and Fatty Oils. Ullmann's Encyclopedia of Industrial Chemistry. Wiley-VCH Verlag GmbH.
- Transportation Energy Data Book: Edition 26, 2007. Department of Energy, Energy's Office of Energy Efficiency and Renewable Energy.
- Tyurin, M.V., et al., 2004. Electroporation of *Clostridium thermocellum*. Appl. Environ. Microbiol. 70, 883–890.
- Umemoto, A., et al., 1996. The effect of the crp genotypes on the transformation efficiency in *Escherichia coli*. DNA Res. 3, 93–94.
- van Maris, A.J.A., et al., 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 90, 391–418.
- Varela, C.A., et al., 2004. Osmotic stress response: quantification of cell maintenance and metabolic fluxes in a lysine-overproducing strain of *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 70, 4222–4229.
- Voelker, T.A., Davies, H.M., 1994. Alteration of the specificity and regulation of fatty acid synthesis of *Escherichia coli* by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J. Bacteriol. 176, 7320–7327.
- Watanabe, M., et al., 2007. Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol-tolerance sake yeast Kyokai no. 11. J. Biosci. Bioeng. 104, 163–170.
- Wei, P., et al., 2008. Genome shuffling of ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. Biotechnol. Appl. Biochem. 49, 113–120.
- Welte, M.A., et al., 1993. A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. Curr. Biol. 3, 842–853.
- Wilkinson, S.R., et al., 1995. Molecular genetics and the initiation of solventogenesis in *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052. FEMS Microbiol. Rev. 17, 275–285.
- Withers, S.T., et al., 2007. Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. Appl. Environ. Microbiol. 73, 6277–6283.
- Yazawa, H., et al., 2007. Disruption of URA7 and GAL6 improves the ethanol tolerance and fermentation capacity of *Saccharomyces cerevisiae*. Yeast 24, 551–560.
- Yergin, D., et al., 2006. Gasoline and the American People 2007. Cambridge Energy Research Associates, Inc.
- Yomano, L.P., et al., 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20, 132–138.
- Zhang, Y., et al., 2003. Biodiesel production from waste cooking oil: 1. Process design and technological assessment. Bioresour. Technol. 89, 1–16.
- Zhang, Y.H.P., et al., 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24, 452–481.

LETTERS

Production of the antimalarial drug precursor artemisinic acid in engineered yeast

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Malaria is a global health problem that threatens 300–500 million people and kills more than one million people annually¹. Disease control is hampered by the occurrence of multi-drug-resistant strains of the malaria parasite *Plasmodium falciparum*^{2,3}. Synthetic antimalarial drugs and malaria vaccines are currently being developed, but their efficacy against malaria awaits rigorous clinical testing^{4,5}. Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* L (family Asteraceae; commonly known as sweet wormwood), is highly effective against multi-drug-resistant *Plasmodium* spp., but is in short supply and unaffordable to most malaria sufferers⁶. Although total synthesis of artemisinin is difficult and costly⁷, the semi-synthesis of artemisinin or any derivative from microbially sourced artemisinic acid, its immediate precursor, could be a cost-effective, environmentally friendly, high-quality and reliable source of artemisinin^{8,9}. Here we report the engineering of *Saccharomyces cerevisiae* to produce high titres (up to 100 mg l⁻¹) of artemisinic acid using an engineered mevalonate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (*CYP71AV1*) from *A. annua* that performs a three-step oxidation of amorphha-4,11-diene to artemisinic acid. The synthesized artemisinic acid is transported out and retained on the outside of the engineered yeast, meaning that a simple and inexpensive purification process can be used to obtain the desired product. Although the engineered yeast is already capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua*, yield optimization and industrial scale-up will be required to raise artemisinic acid production to a level high enough to reduce artemisinin combination therapies to significantly below their current prices.

We engineered artemisinic-acid-producing yeast in three steps, by (1) engineering the farnesyl pyrophosphate (FPP) biosynthetic pathway to increase FPP production and decrease its use for sterols, (2) introducing the amorphadiene synthase gene (*ADS*) from *A. annua* into the high FPP producer to convert FPP to amorphadiene, and (3) cloning a novel cytochrome P450 that performs a three-step oxidation of amorphadiene to artemisinic acid from *A. annua* and expressing it in the amorphadiene producer (Fig. 1). The first committed reaction in artemisinin biosynthesis is catalysed by *ADS*¹⁰, which has been characterized and used for *de novo* production of amorphadiene in *Escherichia coli*¹¹. To test for improvements in FPP production, we expressed *ADS* under the control of the *GAL1* promoter on the pRS425 plasmid (see Supplementary Information for details). Yeast engineered with *ADS* alone produced a low quantity of amorphadiene (Fig. 2, strain EPY201, 4.4 mg l⁻¹ amorphadiene).

To increase FPP production in *S. cerevisiae*, the expression of several genes responsible for FPP synthesis was upregulated, and one gene responsible for FPP conversion to sterols was downregulated. All of these modifications to the host strain were made by chromosomal integration to ensure the genetic stability of the host strain. Overexpression of a truncated, soluble form of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*tHMGR*)¹² improved amorphadiene production approximately fivefold (Fig. 2, strain EYP208). Downregulation of *ERG9*, which encodes squalene synthase (the first step after FPP in the sterol biosynthetic pathway), using a methionine-repressible promoter (*P_{MET3}*)¹³ increased amorphadiene production an additional twofold (Fig. 2, strain EPY225). Although *upc2-1*, a semi-dominant mutant allele that enhances the activity of *UPC2* (a global transcription factor regulating the biosynthesis of sterols in *S. cerevisiae*)¹⁴, had only a modest effect on amorphadiene production when overexpressed in the EPY208 background (Fig. 2, strain EPY210), the combination of downregulating *ERG9* and overexpressing *upc2-1* increased amorphadiene production to 105 mg l⁻¹ (Fig. 2, strain EPY213). Integration of an additional copy of *tHMGR* into the chromosome further increased amorphadiene production by 50% to 149 mg l⁻¹ (Fig. 2, strain EPY219). Although overexpression of the gene encoding FPP synthase (*ERG20*) had little effect on total amorphadiene production (Fig. 2, strain EPY224), the specific production increased by about 10% owing to a decrease in cell density. Combining all of these modifications resulted in a strain (EPY224) able to produce 153 mg l⁻¹ amorphadiene, a sesquiterpene production level nearly 500-fold higher than previously reported¹⁵.

To create a strain that produced artemisinic acid from amorphadiene, we isolated genes encoding enzymes responsible for oxidizing amorphadiene to artemisinic acid in *A. annua*. Artemisinin is a sesquiterpene lactone derivative, which is the most widespread and characteristic class of secondary metabolites found in Asteraceae (also known as Compositae)¹⁶. We hypothesized that plants belonging to the Asteraceae family would share common ancestor enzymes for the early steps in the biosynthesis of sesquiterpene lactones, and therefore undertook a comparative genomic analysis of plants in the Asteraceae family. Previous cell-free assays have indicated that a cytochrome P450 monooxygenase (P450) catalyses the first region-specific hydroxylation of amorphadiene (Fig. 1) in *A. annua*¹⁷. We thus retrieved P450-expressed-sequence tags (ESTs) from the Asteraceae EST-database generated from two Asteraceae crops, sunflower and lettuce (<http://www.cgpdb.ucdavis.edu>). Use of degenerate primers highly specific to the Asteraceae CYP71 and CYP82 subfamilies (the most abundant P450 subfamilies in Asteraceae) enabled the isolation

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of several unique P450 fragments from an *A. annua* trichome-enriched complementary DNA pool. Using BLAST¹⁸ analyses of these P450 gene fragments against sunflower and lettuce ESTs, we identified a single *A. annua* P450 gene fragment that had surprisingly high sequence identity (85–88% at the amino-acid level) to ESTs of unknown function from both sunflower and lettuce. Sequence identity of this *A. annua* P450 fragment to other P450 fragments outside the Asteraceae family was much lower (~50% at the amino-acid level), indicating that this P450 is highly conserved in three distantly related genera in the Asteraceae family, but not in plants outside the Asteraceae family. This P450 gene was therefore a good candidate for a conserved Asteraceae sesquiterpene lactone biosynthetic enzyme.

The corresponding full-length P450 cDNA (*CYP71AV1*), encoding an open reading frame of 495 amino acids, was recovered from *A. annua*. Phylogenetic analysis showed that *CYP71AV1* shares a close lineage with other P450s that catalyse the hydroxylation of

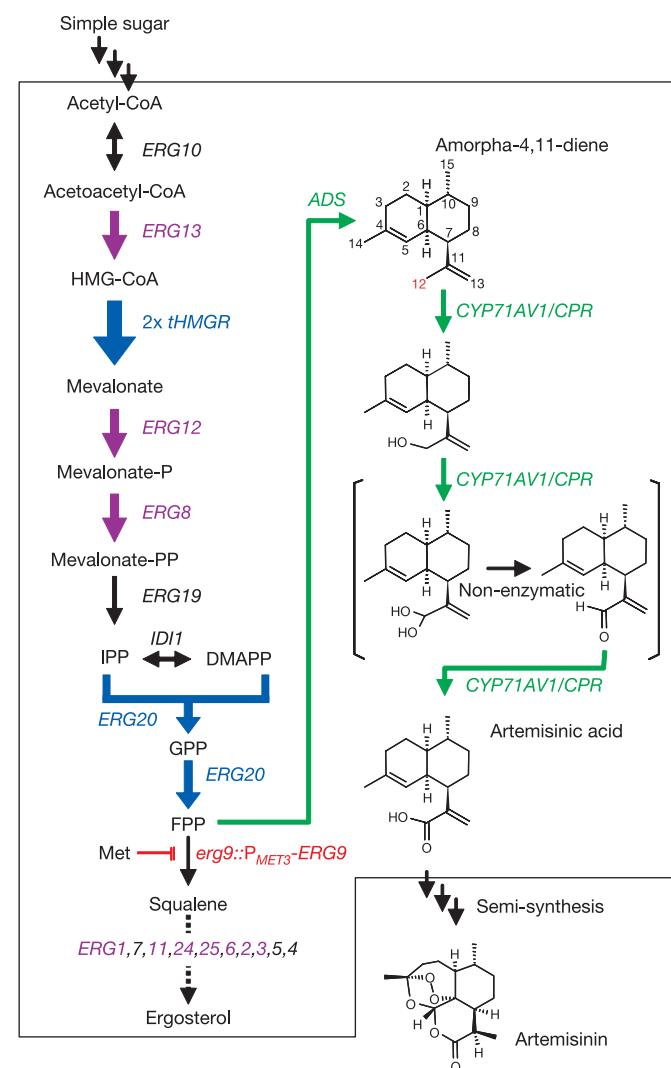


Figure 1 | Schematic representation of the engineered artemisinic acid biosynthetic pathway in *S. cerevisiae* strain EPY224 expressing *CYP71AV1* and *CPR*. Genes from the mevalonate pathway in *S. cerevisiae* that are directly upregulated are shown in blue; those that are indirectly upregulated by *upc2-1* expression are in purple; and the red line denotes repression of *ERG9* in strain EPY224. The pathway intermediates IPP, DMAPP and GPP are defined as isopentenyl pyrophosphate, dimethyl allyl pyrophosphate and geranyl pyrophosphate, respectively. Green arrows indicate the biochemical pathway leading from farnesyl pyrophosphate (FPP) to artemisinic acid, which was introduced into *S. cerevisiae* from *A. annua*. The three oxidation steps converting amorphadiene to artemisinic acid by *CYP71AV1* and *CPR* are shown.

monoterpenoids (CYP71D13/18; ref. 19), sesquiterpenoids (CYP71D20; ref. 20) or diterpenoids (CYP71D16; ref. 21), further suggesting the potential involvement of this P450 in terpenoid metabolism (Supplementary Information). For functional, heterologous expression of *CYP71AV1*, its native redox partner, NADPH: cytochrome P450 oxidoreductase (*CPR*), was also isolated from *A. annua*, and its biochemical function was confirmed *in vitro*. Michaelis–Menten constants (*K_m*) for cytochrome *c* and NADPH were determined to be $4.3 \pm 0.7 \mu\text{M}$ and $23.0 \pm 4.4 \mu\text{M}$ (mean \pm s.d., $n = 3$), respectively.

Using *A. annua* *CPR* as a redox partner for *CYP71AV1*, we then investigated whether *CYP71AV1* could catalyse the conversion of amorphadiene to more oxygenated products *in vivo*. The transgenic yeast strain EPY224 was transformed with a vector harbouring *CPR* and *CYP71AV1* under the control of galactose-inducible promoters. After galactose induction, the ether-extractable fraction of the yeast culture medium and cell pellet were analysed by gas chromatography mass spectrometry (GC–MS). A single chromatographic peak unique to EPY224 co-expressing *CYP71AV1* and *CPR* was detected in both the yeast culture medium and cell pellet, but was not present in control yeast (EPY224 expressing *CPR* only). However, more than 95% of this novel compound was associated with the cell pellet. In GC–MS analysis, the electron-impact mass spectrum and retention time of this compound were identical to those of artemisinic acid isolated from *A. annua* (Fig. 3). In a shake-flask culture, $32 \pm 13 \text{ mg l}^{-1}$ (mean \pm s.d., $n = 7$) artemisinic acid was produced from EPY224 expressing *CYP71AV1* and *CPR*. Notably, the pathway intermediates, artemisinic alcohol and artemisinic aldehyde, were present at negligible levels in the culture medium and cell pellets of EPY224 engineered with *CYP71AV1* and *CPR*. (Artemisinic alcohol was present at less than 5% of the artemisinic acid in the cell pellet, and no artemisinic aldehyde was detected.)

Almost all (>96%) of the synthesized artemisinic acid was removed from the cell pellet by washing with alkaline buffer (pH 9 Tris-HCl buffer supplemented with 1.2 M sorbitol), with less than 2% remaining in the washed cell pellet or culture medium. Thus, it seems that artemisinic acid is efficiently transported out of yeast cells but remains bound to the cell surface when it is protonated under acidic culture conditions. We used this feature to develop a one-step purification method: a single silica gel column chromatographic separation of ether-extracted artemisinic acid from the wash buffer routinely yielded >95% pure artemisinic acid. In a one-litre aerated bioreactor, 115 mg of artemisinic acid was produced, of which 76 mg was purified using this method. The ¹H and ¹³C nuclear magnetic resonance spectra of this yeast-derived artemisinic acid were identical to those of artemisinic acid isolated from the leaves of *A. annua*, and are consistent with previously reported values^{22,23}. We can therefore confirm that structurally authentic artemisinic acid is synthesized by

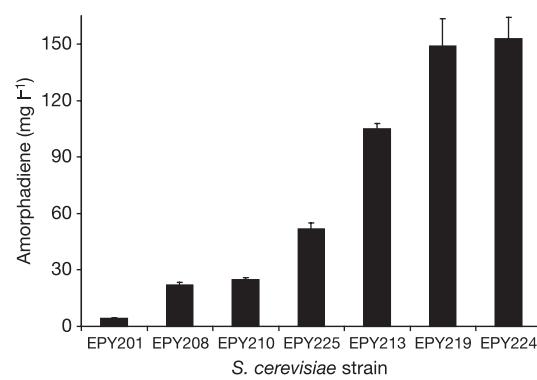


Figure 2 | Production of amorphadiene by *S. cerevisiae* strains. The various *S. cerevisiae* strains are described in the text. Cultures were sampled after 144 h of growth, and amorphadiene levels were quantified. Data, shown as total production, are mean \pm s.d. ($n = 3$).

transgenic yeast *de novo*. The transgenic yeast produced artemisinic acid at a biomass fraction comparable to that produced by *A. annua* (4.5% dry weight in yeast compared to 1.9% artemisinic acid and 0.16% artemisinin in *A. annua*) but over a much shorter time (4–5 days for yeast versus several months for *A. annua*). As such, the specific productivity of the engineered yeast strain is nearly two orders of magnitude greater than *A. annua*.

Three-step oxidations by P450 enzymes have been previously reported in plant hormone gibberellin biosynthetic pathways^{24,25}. We conducted *in vitro* enzyme assays to identify whether CYP71AV1 catalyses all three oxidation reactions from amorphadiene to artemisinic acid. Microsomes were isolated from *S. cerevisiae* strain YPH499 expressing either CPR alone or CPR and CYP71AV1, and incubated with pathway intermediates (amorphadiene, artemisinic alcohol or artemisinic aldehyde) (Fig. 4). Microsomes from the CPR control did not catalyse the conversion of any pathway intermediate to more oxidized products, whereas efficient conversion of amorphadiene, artemisinic alcohol and artemisinic aldehyde to the final product artemisinic acid was detected in microsomes containing

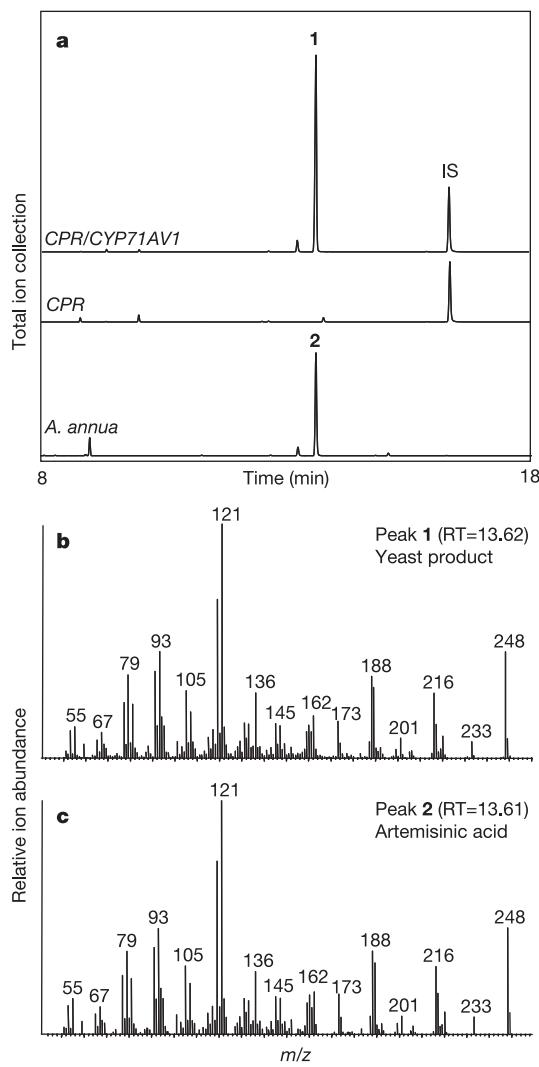


Figure 3 | GC-MS analysis of artemisinic acid produced from *A. annua* and transgenic yeast. **a**, Cell pellets from *S. cerevisiae* strain EPY224 expressing CPR or CPR and CYP71AV1 were washed using an alkaline buffer followed by acidification and ether extraction. Artemisinic acid was extracted from *A. annua* leaves using hexane. Methyl esters of both samples were prepared with trimethylsilyl-diazomethane before GC-MS analysis. The internal standard (IS) is the methyl ester of 4-octylbenzoic acid. **b, c**, Mass spectra and retention times of artemisinic acid from yeast (**b**) and *A. annua* (**c**). RT, retention time (in min).

CYP71AV1 and CPR. These *in vitro* assays demonstrate unambiguously that recombinant CYP71AV1 is able to catalyse three oxidation reactions at the C12 position of amorphadiene. Previous *in vitro* enzyme assays using *A. annua* protein extract have suggested that soluble alcohol and aldehyde dehydrogenases and a C11,13 double-bond reductase (which acts on the aldehyde) are involved in artemisinin biosynthesis¹⁷. Although we cannot exclude a catalytic role for additional alcohol and aldehyde dehydrogenases in artemisinin synthesis in *A. annua*, the efficient *in vivo* conversion of amorphadiene to artemisinic acid by recombinant CYP71AV1 indicates that the membrane-bound, multifunctional CYP71AV1 is a key contributor to artemisinin biosynthesis.

In summary, we have created a strain of *S. cerevisiae* capable of producing high levels of artemisinic acid by engineering the FPP biosynthetic pathway to increase FPP production and by expressing amorphadiene synthase, a novel cytochrome P450 and its redox partner from *A. annua*. Given the existence of known, relatively high-yielding chemistry for the conversion of artemisinic acid to artemisinin or any other derivative that might be desired^{8,9}, microbially produced artemisinic acid is a viable source of this potent family of antimalarial drugs. Upon optimization of product titres, a conservative analysis suggests that artemisinin combination therapies could be offered significantly below their current prices (see Supplementary Information). In addition to cost savings, this bioprocess should not be subject to factors like weather or political climates that may affect plant cultivation. Furthermore, artemisinic acid from a microbial source can be extracted using an environmentally friendly process without worrying about contamination by other terpenes that are produced by plants, thereby increasing the ease with which it can be produced while reducing purification costs.

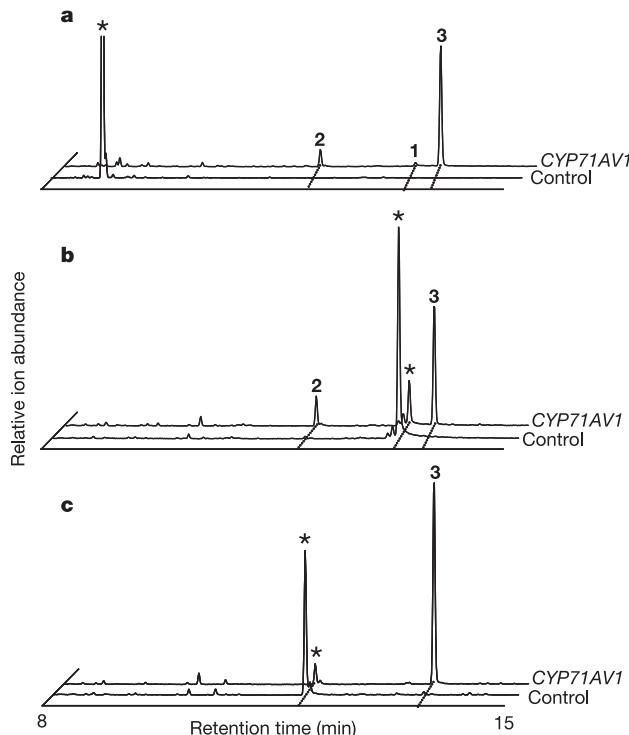


Figure 4 | In vitro enzyme assays for CYP71AV1 activities. Microsomes were isolated from *S. cerevisiae* strain YPH499 expressing CPR (control) or CPR and CYP71AV1 (CYP71AV1). **a–c**, For each enzyme assay, 10 μ M amorphadiene (**a**), 25 μ M artemisinic alcohol (**b**) or 25 μ M artemisinic aldehyde (**c**) was used. Chromatographic peaks for the substrates used are indicated with asterisks. Ether-extractable fractions were derivatized and analysed by GC-MS in selective ion mode (m/z : 121, 189, 204, 218, 220 and 248). Enzymatic products are as indicated: 1, artemisinic alcohol (retention time 13.20 min); 2, artemisinic aldehyde (retention time 11.79 min); 3, artemisinic acid (retention time 13.58 min, detected as methyl ester).

METHODS

Detailed descriptions of the methods used in the generation and characterization of *S. cerevisiae* EPY strains, the cloning of *CYP71AV1* and *CPR*, and the semi-synthesis of artemisinic alcohol and artemisinic aldehyde are provided in Supplementary Information.

Chemicals and plant material. Authentic artemisinic acid was purchased from Apin Chemicals or extracted from *A. annua* leaves with hexane, as described in Supplementary Information. *A. annua* plants were started from seeds (Sandeman Seeds) and grown in a greenhouse at the University of California, Berkeley.

GC-MS analysis of amorphadiene. Amorphadiene production by the various strains was measured by GC-MS using a dodecane layer to trap volatile amorphadiene (see Supplementary Information for details). Amorphadiene (90% pure) was prepared by fermentation using an *E. coli* strain¹¹, and was used to construct a standard curve to determine amorphadiene production levels.

In vivo production, purification and chemical analysis of artemisinic acid.

Pre-cultured EPY224 strains transformed with pESCURA::CPR or pESCURA::CPR/CYP71AV1 were inoculated at an absorbance of 0.05 at 600 nm (A_{600}) in 25 ml synthetic defined medium lacking histidine, leucine, methionine and uracil, and supplemented with 0.2% dextrose, 1.8% galactose and 1 mM methionine. After 120 h of culture at 30 °C, the cells were centrifuged and the cell pellet was washed using 50 mM Tris-HCl buffer (pH 9). The buffer was acidified to pH 2 using 2 M HCl, and extracted with ethyl acetate spiked with 4-octyl benzoic acid (10 µg ml⁻¹). The extracts were derivatized by 50 µl of 2 M TMS-diazomethane (Aldrich) with 10% methanol. For qualitative analysis by GC-MS, the product was purified by silica gel column chromatography eluted with ether and pentane (1:1).

Products were analysed using a gas chromatography mass spectrometer (70 eV, Agilent Technologies) equipped with a DB5 capillary column (0.25 mm internal diameter × 0.25 µm × 30 m, J&W Scientific). The gas chromatography oven programme used was 80 °C (held for 2 min), 20 °C min⁻¹ ramp to 140 °C, product separation by a 5 °C min⁻¹ increment up to 220 °C. For quantification by gas chromatography-flame ionization detection, samples were analysed without column purification using the same gas chromatography oven programme.

Fermentation and product analyses. A one-litre bioreactor (New-Brunswick Scientific) was used to culture the transgenic yeast strain for 93 h at 30 °C. Yeast cells were induced with 2% galactose at an A_{600} of 1.7, and the final cell density reached an A_{600} of 5.0. The dissolved oxygen level was maintained at 40% by altering agitation speed from 100 to 500 r.p.m. and sparging air at 0.51 min⁻¹. Artemisinic acid was removed from the cell pellet by an alkaline wash as before, and purified through a silica column eluted with 78% hexane, 20% ethyl acetate and 2% acetic acid. The structure of the isolated artemisinic acid (>95% purity) was analysed by ¹H and ¹³C NMR using a 500 MHz NMR spectrometer (Bruker DRX-500) in the College of Chemistry NMR Facility at the University of California.

In vitro enzyme assays. A one-litre culture of *S. cerevisiae* YPH499 transformed with pESCURA::CPR or pESCURA::CPR/CYP71AV1 was induced with 2% galactose for 24 h. Microsomes were purified by polyethylene glycol precipitation followed by an additional ultracentrifugation step to remove cytosolic protein contamination, as previously described²⁶. Approximately 500 µg of total microsomal protein was used in a 1-ml reaction containing 100 mM potassium phosphate buffer pH 7.5, 10 or 25 µM substrate, 100 µM NADPH and an NADPH regeneration system (5 mM glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase). Reactions were incubated for 2 h at 24 °C with gentle agitation, acidified to pH 2, and extracted with ethyl ether. Products were separated using the same gas chromatography oven programme as above. Selective ion mode (SIM), including six ions characteristic to the products (121, 189, 204, 218, 220 and 248), was used for detection.

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1. World Health Organization. *World Malaria Report 2005*. (WHO, Geneva, 2005).
2. Korenromp, E. L., Williams, B. G., Gouws, E., Dye, C. & Snow, R. W. Measurement of trends in childhood malaria mortality in Africa: An assessment of progress toward targets based on verbal autopsy. *Lancet Infect. Dis.* 3, 349–358 (2003).
3. Marsh, K. Malaria disaster in Africa. *Lancet* 352, 924 (1998).
4. Hoffman, S. Save the children. *Nature* 430, 940–941 (2004).
5. Vernerstrom, J. L. et al. Identification of an antimalarial synthetic trioxolane drug development candidate. *Nature* 430, 900–904 (2004).
6. Enserink, M. Infectious diseases: Source of new hope against malaria is in short supply. *Science* 307, 33 (2005).
7. Schmid, G. & Hofheinz, W. Total synthesis of Qinghaosu. *J. Am. Chem. Soc.* 105, 624–625 (1983).
8. Acton, N. & Roth, R. J. On the conversion of dihydroartemisinic acid into artemisinin. *J. Org. Chem.* 57, 3610–3614 (1992).
9. Haynes, R. K. & Vonwiller, S. C. Cyclic peroxyacetal lactone, lactol and

ether compounds. US patent 5,310,946 (1994).

10. Mercke, P., Bengtsson, M., Bouwmeester, H. J., Posthumus, M. A. & Brodelius, P. E. Molecular cloning, expression, and characterization of amorph-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Arch. Biochem. Biophys.* 381, 173–180 (2000).
11. Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D. & Keasling, J. D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnol.* 21, 796–802 (2003).
12. Donald, K., Hampton, R. & Fritz, I. Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 63, 3341–3344 (1997).
13. Gardner, R. G. & Hampton, R. Y. A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. *J. Biol. Chem.* 274, 31671–31678 (1999).
14. Davies, B. S. J., Wang, H. S. & Rine, J. Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: Similar activation/regulatory domains but different response mechanisms. *Mol. Cell. Biol.* 25, 7375–7385 (2005).
15. Jackson, B. E., Hart-Wells, E. A. & Matsuda, S. P. T. Metabolic engineering to produce sesquiterpenes in yeast. *Org. Lett.* 5, 1629–1632 (2003).
16. Seaman, F. C. Sesquiterpene lactones as taxonomic characters in the Asteraceae. *Bot. Rev.* 48, 121–592 (1982).
17. Bertea, C. M. et al. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med.* 71, 40–47 (2005).
18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410 (1990).
19. Lupien, S., Karp, F., Wildung, M. & Croteau, R. Regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (–)-4S-limonene-3-hydroxylase and (–)-4S-limonene-6-hydroxylase. *Arch. Biochem. Biophys.* 368, 181–192 (1999).
20. Ralston, L. et al. Cloning, heterologous expression, and functional characterization of 5-epi-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*). *Arch. Biochem. Biophys.* 393, 222–235 (2001).
21. Wang, E. et al. Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product-based aphid resistance. *Nature Biotechnol.* 19, 371–374 (2001).
22. Elmarakby, S. A., El-Feraly, F. S., Elsohly, H. N., Croom, E. M. & Hufford, C. D. Microbiological transformations of artemisinic acid. *Phytochemistry* 27, 3089–3091 (1988).
23. Kim, S., Han, J. & Lim, Y. Revised assignment of ¹H-NMR signals of artemisinic acid. *Planta Med.* 62, 480–481 (1996).
24. Hellierwell, C. A., Poole, A., Peacock, W. J. & Dennis, E. S. *Arabidopsis* ent-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. *Plant Physiol.* 119, 507–510 (1999).
25. Hellierwell, C. A., Chandler, P. M., Poole, A., Dennis, E. S. & Peacock, W. J. The CYP88A cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proc. Natl. Acad. Sci. USA* 98, 2065–2070 (2001).
26. Pompon, D., Louerat, B., Bronine, A. & Urban, P. Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol.* 272, 51–64 (1996).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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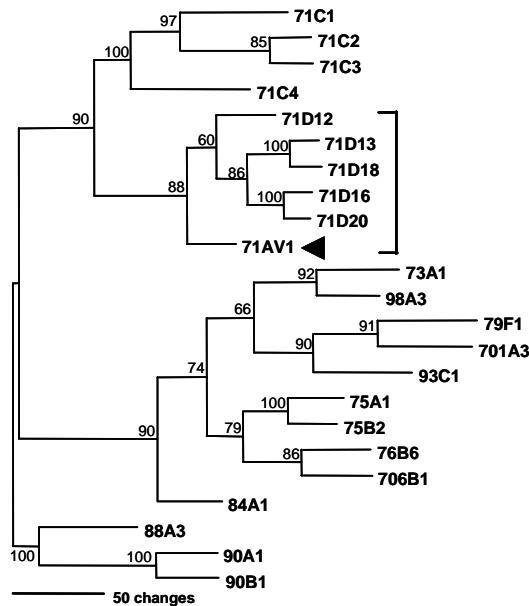
Author Contributions D.-K.R., E.M.P. and J.D.K. designed the project and experiments. D.-K.R., E.M.P., Y.S., M.C.Y.C., S.T.W. and J.K. performed experiments. K.J.F. conducted NMR analysis of artemisinic acid. J.M.N. and R.S. semi-synthesized artemisinic alcohol and artemisinic aldehyde. T.S.H. performed bioinformatics analysis of the Compositae EST-database. M.O., R.A.E. and K.A.H. provided technical assistance. D.-K.R., E.M.P., K.L.N. and J.D.K. wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information *Artemisia annua* CYP71AV1 and *CPR* gene sequence information has been deposited in GenBank under accession numbers DQ268763 and DQ318192, respectively. Reprints and permissions information is available at www.ncbi.nlm.nih.gov/reprintsandpermissions. The authors declare competing financial interests: details accompany the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.D.K. (keasling@berkeley.edu).

Supplementary Information for

Production of the anti-malarial drug precursor artemisinic acid in engineered yeast

Supplementary Figure 1.



Legend for Supplementary Figure 1. Phylogenetic reconstruction of *CYP71AV1* with other plant P450s of known functions.

Tree reconstruction was performed with the tree-bisection-reconnection heuristic search algorithm. Selected P450s were plant specific P450s (A-type) of known functions and three non-A-type P450s (88A3, 90A1, and 90B1)¹ which served as an outgroup. An arrow-head indicates the *A. annua* CYP71AV1, amorphadiene oxidase. The bracket indicates the plant CYP71D subfamily, which comprises tobacco cembratrienol hydroxylase (71D16), tobacco 5-epi-aristolochene hydroxylase (71D20), mint limonene 3-hydroxylase (71D13), mint

limonene 6-hydroxylase (71D18), and Madagascar periwinkle tabersonine 16-hydroxylase (71D12). Bootstrap values (percent of 1,000 replicates) for each cluster are given at the nodes. GenBank accession numbers or Arabidopsis Genome Initiative (AGI) numbers are given. 71C1 to 71C4, four P450s involved in DIMBOA (cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one) biosynthesis from *Z. mays* (X81827, Y11404, Y11403, and Y11368); 71D20, aristolochene-1,3-dihydroxylase from *N. tabacum* (AF368376); 71D16, cembratrienol hydroxylase from *N. tabacum* (AF166332); 71D13, limonene 3-hydroxylase from *Mentha x Piperita* (AF124816); 71D18, limonene 6-hydroxylase from *M. spicata* (AF124815); 71D12, tabersonine 16-hydroxylase from *C. roseus* (AJ238612); 73A1, cinnamate-4-hydroxylase from *H. tuberosus* (Z17369); 75A1, flavonoid 3',5'-hydroxylase from *P. hybrida* (Z22544); 75B2, flavonoid 3'-hydroxylase from *P. hybrida* (AF155332); 76B6, geraniol 10-hydroxylase from *C. roseus* (AJ251269); 79F1, aliphatic glucosinolate biosynthesis from *A. thaliana* (At1g16410); 84A1, coniferaldehyde hydroxylase from *A. thaliana* (At4g36220); 93C1, isoflavone synthase from *G. max* (AF135484); 98A3, 5-O-(4-coumaroyl shikimate)-3'-hydroxylase from *A. thaliana* (At2g40890); 701A3, ent-kaurene oxidase from *A. thaliana* (AF047719); 706B1, cadinene 8-hydroxylase from *G. arboreum* (AF332974); 88A3, ent-kaurenoic acid oxidase from *A. thaliana* (AF318501); 90A1, brassinosteroid 23-hydroxylase from *A. thaliana* (X87367); 90B1, brassinosteroid 22-hydroxylase from *A. thaliana* (AF044216).

Supplementary Methods

Cloning of *CYP71AV1* and *CPR* cDNA. A cDNA pool was prepared by Super SMART PCR cDNA synthesis kit (BD Bioscience) using 50 ng of total RNA purified from *A. annua* trichome-enriched cells. Degenerate P450 primers were designed from a conserved amino acid motif of lettuce and sunflower CYP71 subfamily; primer 1 from [Y/Q]G[E/D][H/Y]WR (forward) and primer 2 from FIPERF (reverse) (see, Supplementary Table I for sequence information). Polymerase chain reaction (PCR) using these primers and *A. annua* cDNAs yielded a 1-kb DNA fragment. The PCR-program used was 7 cycles with 48 °C annealing temperature and additional 27 cycles with 55 °C annealing temperature. The deduced amino acids from the amplified gene fragment showed 85% and 88% amino acid identity to the sunflower (QH_CA_Contig1442) and lettuce (QG_CA_Contig7108) contigs, respectively. The Compositae EST-database can be found at cgpdb.ucdavis.edu. *A. annua CPR* fragment was isolated using a forward primer (primer 3), and a reverse primer (primer 4), designed from the conserved QYEHFNKI and CGDAKGMA motifs, respectively. The PCR-program used was 30 cycles with 50 °C annealing temperature. Both 5'- and 3'-end sequences for *CYP71AV1* and *CPR* were determined using an RLM-RACE kit (Ambion) followed by full-length cDNA recovery from *A. annua* leaf cDNAs. The open reading frames of *CYP71AV1* and *CPR* were amplified by PCR and ligated into the *SpeI* and *BamHI/SalI* sites of pESC-URA (Stratagene) in FLAG and cMyc tagging, respectively. For PCR-

amplification of *CYP71AV1*, primers 5 and 6 were used; for PCR-amplification of *CPR*, primers 7 and 8 were used. The PCR-program used was 35 cycles with 55 °C annealing temperature. All clones were sequenced to confirm sequences.

Plant extract analysis. *A. annua* leaf (100 to 200 mg fresh weight) was vigorously shaked in 1 mL hexane spiked with 5.8 µM octadecane as an internal standard for 2 hours. The hexanolic extracts were concentrated to 200 µL, and 1 µL sample was used for the GC-MS analysis using a DB-XLB column (0.25 mm i.d. x 0.25 µm x 30 m, J & W Scientific) to determine artemisinin content from 14 plant samples as described². GC oven program used was 100 °C to 250 °C in 5 °C min⁻¹ increment. The plant hexanolic extracts were derivatized by TMS-diazomethane to determine artemisinic acid content by the GC-FID equipped with DB5 column (n = 8). The GC oven program used was 80 °C (hold 2 min), 20 °C min⁻¹ ramp to 140 °C, product separation by 5 °C min⁻¹ increment up to 220 °C. Authentic artemisinin standards were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of artemisinic alcohol. Artemisinic acid (100.0 mg, 0.43 mmol) was dissolved in THF (10.0 mL) and LiAlH₄ (17.0 mg, 0.45 mmol) was added. The heterogeneous mixture was held at reflux (70°C) for 15 h. After cooling, the reaction was quenched with water (3.0 mL) and 15% aqueous NaOH (3.0 mL), stirred for 10 min and filtered through celite. The organic phase was separated, dried over MgSO₄, and concentrated using a rotary evaporator. The product was purified by column chromatography (2:1 hexanes/EtOAc) to

give 61.0 mg (65% yield) of the alcohol as a colorless oil. A minor amount of artemisinic acid contaminant was further removed by column chromatography over neutral alumina (Brockman activity 1). Characterization data was consistent with literature values³.

Synthesis of artemisinic aldehyde. Artemisinic alcohol was oxidized to artemisinic aldehyde following a procedure reported in the literature⁴. In a flame-dried 10-mL flask containing RuCl₂(PPh₃)₃ (17.0 mg, 0.018 mmol) and N-methyl morpholine N-oxide (60.0 mg, 0.51 mmol) under an atmosphere of argon was added acetone (4.0 mL). To the solution was added artemisinic alcohol (55.0 mg, 0.25 mmol) dissolved in acetone (1.0 mL) via syringe. The mixture was stirred at 23 °C for 2 h and concentrated *in vacuo*. The crude product was purified by column chromatography (4:1 hexanes/EtOAc) to give 32.0 mg (59% yield) of artemisinic aldehyde as a colorless oil. Characterization data was consistent with literature report³.

EPY strain generation and characterization

Chemicals. Dodecane and caryophyllene were purchased from Sigma-Aldrich (St. Louis, MO). 5-fluoroortic acid (5-FOA) was purchased from Zymo Research (Orange, CA). Complete Supplement Mixtures for formulation of Synthetic Defined (SD) media were purchased from Qbiogene (Irvine, CA). All other media components were purchased from either Sigma-Aldrich or Becton, Dickinson (Franklin Lakes, NJ).

Strains and media. *Escherichia coli* strains DH10B and DH5 α were used for bacterial transformation and plasmid amplification in the construction of the expression plasmids used in this study. The strains were cultivated at 37 °C in Luria-Bertani medium with 100 mg L⁻¹ ampicillin with the exception of p δ -UB-based plasmids which were cultivated with 50 mg L⁻¹ ampicillin using DH5 α .

Saccharomyces cerevisiae strain BY4742⁵, a derivative of S288C, was used as the parent strain for all yeast strains. This strain was grown in rich YPD medium⁶. Engineered yeast strains were grown in SD medium⁶ with leucine, uracil, histidine, and/or methionine dropped out where appropriate. For induction of genes expressed from the *GAL1* promoter, *S. cerevisiae* strains were grown in 2% galactose as the sole carbon source.

Plasmid construction. To create plasmid pRS425ADS for expression of *ADS* with the *GAL1* promoter, *ADS* was PCR amplified from pADS⁷ using primer pair 9 and 10. (Supplementary Table I). Using these primers the nucleotide sequence 5'-AAAACA-3' was cloned immediately upstream of the start codon of *ADS*. This consensus sequence was used for efficient translation^{8,9} of *ADS* and the other galactose-inducible genes used in this study. The amplified product was cleaved with *SpeI* and *HindIII* and cloned into *SpeI* and *HindIII* digested pRS425GAL1¹⁰.

For integration of an expression cassette for *tHMGR*, plasmid p δ -HMGR was constructed. First *SacII* restriction sites were introduced into pRS426GAL1¹⁰ at the 5' end of

the *GAL1* promoter and 3' end of the *CYC1* terminator. To achieve this, the promoter-multiple cloning site-terminator cassette of pRS426GAL1 was PCR amplified using primer pair 11 and 12. The amplified product was cloned directly into *Pvu*II-digested pRS426GAL1 to construct vector pRS426-SacII. The catalytic domain of *HMG1* was PCR amplified from plasmid pRH127-3¹¹ with primer pair 13 and 14. The amplified product was cleaved with *Bam*HI and *Sal*I and cloned into *Bam*HI and *Xho*I digested pRS426-SacII. pRS-HMGR was cleaved with *Sac*II and the expression cassette fragment was gel extracted and cloned into *Sac*II digested pδ-UB¹².

The *upc2-1* allele of *UPC2* was PCR amplified from plasmid pBD33 (provided by Jasper Rine) using primer pair 15 and 16. The amplified product was cleaved with *Bam*HI and *Sal*I and cloned into *Bam*HI and *Xho*I digested pRS426-SacII to create plasmid pRS-UPC2. For the integration of *upc2-1*, pδ-UPC2 was created in an identical manner by digesting pRS-UPC2 with *Sac*II and moving the appropriate fragment to pδ-UB.

To replace the *ERG9* promoter with the *MET3* promoter, plasmid pRS-ERG9 was constructed. Plasmid pRH973 (provided by Randy Hampton)¹³ contained a truncated 5' segment of *ERG9* placed behind the *MET3* promoter. pRH973 was cleaved with *Apa*I and *Cla*I and cloned into *Apa*I and *Cla*I digested pRS403 which has a HIS3 selection marker¹⁴.

For expression of *ERG20*, plasmid pδ-ERG20 was constructed. Plasmid pRS-SacII was first digested with *Sal*I and *Xho*I which created compatible cohesive ends. The plasmid

was then self-ligated, eliminating *Sal*I and *Xho*I sites to create plasmid pRS-SacII-DX. *ERG20* was PCR amplified from the genomic DNA of BY4742 using primer pair 17 and 18. The amplified product was cleaved with *Spe*I and *Sma*I and cloned into *Spe*I and *Sma*I digested pRS-SacII-DX. pRS-*ERG20* was then cleaved with *Sac*II and the expression cassette fragment was gel extracted and cloned into *Sac*II digested pδ-UB.

Yeast transformation and strain construction. *S. cerevisiae* strain BY4742⁵, a derivative of S288C was used as the parent strain for all *S. cerevisiae* strains. Transformation of all strains of *S. cerevisiae* was performed by the standard lithium acetate method¹⁵. Three to ten colonies from each transformation were screened for the selection of the highest amorphadiene producing transformant. Strain EPY201 was constructed by the transformation of strain BY4742 with plasmid pRS425ADS and selection on SD-LEU plates. Plasmid pδ-HMGR was digested with *Xho*I before transformation of the DNA into strain EPY201. After initial selection on SD-LEU-URA plates, transformants were cultured and plated on SD-LEU plates containing 1 g L⁻¹ 5-FOA as a selection for the loss of the *URA3* marker. The resulting uracil auxotroph, EPY208 was then transformed with *Xho*I-digested pδ-UPC2 plasmid DNA. After initial selection on SD-LEU-URA plates, transformants were cultured and plated on SD-LEU plates including 1 g L⁻¹ 5-FOA for the construction of EPY210. Plasmid pRS-*ERG9* was cleaved with *Hind*II for the integration of the P_{MET3}-*ERG9* fusion at the *ERG9* loci of EPY208 and EPY210 for the construction of EPY213 and EPY225, respectively. These

strains were selected for on SD-LEU-HIS-MET plates. EPY213 was then transformed with *Xho*I digested p δ -HMGR plasmid DNA. After initial selection on SD-LEU-URA-HIS-MET plates, transformants were cultured and plated on SD-LEU-HIS-MET plates containing 1 g L⁻¹ 5-FOA for the construction of EPY219. EPY219 was transformed with *Xho*I digested p δ -ERG20 plasmid DNA. After initial selection on SD-LEU-URA-HIS-MET plates, transformants were cultured and plated on SD-LEU-HIS-MET plates including 1 g L⁻¹ 5-FOA for the construction of EPY224.

Integration of pRS-ERG9 was verified by PCR analysis using two sets of primers. Each set contained one oligo to bind to the inserted DNA and one to bind to the genomic DNA surrounding the insertion. All other integrations were verified for full length insertion using a primer binding to the 5'-end of the *GAL1* promoter and 3'-end of the fused gene.

Yeast cultivation. All optical densities at 600 nm (OD₆₀₀) measurements were taken using a Beckman DU-640 spectrophotometer. To measure amorphadiene production, culture tubes containing 5 mL of SD (2% galactose) medium (with appropriate amino acid omissions as described above) were inoculated with the strains of interest. These innocula were grown at 30°C to OD₆₀₀ between 1 and 2. Unbaffled culture flasks (250 mL) containing 50 mL SD medium were inoculated to an OD₆₀₀ 0.05 with these seed cultures. Amorphadiene production was measured after 6 days of growth. 1 mM methionine was present in each culture for repression of the P_{MET3}-*ERG9* fusion at the *ERG9* loci. All flasks also contained 5 mL

dodecane. This dodecane layer was sampled and diluted in ethyl acetate for determination of amorphadiene production by GC-MS.

Supplementary Discussion

Process considerations.

With the development of an industrial strain and an optimized fermentation and purification process, we project yields in excess of 25 g L⁻¹ artemisinic acid, well below yields for other high-value commodity chemicals produced by fermentation¹⁶⁻¹⁸. Given production levels of artemisinic acid at 25 g L⁻¹ in fermentation, published yields for chemical transformations (conversion of artemisinic acid to artemisinin or various derivatives)^{19,20}, and production levels in excess of 100 tons annually, we project that artemisinin or its derivatives could be produced at costs significantly below current prices, thereby lowering the cost of an artemisinin combination therapy by a significant amount.

Supplementary References

1. Durst, F. & Nelson, D. R. Diversity and evolution of plant P450 and P450-reductases. *Drug Metab. Drug Interact.* **12**, 189-206 (1995).
2. Woerdenbag, H. J., Pras, N., Bos, R., Visser, J. F., Hendriks, H. & Malingre, T. M. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. *Phytochem. Anal.*, **2**, 215-219 (1991).
3. Berteau, C. M. *et al.* Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med* **71**, 40-47 (2005).

4. Sharpless, K. B., Akashi, K. & Oshima, K. Ruthenium catalyzed oxidation of alcohols to aldehydes and ketones by amine-n-oxides. *Tetrahedron Letters* **17**, 2503-2506 (1976).
5. Brachmann, C. B., Davis, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., Boeke, J. D. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115-132 (1998).
6. Burke, D., Dawson, D. & Stearns, T. *Methods in yeast genetics: a Cold Spring Harbor laboratory course manual* (Cold Spring Harbor Laboratory Press, Plainview, NY, 2000).
7. Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D. & Keasling, J. D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* **21**, 796-802 (2003).
8. Looman, A. C. & Kuivenhoven, J. A. Influence of the 3 nucleotides upstream of the initiation codon on expression of the *Escherichia coli* lacZ gene in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **21**, 4268-4271 (1993).
9. Yun, D. F., Laz, T. M., Clements, J. M. & Sherman, F. mRNA sequences influencing translation and the selection of AUG initiator codons in the yeast *Saccharomyces cerevisiae*. *Molecular Microbiology* **19**, 1225-1239 (1996).
10. Mumberg, D., Muller, R. & Funk, M. Regulatable Promoters of *Saccharomyces cerevisiae* - Comparison of Transcriptional Activity and Their Use for Heterologous Expression. *Nucleic Acids Research* **22**, 5767-5768 (1994).
11. Donald, K., Hampton, R. & Fritz, I. Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **63**, 3341-3344 (1997).
12. Lee, F. & Da Silva, N. Sequential delta-integration for the regulated insertion of cloned genes in *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **13**, 368-373 (1997).
13. Gardner, R. G. & Hampton, R. Y. A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. *J. Biol. Chem.* **274**, 31671-31678 (1999).
14. Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27 (1989).
15. Gietz, R. D. & Woods, R. A. in *Guide to Yeast Genetics and Molecular and Cell Biology, Part B*, 87-96 (Academic Press Inc, San Diego, 2002).
16. Nakamura, C. E. & Whited, G. M. Metabolic engineering for the microbial production of 1,3-propanediol. *Curr. Opin. Biotechnol.* **14**, 454-459 (2003).
17. Zeikus, J. G., Jain, M. K., & Elankovan, P. Biotechnology of succinic acid production

- and markets for derived industrial products. *Appl. Microbiol. Biotechnol.* **51**, 545-552 (1999).
- 18. Lee, S. Y., Hong, S. H., Lee, S. H., & Park, S. J. Fermentative production of chemicals that can be used for polymer synthesis. *Macromol. Biosci.* **4**, 157-164 (2004).
 - 19. Roth, R. J. & Acton, N. On the conversion of dihydroartemisinic acid into artemisinin. *J. Org. Chem.* **57**, 3610-3614 (1992).
 - 20. Haynes, R. K. & Vonwiller, S. C. US Patent #5310946, 1994, May 10.

Supplementary Tables**Table I. Primers used for construction of plasmids**

Primer number	Sequence (5' to 3')
1	TCCGACCA(C/T)ANGNGAN(C/T)A(C/T)TGGAG
2	TCCGACCAAANC(G/T)(C/T)TCNGG(A/G/T)AT(A/G)AA
3	CCAGCACA(A/G)TA(C/T)GA(A/G)CA(C/T)TT(C/T)AA(C/T)AA(A/G)AT
4	CCAGCAGCCATNCC(C/T)TTNGC(A/G)TCNCC(A/G)CA
5	ACGT<u>TAGAAT</u>GAAGAGTATACTAAAAGCAATG
6	ACGT<u>CTAGAG</u>CGAAACTTGGAACGAGTAACAAC
7	ATGG<u>ATCCTAT</u>GCAATCAACAAC TTCCGTTAAGTTAT
8	TAT<u>GTCGACCC</u>CATACATCACGGAGATATCTCCT
9	GG<u>ACTAGT</u>AAAACA<u>ATGGCCCT</u>GACCGAAGAG
10	CCA<u>AGCTTC</u>CAGATGGACATCGGGTAAAC
11	CTGCC<u>CGGGGCG</u>CAAATTAAAGCCTTC
12	CTGCC<u>CGGT</u>AGTACGGATTAGAAGCCGC
13	CGGG<u>ATCCAAACAA</u>AT<u>GGCTGC</u>CAGACCAATTGGTG
14	GCG<u>TCGACT</u>TAGGATTAA<u>ATGC</u>CAGGTGACG
15	CGGG<u>ATCCAAACAA</u>AT<u>GAGCGAAGTCGGT</u>TACAG
16	GCG<u>TCGACT</u>CATAACGAAAA<u>ATCAGAGAA</u>ATTG
17	GG<u>ACTAGT</u>AAAACA<u>ATGGCTTC</u>CAGAAAAAGAAATTAG
18	T<u>CCCCCGGG</u>CTATTGCTTCTCTTGAAAC

Restriction sites are underlined and bold indicates a start or stop codon.

Table II. Description of plasmids used in this study

Name	Gene expressed	Plasmid type	Marker
pRS425ADS	<i>ADS</i>	2-micron replicon	<i>LEU2</i>
pδ-HMGR	<i>tHMGR</i>	Integrating	<i>URA3</i>
pδ-UPC2	<i>upc2-1</i>	Integrating	<i>URA3</i>
pRS-ERG9	<i>P_{MET3}-ERG9</i>	Integrating	<i>HIS3</i>
pδ-ERG20	<i>ERG20</i>	Integrating	<i>URA3</i>

Table III. Yeast strains used in this study and relevant genotypes

BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>
EPY201	BY4742 pRS425ADS
EPY208	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ (ura-)
EPY210	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ $P_{GAL1}\text{-}upc2\text{-}I$ (ura-)
EPY225	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ <i>erg9::PMET3-ERG9</i> (ura-)
EPY213	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ $P_{GAL1}\text{-}upc2\text{-}I$ <i>erg9::P_{MET3}\text{-}ERG9</i> (ura-)
EPY219	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ $P_{GAL1}\text{-}upc2\text{-}I$ <i>erg9::P_{MET3}\text{-}ERG9</i> $P_{GAL1}\text{-}tHMGR$ (ura-)
EPY224	BY4742 pRS425ADS $P_{GAL1}\text{-}tHMGR$ $P_{GAL1}\text{-}upc2\text{-}I$ <i>erg9::P_{MET3}\text{-}ERG9</i> $P_{GAL1}\text{-}tHMGR$ $P_{GAL1}\text{-}ERG20$ (ura-)

Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids

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Isoprenoids are the most numerous and structurally diverse family of natural products. Terpenoids, a class of isoprenoids often isolated from plants, are used as commercial flavor and fragrance compounds and antimalarial or anticancer drugs. Because plant tissue extractions typically yield low terpenoid concentrations, we sought an alternative method to produce high-value terpenoid compounds, such as the antimalarial drug artemisinin, in a microbial host. We engineered the expression of a synthetic amorphadiene synthase gene and the mevalonate isoprenoid pathway from *Saccharomyces cerevisiae* in *Escherichia coli*. Concentrations of amorphadiene, the sesquiterpene olefin precursor to artemisinin, reached 24 µg caryophyllene equivalent/ml. Because isopentenyl and dimethylallyl pyrophosphates are the universal precursors to all isoprenoids, the strains developed in this study can serve as platform hosts for the production of any terpenoid compound for which a terpene synthase gene is available.

Terpenoids comprise a highly diverse class of natural products from which numerous commercial flavors, fragrances and medicines are derived. These valuable compounds are commonly isolated from plants, microbes and marine organisms. For example, terpenoids extracted from plants are used as anticancer and antimalarial drugs^{1,2}. Because these compounds are naturally produced in small quantities, purification from biological material suffers from low yields, impurities and consumption of large amounts of natural resources. Furthermore, because of the complexity of these molecules, the chemical syntheses of terpenoids are inherently difficult and expensive and produce relatively low yields^{3–5}. For these reasons, the engineering of metabolic pathways to produce large quantities of complex terpenoids in a tractable biological host presents an attractive alternative to extractions from environmental sources or chemical syntheses.

Here we describe the production of amorphadiene from the bacterium *E. coli*. Amorphadiene is the sesquiterpene olefin precursor to artemisinin, a valuable and powerful antimalarial natural product first isolated from sweet wormwood or *Artemisia annua*. In certain regions of the world, strains of *Plasmodium* have emerged that are resistant to the traditional antimalarial drugs of choice, such as chloroquine, mefloquine, halofantrine, quinine and the sulfadoxine-pyrimethamine combination. Artemisinins have been acclaimed as the next generation of antimalarial drugs because they show little or no cross-resistance with existing antimalarials^{6–8}. Commercial production of artemisinin currently relies on its extraction and purification from plant material and, as would be expected, the yields are low⁹. Artemisinin is but one example of a group of terpene-based natural products that have been used in treating human disease. These include Taxol, a diterpene extracted from the Pacific yew that is extremely effective in the treatment of

certain cancers^{10,11}, and irufloven, a third-generation semisynthetic analog of the sesquiterpene illudin S that are in late-stage clinical trials for the treatment of various refractory and relapsed cancers^{12,13}. In general, these drugs are extracted from the host plant, in which they accumulate in very small amounts, before further derivatization or use.

To eliminate the need for plant extraction, we sought to produce terpenoid compounds at high yields in a microbial host by introducing a heterologous, high-flux isoprenoid pathway into *E. coli*. Although most terpene olefins are active when derivatized, the ability to produce the olefin backbone in large quantities in a genetically and metabolically tractable host represents an important step toward producing terpenoid-based drugs in large-scale fermentations. Because all terpenoids are produced from the same universal precursors, host microbes engineered to produce copious quantities of these precursors may be used to biosynthesize any terpene.

Two isoprenoid biosynthetic pathways exist that synthesize the precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Fig. 1). Eukaryotes other than plants use the mevalonate-dependent (MEV) isoprenoid pathway exclusively to convert acetyl-coenzyme A (acetyl-CoA) to IPP, which is subsequently isomerized to DMAPP. Plants use both the MEV and the mevalonate-independent, or deoxyxylulose 5-phosphate (DXP), pathways for isoprenoid synthesis. Prokaryotes, with some exceptions¹⁴, use the DXP pathway to produce IPP and DMAPP separately through a branch point¹⁵ (Fig. 1). IPP and DMAPP precursors are essential to *E. coli* for the prenylation of tRNAs¹⁶ and the synthesis of farnesyl pyrophosphate (FPP), which is used for quinone and cell wall biosynthesis.

Several groups have described the engineering of the DXP pathway to increase the supply of isoprenoid precursors needed for

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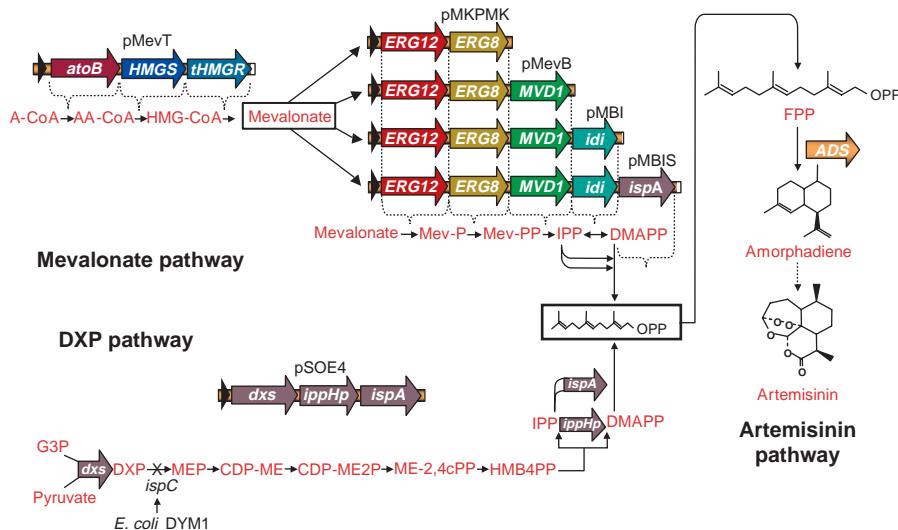


Figure 1 Production of amorphadiene via the DXP or mevalonate isoprenoid pathways and depiction of the synthetic operons used in this study. Black triangles represent the P_{LAC} promoter and tHMGR refers to an N-terminal truncated product of the native *HMGR* gene. Gene symbols and the enzymes they encode (all genes were isolated from *S. cerevisiae* except where noted): *atoB*, acetoacetyl-CoA thiolase from *E. coli*; *HMGS*, HMG-CoA synthase; *tHMGR*, truncated HMG-CoA reductase; *ERG1*, mevalonate kinase; *ERG8*, phosphomevalonate kinase; *MVD1*, mevalonate pyrophosphate decarboxylase; *idl*, IPP isomerase from *E. coli*; *ippH*, IPP isomerase from *Haematococcus pluvialis*; *dxs*, 1-deoxy-D-xylulose 5-phosphate synthase; *ispC*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *ispA*, FPP synthase from *E. coli*; *ADS*, amorphadiene synthase. Pathway intermediates: G3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-2,4cPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; HMB4PP, 1-hydroxy-2-methyl-2-(*E*-butenyl) 4-pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; A-CoA, acetyl-CoA; AA-CoA acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; Mev-P, mevalonate 5-phosphate; Mev-PP, mevalonate pyrophosphate.

high-level production of carotenoids in *E. coli*^{17–19}. Balancing the pool of glyceraldehyde-3-phosphate and pyruvate, or increasing the expression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS; encoded by the gene *dxs*) and IPP isomerase (encoded by *idi*), resulted in increased carotenoid buildup in the cell. Though improvements in isoprenoid production were noted, this approach most likely suffered from limitations owing to control mechanisms present in the native host. Because the DXP pathway may be tied to unknown physiological control elements in *E. coli*, we chose to bypass this pathway by engineering the expression of the *S. cerevisiae* mevalonate-dependent pathway in *E. coli*. We found that expression of this heterologous pathway in *E. coli* led to such an abundance of isoprenoid precursors that cells either ceased to grow or mutated to overcome the toxicity. The simultaneous expression of a synthetic amorphadiene synthase gene²⁰ in our engineered strain resulted in high-level production of amorphadiene and alleviated growth inhibition. Because IPP and DMAPP are the universal precursors to all isoprenoids, the strains reported here can serve as platform hosts for the production of any terpenoid compound for which the biosynthetic genes are available.

RESULTS

Synthase gene assembly and amorphadiene production

Previous studies on the production of sesquiterpenes using native plant genes established that poor expression of the plant genes in *E. coli* restricted the terpene yields²¹. To overcome the difficulties in express-

ing terpene synthases and to achieve high-level production of the artemisinin precursor amorphadiene, we synthesized and expressed a codon-optimized variant of *ADS*, the gene encoding amorphadiene synthase, designed for high-level expression in *E. coli*. By expressing a codon-optimized synthase, we hoped to shift the limitation of microbial terpene synthesis from expression of the synthase gene to supply of the precursor (FPP) by the isoprenoid pathway. The *ADS* gene synthesis, which used a two-step assembly and a one-step amplification PCR, yielded the expected 1.7 kb product. Sequence analysis of three *ADS* genes from independent clones identified two mutations or more in each of the genes. A functional *ADS* gene was assembled from two clones and by means of two site-directed mutagenesis reactions. Expression of the synthetic *ADS* gene in *E. coli* DH10B resulted in a peak concentration of amorphadiene of 0.086 µg caryophyllene equivalent/ml/OD₆₀₀ after 10 h of growth in LB medium (Fig. 2a). The peak concentration of amorphadiene increased to 0.313 µg caryophyllene equivalent/ml/OD₆₀₀ (Fig. 2a) upon coexpression with the SOE4 operon encoding DXS, IPPH_p and IspA (Fig. 1), which are rate-limiting enzymes of the native DXP isoprenoid pathway. Given this 3.6-fold increase in amorphadiene concentration upon coexpression, we suspected that FPP synthesis and not *ADS* expression limited amorphadiene production in this engineered host.

Engineering the mevalonate-dependent pathway in *E. coli*

To increase the intracellular concentration of FPP substrate supplied to the amorphadiene synthase, we assembled the genes encoding the mevalonate-dependent isoprenoid pathway from *S. cerevisiae* into operons and expressed them in *E. coli*. To simplify the task of engineering an eight-gene biosynthetic pathway, we divided the genes into two operons, referred to as ‘top’ and ‘bottom.’ The ‘top’ operon, MevT, transforms the ubiquitous precursor acetyl-CoA to (*R*)-mevalonate in three enzymatic steps (Fig. 1). The ‘bottom’ operon converts the (*R*)-mevalonate to IPP, DMAPP and/or FPP depending on the construct (Fig. 1). To test the functionality of the heterologous pathway, an *E. coli* strain deficient in isoprenoid synthesis (strain DYM1) was transformed with plasmids expressing the three different bottom operon constructs pMevB, pMBI and pMBIS (Fig. 1). Strain DYM1 has a deletion in the *ispC* gene²² and therefore cannot synthesize 2-C-methyl-D-erythritol 4-phosphate, an intermediate in the endogenous isoprenoid biosynthetic pathway (Fig. 1). As expected, all strains grew in the presence of 2-C-methyl-D-erythritol, but only the strains harboring pMBI or pMBIS, and not pMevB, grew on plates supplemented with 1 mM mevalonate in the absence of methylerythritol (data not shown). These results established that the synthetic MBI and MBIS operons were functional and capable of supplying IPP and DMAPP required for the growth of *E. coli*. Because the DXP pathway supplies the cells with IPP and DMAPP from a branch point¹⁵, a mutation in *ispC* prohibits the synthesis of both precursors. Although *E. coli* maintains a nonessential copy of the IPP isomerase gene on its chromosome²³, the

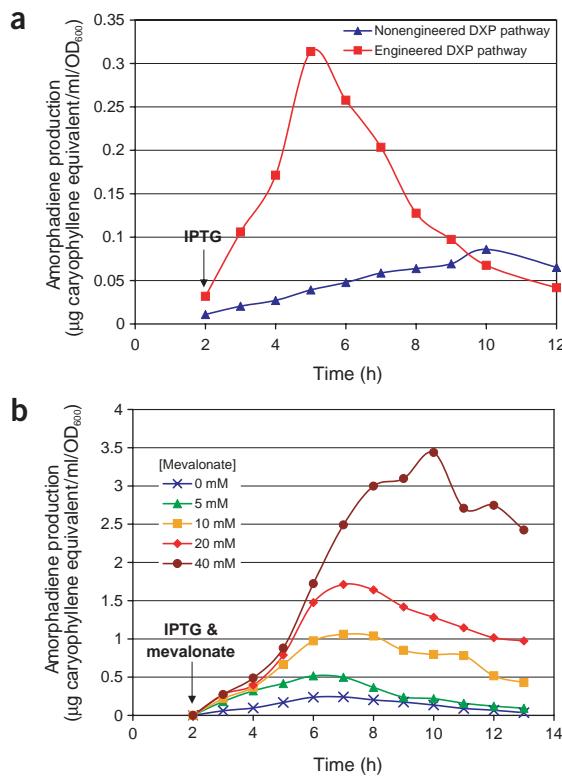


Figure 2 Comparison of the production of amorphadiene in LB medium. (a) Amorphadiene production by the synthetic amorphadiene synthase was measured from *E. coli* DH10B (nonengineered DXP pathway) and *E. coli* DH10B harboring the pSOE4 plasmid (engineered DXP pathway, Fig. 1) and (b) from *E. coli* DH10B expressing the mevalonate bottom operon (pMBIS, Fig. 1) in cultures supplemented with increasing amounts of DL-mevalonate. Because amorphadiene was not available commercially, its concentrations were reported as equivalents of caryophyllene, another sesquiterpene olefin, using a standard curve and the relative abundance of ions 189 and 204 m/z of the two compounds.



gene's expression seems to be too low to support the growth of *E. coli* when only IPP is supplied by the MevB operon.

To complete the mevalonate pathway and allow the synthesis of sesquiterpene precursors from a simple and inexpensive carbon source, the pMevT plasmid expressing the remaining three genes (*atoB*, *HMG* and *tHMG*) of the mevalonate isoprenoid pathway was transformed with either pMBI or pMBIS. Coexpression of the two operons, which together encode a complete pathway for the synthesis of isoprenoids from acetyl-CoA, complemented the *ispC* deletion even in the absence of mevalonate, indicating that the MevT operon was functional (data not shown).

Amorphadiene synthesis from mevalonate

To achieve high-level production of amorphadiene and to determine if the supply of FPP to the terpene synthase was limiting amorphadiene yields, the mevalonate pathway was coupled to amorphadiene synthesis in *E. coli*. Cells harboring the *ADS* gene coexpressed with the MBIS operon were grown in medium supplemented with exogenous mevalonate. Gas chromatography-mass spectrometry (GC-MS) analysis of the culture extracts showed that the peak amorphadiene concentration from these cultures was proportional to the amount of mevalonate added to the medium, up to a concentration of 40 mM mevalonate (Fig. 2b). These results indicated that flux from the MBIS

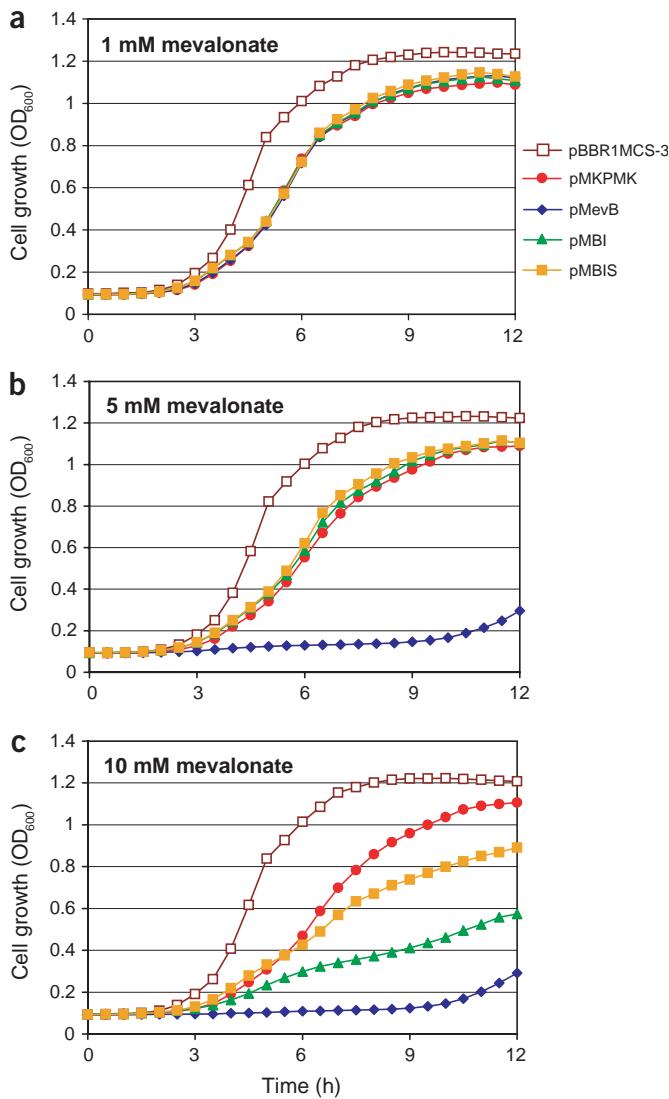


Figure 3 Growth curves of *E. coli* showing the inhibition effect caused by increasing concentrations of DL-mevalonate in the LB medium. The *E. coli* strains harbor either the pBBR1MCS-3 (empty plasmid control), pMKPMK, pMevB, pMBI or pMBIS plasmids expressing the various mevalonate operons described in Figure 1.

operon did not limit amorphadiene production at the highest mevalonate concentration used. Cultures supplemented with 40 mM mevalonate produced a peak concentration of 3.4 μg caryophyllene equivalents/ml/OD₆₀₀, which is a 40- and 11-fold increase over the endogenous and engineered DXP pathway, respectively. The drop in amorphadiene concentration with time was due to the loss of the volatile terpene to the headspace, which means that these reported production values are certainly underestimated.

We observed severe growth inhibition upon addition of more than 10 mM mevalonate in the control cultures where the amorphadiene synthase was not expressed (Fig. 3). To investigate the cause of this inhibition, we measured the growth of *E. coli* DH10B from strains harboring either the pMKPMK, pMevB, pMBI or pMBIS plasmid in media supplemented with increasing concentrations of exogenous mevalonate. Although the addition of 5 mM mevalonate to the medium inhibited the growth of cells harboring pMevB, this concen-

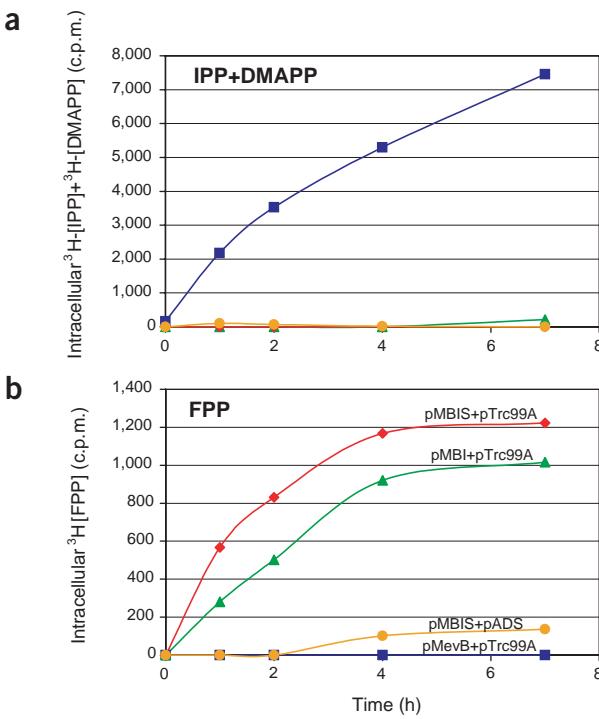


Figure 4 Prenyl pyrophosphate accumulation in resting cells harboring various mevalonate operons. **(a)** Intracellular accumulation of $[^3\text{H}]$ isopentenyl pyrophosphate (IPP) and $[^3\text{H}]$ dimethylallyl pyrophosphate (DMAPP). **(b)** Intracellular accumulation of $[^3\text{H}]$ farnesyl pyrophosphate (FPP) from cell suspensions of *E. coli* harboring pMevB+pTrc99A, pMBI+pTrc99A, pMBIS+pTrc99A or pMBIS+pADS. The HPLC method used to analyze IPP and DMAPP could not resolve the two intermediates. Therefore, the counts per minute (c.p.m.) reported as IPP+DMAPP are from a single HPLC peak.

tration of mevalonate did not affect the growth of cells harboring pMKPMK, pMBI or pMBIS (Fig. 3). Expression of the operons in the absence of mevalonate or in media supplemented with 1 mM mevalonate resulted in only a slight decrease in growth. Thus, from these data we hypothesized that the accumulation of IPP, which occurs in cells with high flux through the mevalonate pathway, is toxic and inhibits normal cell growth. To compare the intracellular prenyl pyrophosphate pools in the same strains, resting cells harboring the different mevalonate operon constructs were fed radiolabeled mevalonate and the labeled metabolites were tracked. As predicted, the strain expressing MevB accumulated IPP but not FPP, whereas the MBI and MBIS strains accumulated FPP but did not build up measurable levels of intracellular IPP (Fig. 4). Simultaneous expression of the amorphadiene synthase consumed the excess FPP pool that accumulated in the MBIS host, as shown by a decrease in intracellular FPP.

Because cells expressing MBIS accumulated FPP and exhibited growth inhibition in the presence of 10 mM mevalonate, we suspected that coexpression of the amorphadiene synthase would alleviate the growth inhibition by channeling the intracellular prenyl pyrophosphate intermediates to the volatile terpene olefin. As expected, approximately 2 h after addition of 10–40 mM mevalonate and IPTG, growth inhibition was observed only in strains lacking the ADS gene (Fig. 5). In contrast, cells coexpressing the MBIS operon and the synthase gene, both under control of IPTG-inducible promoters, exhibited normal growth rates at all mevalonate concentrations (Fig. 5). As shown previ-

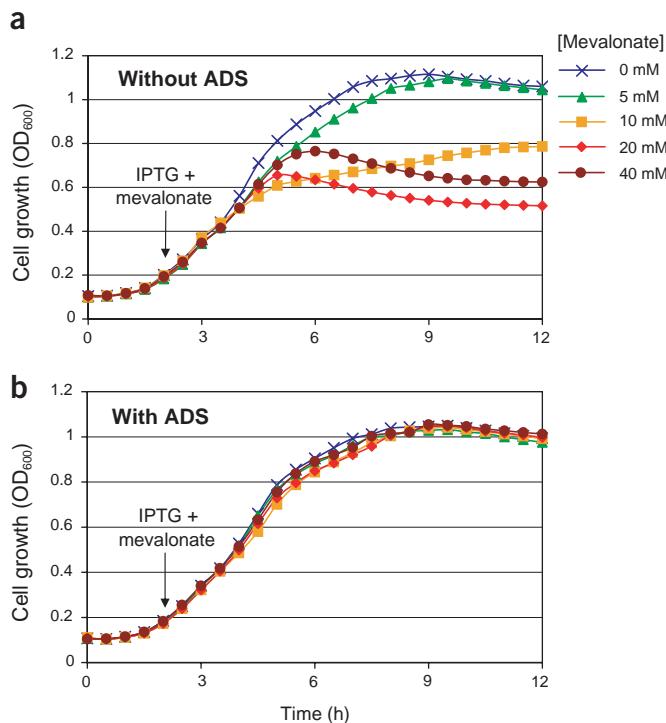


Figure 5 Effect of amorphadiene synthase (ADS) expression on the growth of *E. coli* harboring pMBIS. **(a)** pMBIS and the empty expression vector pTrc99A (without ADS) or **(b)** pADS expressing the amorphadiene synthase. LB medium was supplemented with 0 mM, 5 mM, 10 mM, 20 mM or 40 mM of DL-mevalonate.

ously, amorphadiene production from these cultures increased proportionally with the addition of exogenous mevalonate (Fig. 2b), further supporting the conclusion that the conversion of FPP to amorphadiene has a key role in minimizing growth inhibition. Taken together, these data strongly suggest that the engineered mevalonate pathway produces high levels of the prenyl pyrophosphate precursors. However, in the absence of the IPP isomerase, FPP synthase and terpene synthase to channel the pathway intermediates to the terpene olefin, toxic levels of intracellular prenyl pyrophosphates, especially IPP, may accumulate.

Amorphadiene synthesis from acetyl-CoA

To achieve amorphadiene production from a simple and inexpensive carbon source, the pMevT plasmid was introduced into *E. coli* harboring the pMBIS and pADS plasmids. This strain was tested for its ability to produce amorphadiene in the absence of exogenous mevalonate. Peak amorphadiene production from the complete mevalonate pathway reached 3.1 μg caryophyllene equivalent/ml/OD₆₀₀ after 9 h of growth in LB medium. This represents 36- and 10-fold improvements over the peak production for the strains with the native and engineered DXP pathway (0.086 and 0.313 μg caryophyllene equivalents/ml/OD₆₀₀, respectively, as described above) (Fig. 6). From the comparison of the amorphadiene production between the complete (Fig. 6) and the 'bottom' (Fig. 2b) mevalonate pathways, we estimated that the MevT pathway produced the equivalent of approximately 40 mM of exogenous mevalonate. Glycerol was amended in the cultures to investigate the effect on amorphadiene yields of supplying an additional carbon source. The addition of 0.8% glycerol to the LB medium led to higher biomass yields and prolonged amorphadiene production well into the stationary phase of growth. The glycerol-amended culture reached

optical densities of 3.7 and amorphadiene concentrations of 24 µg caryophyllene equivalents/ml. Using the rate of amorphadiene loss from the LB culture (Fig. 6) and assuming that the cells no longer produced amorphadiene after 11 h, we estimated a mass transfer coefficient of 0.87/h. By using this coefficient to account for the loss of amorphadiene to the headspace, we estimated a total production of approximately 22.6 and 112.2 mg/l from the LB and LB + 0.8% glycerol cultures, respectively. From these data, it is clear that the expression of the mevalonate-dependent isoprenoid biosynthetic pathway delivers high levels of isoprenoid precursor for the production of sesquiterpenes from a simple carbon source and that optimization of fermentation conditions should result in terpene production in the g/l range.

DISCUSSION

The development of artemisinin, a promising and potent antimalarial drug, has been limited by the costs associated with extracting the compound from its natural sources and the complexity of the alternative chemical synthesis^{2,7,24}. Classical plant breeding and selection combined with improved agricultural practices may not be adequate to lower the costs of artemisinin production to a price affordable for those most affected by the pathogen. Several laboratories have focused on the isolation of *Artemisia annua* L. genes involved in artemisinin synthesis in the hope of lowering the cost of artemisinin production by improving the yields from genetically engineered plants^{20,25–27}. The first gene discovered encoded the amorphadiene synthase, which converts FPP to amorphadiene. We sought to capitalize on this discovery by expressing the terpene synthase gene in a microbial host engineered to produce high levels of the FPP precursor for enhanced yields of the sesquiterpene olefin.

From our previous work on the microbial production of plant sesquiterpenes in *E. coli*, we concluded that poor expression of the synthase genes limits high terpene olefin yields from this host²¹. To circumvent this limitation, we chose to synthesize an amorphadiene synthase gene from oligonucleotides using the *E. coli* codon preferences, which can greatly improve protein expression²⁸. Comparison of sesquiterpene production in *E. coli* expressing native sesquiterpene synthase genes²¹ and *E. coli* expressing the synthetic ADS gene showed from a 10- to 300-fold improvement in terpene synthesis in the latter.

Insufficient supply of the prenyl pyrophosphate precursor by the native DXP pathway was shown to limit carotenoid²⁹ and taxadiene³⁰ yields in *E. coli*. Metabolic engineering of the DXP pathway^{17,18,31–34} increased the flux in carotenoid accumulation by 2- to 40-fold over incubation periods of 20–50 h. Likewise, in this study we observed a threefold increase in sesquiterpene accumulation after 5 h using a similar engineering strategy (Fig. 6). These observations imply that this approach to engineering the DXP pathway results in only a modest increase in flux that may be detectable only by using carotenoid biosynthesis as a reporter and long incubation periods. In this work, we demonstrate that the mevalonate isoprenoid pathway is a superior biosynthetic route for delivering high-level isoprenoid precursors to terpene synthases for large-scale production. By engineering the *S. cerevisiae* mevalonate-dependent isoprenoid pathway into *E. coli*, we circumvent the mevalonate pathway's native regulatory elements found in yeast while bypassing those of *E. coli*'s native DXP pathway. In fact, the heterologous pathway leads to such a vast excess of prenyl pyrophosphates that cell growth is inhibited. Coexpression of a synthetic sesquiterpene synthase consumes the excess pool of precursor, thereby eliminating growth inhibition and providing high yields of amorphadiene. Although total biosynthesis of artemisinin was not achieved in this study, the engineered biochemical pathway could be extended to produce artemisinic acid. Artemisinic acid can then be

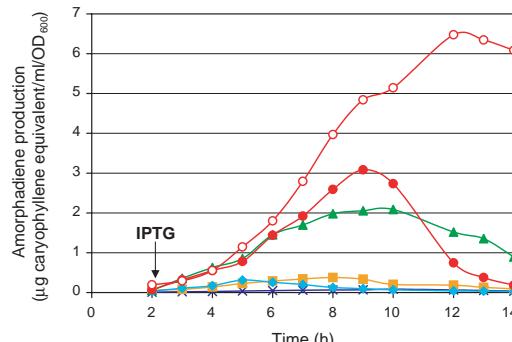


Figure 6 Comparison of amorphadiene production between *E. coli* expressing the native DXP pathway and the engineered isoprenoid pathways. The symbols represent amorphadiene production from cells supplying FPP to synthase using the native DXP pathway (pLac33, pBBR1MCS-3, dark blue x); the engineered DXP pathway (pSOE4, pBBR1MCS-3, light blue diamond); the mevalonate bottom pathway in the absence of DL-mevalonate (pLac33, pMBIS, yellow square); the mevalonate bottom pathway in medium supplemented with 30 mM DL-mevalonate (pLac33, pMBIS, green triangle); the complete mevalonate pathway (pMevT, pMBIS, filled red circle) and the complete mevalonate pathway in medium supplemented with 0.8% glycerol (open red circle).

converted to high yields (40%) of artemisinin or one of the derivatives via a photo-oxidation cyclization reaction³⁵.

In summary, the production of terpene-based compounds first requires the ability to produce large quantities of the olefin precursor. Our work provides a microbial host capable of producing precursors for the large-scale production of any terpene olefin and represents the first essential step toward the production of a broad range of terpene-based compounds in microorganisms. The use of microbes as platform hosts for the synthesis of terpenes offers several advantages over existing methods because they are better suited for the engineering of enzymes and biochemical pathways. For example, the amorphadiene gene may be easily replaced with any terpene synthase for high-level production of the new terpene. Furthermore, *in vitro* evolution and combinatorial biosynthesis of sesquiterpene biochemical pathways in microbes may lead to artemisinin derivatives or even new sesquiterpene lead compounds. Although greatly improved yields were obtained by combining the expression of a synthetic sesquiterpene synthase with a recombinant mevalonate pathway, the data suggest that a maximum yield was not attained. Therefore, efforts are now directed at identifying the pathway bottlenecks to maximize the flux and optimize expression of the mevalonate pathway.

METHODS

Strains and media. *E. coli* DH10B was used as the cloning and isoprenoid expression strain (see Supplementary Table 1 online for a summary of the strains and plasmids used in this study). For the growth studies, the optical density of cultures expressing the various recombinant pathways was measured with a microtiter plate reader (SpectraMax, Molecular Devices) from 200 µl cultures of LB broth in 96-well plates incubated at 37 °C with continuous shaking. DL-Mevalonolactone was purchased from Sigma-Aldrich and 2-C-methyl-D-erythritol was synthesized from citraconic anhydride according to the protocol of Duvold *et al.*³⁶. The *ispC* mutant *E. coli* strain DYM1²² (kindly provided by Haruo Seto, University of Tokyo) was used to test the functionality of the synthetic mevalonate operons. The DYM1 strain was propagated on LB medium containing 0.5 mM methylerythritol and transformed DYM1 cells were first allowed to recover on plates supplemented with methylerythritol before being streaked on test media. Media used to test the functionality of the operons were supplemented with 1 mM DL-mevalonate prepared by mixing 1 volume

of 2 M DL-mevalonolactone with 1.02 volumes of 2 M KOH and incubating at 37 °C for 30 min³⁷.

Synthesis of amorphadiene synthase gene. The synthetic *ADS* gene was designed using Calcgene²⁸ and the protein sequence of the synthase isolated by Mercke *et al.*²⁰ (see Supplementary Fig. 1 online for gene sequence and list of oligonucleotides). To assemble the *ADS* gene, each of the 84 overlapping oligonucleotides (Gibco-BRL) was dissolved in distilled, deionized H₂O to a final concentration of 100 μM. A mixture was prepared by combining 10 μl of each of the individual oligonucleotides. The first PCR reaction in the two-step PCR assembly of *ADS* contained in 100 μl, 1× *Pfu* polymerase buffer (20 mM Tris-HCl, pH 8.8, 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1% mg/ml BSA), 0.25 mM of each dNTP, 1 μM of oligonucleotides mixture and 5 U *Pfu* polymerase (Stratagene). The PCR cycling program was 94 °C for 30 s, 40 °C for 2 min, 72 °C for 10 s followed by 40 cycles at 94 °C for 15 s, 40 °C for 30 s, 72 °C for 20 s + 3 s per cycle. The second PCR reaction contained in 100 μl, 33 μl of the first assembly reaction, 1× *Pfu* buffer, 0.25 mM of each dNTP and 5 U *Pfu* polymerase. The PCR program for the second step of the assembly was as follows: 94 °C for 30 s, 40 °C for 10 s, 72 °C for 10 s followed by 25 cycles at 94 °C for 15 s, 40 °C for 30 s, 72 °C for 45 s + 1 s per cycle. The DNA smear in the range of 1.7 kb was gel purified and used as template for a third and final PCR reaction containing in 100 μl, 1X *Pfu* buffer, 0.25 mM of each dNTP, 250 nM each of the two outside primers (T-1 and B-42), 10 μl of the gel purified DNA and 5 U *Pfu* polymerase. The PCR program was 40 cycles of 94 °C for 45 s and 72 °C for 4 min followed by a final step at 72 °C for 10 min. The expected 1.7-kb band was gel purified and ligated into pTrc99A using 5' *Nco*I and 3' *Xma*I sites designed into the gene sequence, thereby generating pADS. Two rounds of site-directed mutagenesis were needed to eliminate point mutations and generate a functional gene.

Construction of the DXP pathway operon. The *dxs* gene of *E. coli* was spliced to the IPP isomerase gene (*ippHp*) from pAC-LYC04 (ref. 38) using overlapping extensions and PCR primers dxs1, dxs2, *ippHp*1 and *ippHp*2 (see Supplementary Table 2 online for primer sequences). The *E. coli* *ispA* gene was isolated by PCR using primers *ispA*1 and *ispA*2 and ligated to the *Nco*I site 3' to *ippHp*. The three-gene DXP operon was amplified with primers SOE-f and *ispA*2 and ligated into the *Kpn*I-*Pst*I sites of pMevT, thereby replacing the MevT operon with the SOE4 operon.

Construction of the mevalonate pathway operons. The *S. cerevisiae* mevalonate pathway was engineered as two separate, independently expressed operons. The genes encoding the last three enzymes of the biosynthetic pathway, mevalonate kinase (MK; gene, *ERG12*), phosphomevalonate kinase (PMK; gene, *ERG8*) and mevalonate pyrophosphate decarboxylase (MPD; gene, *MVD1*, also known as *ERG19*), were isolated by PCR from chromosomal DNA preparations of *S. cerevisiae*. The individual genes were spliced together (MevB, Fig. 1) using overlapping extensions from primers MK-f, MK-r, PMK-f, PMK-r, MPD-f and MPD-r. The genes encoding the first three enzymes of the mevalonate pathway, the acetoacetyl-CoA thiolase from *E. coli* (AACT or *atoB*), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS or *ERG13*) and a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase³⁹ (tHMGR1), were isolated and spliced together as a single operon (MevT, Fig. 1) using the following primers: *atoB*-f, *atoB*-r, HMGS-f, HMGS-r, tHMGR-f and tHMGR-r. Individual genes were isolated by PCR using *Pfu* DNA polymerase and a standard PCR protocol. The synthetic operons were ligated into pCR4 (TA vector from Invitrogen), after the addition of 3' A overhangs, and sequenced to ensure accuracy. The MevB operon was ligated into the *Pst*I site of pBBR1MCS-1 (ref. 40), generating pMevBCm. The *idi* gene was ligated into the *Xma*I site, 3' to MevB using primers *idi*-f and *idi*-r and the MBI operon was moved to the *Sal*I-*Sac*I sites of pBBR1MCS-3 to generate pMBI. The *idi* gene was excised from pMBI using *Xma*I, thereby generating pMevB. The *ispA* gene from *E. coli* was ligated into the *Sac*I-*Sac*II sites of pMBI using primers *ispA*-f and *ispA*-r, thereby producing pMBIS. The MevT operon was ligated into the *Xma*I-*Pst*I sites of pBAD33 (ref. 41). To place the operon under control of the P_{LAC} promoter, the *araC*-P_{BAD} *Nsi*I-*Xma*I fragment was replaced with the *Nsi*I-*Xma*I fragment of pBBR1MCS, thereby generating pMevT. To generate pLac33, the MevT operon was excised from pMevT with *Sal*I.

GC-MS analysis of amorphadiene. Amorphadiene production by the various strains was measured by GC-MS as previously described²¹ by scanning only for two ions, the molecular ion (204 m/z) and the 189 m/z ion. Cells were grown in LB medium at 37 °C for 2 h and induced to express the *ADS* and the mevalonate pathway by the simultaneous addition of 0.5 mM IPTG and varying concentrations of mevalonate. Amorphadiene concentrations were converted to caryophyllene equivalents using a caryophyllene standard curve and the relative abundance of ions 189 and 204 m/z to their total ions. The sesquiterpene caryophyllene was purchased from Sigma-Aldrich.

Radio-HPLC analysis of intracellular prenyl pyrophosphates. Intracellular IPP+DMAPP and FPP levels were measured using a resting cell suspension assay supplemented with (R)-[5-³H]mevalonate (39 Ci/mmol; Perkin-Elmer Life Sciences). Cells induced with 0.5 mM IPTG were grown in LB broth at 37 °C to an OD₆₀₀ of ~0.6, harvested, washed once and suspended to 20× concentration in 100 mM KPO₄ buffer (pH 7.4). Unlabeled DL-mevalonate (10 mM) and ³H-radiolabeled (R)-mevalonate (60 μCi) were added to 8 ml of cell suspension and incubated at 37 °C. Cells from 1.5-ml aliquots were washed twice with cold KPO₄ buffer and the intracellular IPP+DMAPP and FPP were extracted from cell pellets with 1 ml of 2:1 (vol/vol) methanol/chloroform. The cell extracts were dephosphorylated using potato acid phosphatase as previously described by Fujii *et al.*⁴² The prenyl alcohols were resolved on a reverse phase C-18 column (4.5 mm × 250 mm, 5 μm particle size; Alltech) by HPLC (Agilent Technologies model 1100) using the method of Zhang and Poultier⁴³ and detected with a flow-through scintillation counter (Packard BioScience, Radiomatic model 500TR).

Note: Supplementary information is available on the *Nature Biotechnology* website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Cragg, G.M. Paclitaxel (Taxol): a success story with valuable lessons for natural product drug discovery and development. *Med. Res. Rev.* **18**, 315–331 (1998).
2. Dhingra, V., Rao, K.V. & Narasu, M.L. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci.* **66**, 279–300 (2000).
3. Danishefsky, S.J. *et al.* Total synthesis of baccatin III and taxol. *J. Amer. Chem. Soc.* **118**, 2843–2859 (1996).
4. Nicolaou, K.C. *et al.* Total synthesis of eleutherobin. *Angew. Chem. Int. Ed.* **36**, 2520–2524 (1997).
5. Avery, M.A., Chong, W.K.M. & Jennings-White, C. Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. *J. Amer. Chem. Soc.* **114**, 974–979 (1992).
6. White, N.J. Artemisinin—Current status. *Trans. R. Soc. Trop. Med. Hyg. Suppl.* **88**, 53–54 (1994).
7. Ridley, R.G. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature* **415**, 686–693 (2002).
8. Haynes, R.K. Artemisinin and derivatives: the future for malaria treatment? *Curr. Opin. Infect. Dis.* **14**, 719–726 (2001).
9. Wallaart, T.E., Pras, N., Beekman, A.C. & Quax, W.J. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Med.* **66**, 57–62 (2000).
10. Jennewein, S. & Croteau, R. Taxol: biosynthesis, molecular genetics, and biotechnological applications. *Appl. Microbiol. Biotechnol.* **57**, 13–19 (2001).
11. Skeel, R.T. *Handbook of Cancer Chemotherapy*, edn. 5 (Lippincott Williams & Wilkins, Philadelphia, 1999).
12. Baekelandt, M. Irofulven (MGI Pharma). *Curr. Opin. Investig. Drugs* **3**, 1517–1526 (2002).
13. Amato, R.J., Perez, C. & Pagliaro, L. Irofulven, a novel inhibitor of DNA synthesis, in metastatic renal cell cancer. *Invest. New Drugs* **20**, 413–417 (2002).
14. Boucher, Y. & Doolittle, W.F. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol. Microbiol.* **37**, 703–716 (2000).
15. Rohdich, F. *et al.* Studies on the nonmevalonate terpene biosynthetic pathway: meta-



- bolic role of IspH (LytB) protein. *Proc. Natl. Acad. Sci. USA* **99**, 1158–1163 (2002).
16. Connolly, D.M. & Winkler, M.E. Genetic and physiological relationships among the *miaA* gene, 2-methylthio-N6-(Δ2-isopentenyl)adenosine transfer RNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. *J. Bacteriol.* **171**, 3233–3246 (1989).
 17. Farmer, W.R. & Liao, J.C. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog.* **17**, 57–61 (2001).
 18. Kajiwara, S., Fraser, P.D., Kondo, K. & Misawa, N. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.* **324**, 421–426 (1997).
 19. Kim, S.-W. & Keasling, J.D. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng.* **72**, 408–415 (2001).
 20. Mercke, P., Bengtsson, M., Bouwmeester, H.J., Posthumus, M.A. & Brodelius, P.E. Molecular cloning, expression, and characterization of amorpho-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Arch. Biochem. Biophys.* **381**, 173–180 (2000).
 21. Martin, V.J.J., Yoshikuni, Y. & Keasling, J.D. The *in vivo* synthesis of plant sesquiterpenes by *Escherichia coli*. *Biotechnol. Bioeng.* **75**, 497–503 (2001).
 22. Kuzyuama, T., Takahashi, S. & Seto, H. Construction and characterization of *Escherichia coli* disruptants defective in the *yaeM* gene. *Biosci. Biotechnol. Biochem.* **63**, 776–778 (1999).
 23. Hahn, F.M., Hurlburt, A.P. & Poulter, C.D. *Escherichia coli* open reading frame 696 is id1, a nonessential gene encoding isopentenyl diphosphate isomerase. *J. Bacteriol.* **181**, 4499–4504 (1999).
 24. Van Geldre, E., Vergauwe, A. & Van den Eeckhout, E. State of the art of the production of the antimalarial compound artemisinin in plants. *Plant Mol. Biol.* **33**, 199–209 (1997).
 25. Bouwmeester, H.J. et al. Amorpho-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochem.* **52**, 843–854 (1999).
 26. Wallaart, T.E., Bouwmeester, H.J., Hille, J., Poppinga, L. & Maijers, N.C.A. Amorpho-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta* **212**, 460–465 (2001).
 27. Chang, Y.J., Song, S.H., Park, S.H. & Kim, S.U. Amorpho-4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. *Arch. Biochem. Biophys.* **383**, 178–184 (2000).
 28. Hale, R.S. & Thompson, G. Codon optimization of the gene encoding a domain from human type 1 neurofibromin protein results in a threefold improvement in expression level in *Escherichia coli*. *Protein Exper. Purif.* **12**, 185–188 (1998).
 29. Sandmann, G. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *ChemBioChem.* **3**, 629–635 (2002).
 30. Huang, Q.L., Roessner, C.A., Croteau, R. & Scott, A.I. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorgan. Med. Chem.* **9**, 2237–2242 (2001).
 31. Matthews, P.D. & Wurtzel, E.T. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. *Appl. Microbiol. Biotechnol.* **53**, 396–400 (2000).
 32. Albrecht, M., Misawa, N. & Sandmann, G. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechnol. Lett.* **21**, 791–795 (1999).
 33. Harker, M. & Bramley, P.M. Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett.* **448**, 115–119 (1999).
 34. Wang, C.-W., Oh, M.-K. & Liao, J.C. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol. Bioeng.* **62**, 235–241 (1999).
 35. Jung, M., ElSohly, H.N. & McChesney, J.D. Artemisinic acid: a versatile chiral synthon and bioprecursor to natural products. *Planta Med.* **56**, 624 (1990).
 36. Duvoid, T., Bravo, J.M., Pale-Grosdemange, C. & Rohmer, M. Biosynthesis of 2-C-methyl-D-erythritol, a putative C-5 intermediate in the mevalonate independent pathway for isoprenoid biosynthesis. *Tetrahedron Lett.* **38**, 4769–4772 (1997).
 37. Campos, N. et al. *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. *Biochem. J.* **353**, 59–67 (2001).
 38. Cunningham, F.X., Sun, Z., Chamovitz, D., Hirschberg, J. & Gantt, E. Molecular structure and enzymatic function of lycopene cyclase from the *Cyanobacterium* *synechococcus* sp. strain PCC7942. *Plant Cell* **6**, 1107–1121 (1994).
 39. Polakowski, T., Stahl, U. & Lang, C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. *Appl. Microbiol. Biotechnol.* **49**, 66–71 (1998).
 40. Kovach, M.E. et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176 (1995).
 41. Guzman, L.-M., Belin, D., Carson, M.J. & Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121–4130 (1995).
 42. Fujii, H., Koyama, T. & Ogura, K. Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. *Biochem. Biophys. Acta* **712**, 716–718 (1982).
 43. Zhang, D.L. & Poulter, C.D. Analysis and purification of phosphorylated isoprenoids by reversed-phase HPLC. *Anal. Biochem.* **213**, 356–361 (1993).

Supplementary Figure 1 Gene sequence and oligonucleotide design of synthetic amorphadiene synthase

<p>T-1</p> <pre>GATTAAGGCATGCACCATGCCCTGACCGAAGAGAAACCGATCCGCCGATCGCTAACT</pre>	<p>B-1</p> <pre>CTAATTCCGTACGGTACCGGGACTGGCTCTTTGGCTAGGCGGGTAGCGATTGA</pre>
<p>T-2</p> <pre>TCCCGCCGTCTATCTGGGTGACCAGTCCTGATCTACGAAAAGCAGGTTGAGCAGGGT</pre>	<p>B-2</p> <pre>AGGGCGGCAGATAGACCCCCTGGTCAAGGACTAGATGCTTTCGTCCAACCGTCCCCA</pre>
<p>T-3</p> <pre>GGTGAACAGATCGTAAACGACCTGAAGAAAGAAGTTCGTCAGCTGCTGAAAGAACGCTCT</pre>	<p>B-3</p> <pre>CAACTTGTCTAGCATTTGCTGGACTCTTCCTCAAGCAGTCGACGACTTCTCGAGA</pre>
<p>T-4</p> <pre>GGACATCCCGATGAAACACGCTAACCTGCTGAAACTGATCGACGAGATCCAGCGTCTGG</pre>	<p>B-4</p> <pre>CCTGTAGGGCTACTTGTGCGATTGGACGACTTGACTAGCTGCTCTAGGTGCGCAGACC</pre>
<p>T-5</p> <pre>GTATCCCGTACCACTTCGAAACCGCAAATCGACCGACCGACTGCAGTGCATCTACGAAACC</pre>	<p>B-5</p> <pre>CATAGGGCATGGTGAAGCTTGCGCTTAGCTGGTGCACGTACGTAGATGCTTTGG</pre>
<p>T-6</p> <pre>TACGGCGACAACGGCACCGTTCTCTGTGGTTCGTCTGATGCGTAAACA</pre>	<p>B-6</p> <pre>ATGCCGCTGTTGACCTTGCCGCTGGCAAGAAGAGACACCAAAGCAGACTACGCATTG</pre>
<p>T-7</p> <pre>GGGCTACTACGTTACCTGTGACGTTTTAACAACTACAAGGACAAGAACGGTGCTTCA</pre>	<p>B-7</p> <pre>CCCGATGATGCAATGGACACTGCAAAAATTGTTGATGTTCTGTTGCCACGAAAGT</pre>
<p>T-8</p> <pre>AACAGTCTCTGGCTAACGACGTTGAAGGGCTGCTGGAACGTACGAAGCGACCTCCATG</pre>	<p>B-8</p> <pre>TTGTCAGAGACCGATTGCTGCAACTTCCGGACGACCTTGACATGCTTGGTGGAGGTAC</pre>
<p>T-9</p> <pre>CGTGTACCGGGTGAAATCATCCTGGAGGACGCGCTGGGTTCACCCGTTCTCGTCTGTC</pre>	<p>B-9</p> <pre>GCACATGGCCCACTTAGTAGGACCTCTGCGCGACCCAAAGTGGCAAGAGCAGACAG</pre>
<p>T-10</p> <pre>CATTATGACTAAAGACGCTTCTACTAACCCGGCTCTGTTACCGAAATCCAGCGTG</pre>	<p>B-10</p> <pre>GTAATACTGATTCTGCGAAAGAGATGATTGGGCCGAGACAAGTGGCTTAGGTCGCAC</pre>
<p>T-11</p>	<p>B-11</p>
<p>T-12</p>	<p>B-12</p>
<p>T-13</p>	<p>B-13</p>
<p>T-14</p>	<p>B-14</p>
<p>T-15</p>	<p>B-15</p>

T-16

CTCTGAAACAGCCGCTGTGGAAACGCTCTGCCCGTATCGAAGCAGCACAGTACATTCCG
GAGACTTGTGGCGACACCTTGAGACGGCGCATAGCTCGTGTATGTAAGGC

B-16

T-17

B-17

T-18

TTTTACCAGCAGCAGGACTCTCACACAACAAGACCCGTGCTGAAACTGGCTAAGCTGGAATT
AAAATGGTCGTCGTCCTGAGAGTGTGTTCTGGGACGACTTGACCGATTGACCTTAA

B-18

T-19

CAACCTGCTGCAGTCTCTGCACAAAGAAGAACGTGTCACGTTGTAAGTGGTGGAGG
GTTGGACGACGTCAAGAGACGTGTTCTTCTTGACAGAGTGCAAACATTACACCACCTCC

B-19

T-20

B-20

T-21

CATTTGACATCAAGAAAAACGCGCCGTGCCTGCGTACCGTATCGTTGAATGTTACTTC
GTAAACTGTAGTTCTTTGCGCGGCACGGACGCAGTGGCATAGCAACTTACAATGAAG

B-21

T-22

TGGGGTCTGGGTTCTGGTTATGAACCAACAGTACTCCCCTGCACGTGTGTTCTTCACTAA
ACCCCCAGACCCAAGACCAATACTTGGTGTCACTGAGGGCACGTGCACACAAGAAGTGATT

B-22

T-23

B-23

T-24

AGCTGTAGCTGTTATCACCCCTGATCGATGACACTTACGATGCTTACGGCACCTACGAAG
TCGACATCGACAATAGTGGACTAGCTACTGTGAATGCTACGAATGCCGTGGATGCTTC

B-24

T-25

AACTGAAGATCTTACTGAAGCTGTAGAACGCTGGTCTATCACTTGCCTGGACACTCTG
TTGACTTCTAGAAATGACTTCGACATCTTGCAGACAGATAGTGAAACGGACCTGTGAGAC

B-25

T-26

B-26

T-27

CCGGAGTACATGAAACCGATCTACAAACTGTTCATGGATACCTACACCGAAATGGAGGA
GGCCTCATGTACTTGGCTAGATGTTGACAAGTACCTATGGATGTGGCTTACCTCCT

B-27

T-28

ATT CCTGGCAAAAGAAGGCGTACCGACCTGTTCAACTGCGGTAAAGAGTTGTTAAAG
TAAGGACCGTTCTCCGGCATGGCTGGACAAGTTGACGCCATTCTCAAACAATTTC

B-28

T-29

B-29

AATTCGTACGTAACCTGATGGTTGAAGCTAAATGGCTAACGAAGGCCATATCCCGACT
TTAAGCATGCATTGGACTACCAACTTCGATTACCCGATTGCTCCGGTATAGGGCTGA

B-30

T-31

ACCGAAGAACATGACCCGGTTGTTATCATCACCGCGGTGCAAACCTGCTGACCACAC
TGGCTTCTGTACTGGGCCAACAAATAGTAGTGGCCGCCACGTTGGACGACTGGTGGTG

B-31

T-32

TTGCTATCTGGGTATGTCCGACATCTTACCAAGGAATCTGTTGAATGGGCTGTTCTG
AACGATAGACCCATACAGGCTGTAGAAATGGTCCTTAGACAACTTACCCGACAAAGAC

B-32

B-33

T-33

T-34

CACCGCCGCTGTTCCGTTACTCCGGTATTCTGGGCGTCGTCTGAACGACCTGATGACC
GTGGCGGCACAAGGCAATGAGGCCATAAGACCCAGCAGCAGACTTGCTGGACTACTGG

B-34

T-35

CACAAAGCAGAGCAGGAACGTAAACACTCTTCCTCCTCTGGAAATCCTACATGAAGGA
GTGTTTCTCGTCTCGTCCTGCATTGTGAGAAGGAGGAGACCTTAGGATGTACTTCCT

B-35

B-36

T-36

T-37

ATATAACGTTAACGAGGAGTACGCACAGACTCTGATCTATAAAGAAGTTGAAGACGTAT
TATATTGCAATTGCTCCTCATGCGTGTCTGAGACTAGATATTCTTCAACTTCTGCATA

B-37

T-38

GGAAAGACATCAACCGTGAATACCTGACTACTAAAAACATCCCGGCCGCTGCTGATG
CCTTTCTGTAGTTGGCACTTATGGACTGATGATTGTTAGGGCGCGGGCGACGACTAC

B-38

B-39

T-39

T-40

GCAGTAATCTACCTGTGCCAGTTCTGGAAAGTACAGTACGCTGGTAAAGATAACTTCAC
CGTCATTAGATGGACACGGTCAAGGACCTTCATGTCATGCGACCATTCTATTGAAGTG

B-40

T-41

TCGCATGGCGACGAATACAAACACCTGATCAAATCCCTGCTGGTTACCGATGTCCA
AGCGTACCCGCTGTTATGTTGTGGACTAGTTAGGGACGACCAATGGGCTACAGGT

B-41

B-stop

T-stop

TCTGATCCCAGGATTAGAT
AGACTAGGGCCCTAATCTA

Supplementary Table 1 Summary of strains and plasmids used in this study

Strain or Plasmid	Description	Source
<i>E.coli</i> DH10B		Gibco-Life Technologies
<i>E.coli</i> DYM1	<i>ispC</i> <i>E. coli</i> strain	1
pCR4	TA cloning vector; Ap ^R	Invitrogen
pTrc99A	High-copy expression plasmid; Ap ^R	Pharmacia
pBBR1MCS-3	Low-copy broad-host expression plasmid; Tc ^R	2
pBAD33	Low-copy broad-host expression plasmid; Cm ^R	3
pLac33	Low-copy broad-host expression plasmid; Cm ^R	This study
pSOE4	pLac33 derivative containing the <i>dxs</i> , <i>ippHp</i> and <i>ispA</i> genes; Cm ^R	This study
pAC-LYC04	Plasmid expressing the IPP isomerase (<i>ippHp</i>) isolated from <i>Haematococcus pluvialis</i> ; Cm ^R	4
pMKPMK	pBBR1MCS-3 derivative containing the MK and PMK genes; Tc ^R	This study
pMevB	pBBR1MCS-3 derivative containing the MK, PMK, and MPD genes; Tc ^R	This study
pMBI	pMevB derivative containing the <i>idi</i> gene; Tc ^R	This study
pMBIS	pMevB derivative containing the <i>idi</i> and <i>ispA</i> genes; Tc ^R	This study
pMevT	pLac33 derivative containing the <i>atoB</i> , HMGS and tHMGR genes; Cm ^R	This study
pADS	pTrc99A derivative containing the ADS gene; Ap ^R	This study

1. Kuzuyama, T., Takahashi, S. & Seto, H. Construction and characterization of *Escherichia coli* disruptants defective in the *yaem* gene. *Biosci. Biotechnol. Biochem.* **63**, 776-778 (1999).
2. Kovach, M.E. et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-176 (1995).
3. Guzman, L.-M., Belin, D., Carson, M.J. & Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121-4130 (1995).
4. Cunningham, F.X., Sun, Z., Chamovitz, D., Hirschberg, J. & Gantt, E. Molecular structure and enzymatic function of lycopene cyclase from the *Cyanobacterium synechococcus* sp strain PCC7942. *Plant Cell* **6**, 1107-1121 (1994).

Supplementary Table 2 PCR primers used in this study

MK-f	5'-GATCTGCAGTAGGAGGAATTAAACCATGTCATTACCGTTCTTAAC-3'
MK-r	5'-TTGATCTGCCTCCTATGAAGTCCATGGTAAATT-3'
PMK-f	5'-ACTTCATAGGAGGCAGATCAAATGTCAGAGTTGAGAGCCTTC-3'
PMK-r	5'-GAGTATTACCTCCTATTATCAAGATAAGTTTC-3'
MPD-f	5'-GATAAATAGGAGGTAATACTCATGACCGTTACACAGCATCC-3'
MPD-r	5'-TACCTGCAGTTATTCCCTTGCTAGACAGT-3'
atoB-f	5'-GATGTCGACTAGGAGGAATAAAAATGAAAAATTGTGTCATCGTC-3'
atoB-r	5'-TTAGCTGCCTCCTTAATTCAACCGTTCAATCAC-3'
HMGS-f	5'-GAATTAAAGGAGGACAGCTAAATGAAACTC TCAACTAAACTTG-3'
HMGS-r	5'-AGTGTAAATCCTCCTTATTAAACATCGTAAG-3'
tHMGR-f	5'-GTTAAAAAAATAAGGAGGATTACACTATGGTTTA ACCAATAAAACAG-3'
tHMGR-r	5'-ATCGTCGACTTAGGATTAAATGCAGGTGACGGACC-3'
idi-f	5'-ATCCCGGGAGGAGGATTACTATATGCAAACGGAACACGTC-3'
idi-r	5'-ATCCCGGGTTATTAAAGCTGGTAAATG-3'
ispa-f	5'-AGATCCCGGGAGGAGGAATGAGTAATGGACTTCCGCAGCAC-3'
ispa-r	5'-AGTGAGAGCTTTATTACGCTGGATGATG-3'
dxs1	5'-TTGGGCTAGCAGGAGGAATTACCATGAGTTTGATATT GCCAATAC-3'
dxs2	5'-TCTGAGCAACGAACGAAGCATATATTATGTCCTCCAGG CCTTGATTTG-3'
ippHp1	5'-CAAAATCAAGGCCTGGAGGACATAAATATGCTTCGTT CGTTGCTCAGA-3'
ippHp2	5'-GCATCCATGGTATCATCCTCCGTTGATGTGATG-3'
ispa1	5'-TGATACCATGGACTTCCGCAGCAACTCG-3'
ispa2	5'-GTACATGCATTATTACGCTGGATGATG-3'
SOE-f	5'-TGGGTACCGGGCCCCCCTGCCTCTAGAGTCGACTAGGAGGAATTCA ACCATGAGTTTG-3'

Pyruvate Metabolism in *Saccharomyces cerevisiae*

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In yeasts, pyruvate is located at a major junction of assimilatory and dissimilatory reactions as well as at the branch-point between respiratory dissimilation of sugars and alcoholic fermentation. This review deals with the enzymology, physiological function and regulation of three key reactions occurring at the pyruvate branch-point in the yeast *Saccharomyces cerevisiae*: (i) the direct oxidative decarboxylation of pyruvate to acetyl-CoA, catalysed by the pyruvate dehydrogenase complex, (ii) decarboxylation of pyruvate to acetaldehyde, catalysed by pyruvate decarboxylase, and (iii) the anaplerotic carboxylation of pyruvate to oxaloacetate, catalysed by pyruvate carboxylase. Special attention is devoted to physiological studies on *S. cerevisiae* strains in which structural genes encoding these key enzymes have been inactivated by gene disruption.

KEY WORDS — Yeast; glycolysis; TCA cycle; sugar metabolism; metabolic engineering; pyruvate decarboxylase; pyruvate carboxylase; pyruvate dehydrogenase complex; alcoholic fermentation; Crabtree effect

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		INTRODUCTION	
		Pyruvic acid (from Greek, πυρ=fire; Latin, <i>uva</i> =grape; German Brenztraubensäure) derives its name from the fact that it is formed upon heating of tartaric acid, a major organic acid in wine, as first demonstrated by Erlenmeyer. ³⁹ As	

*Corresponding author.

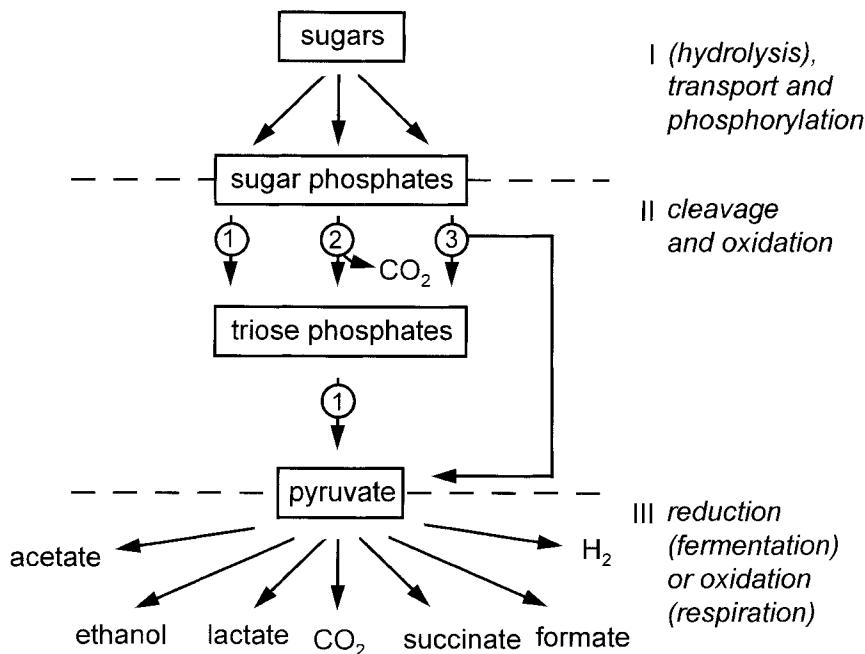


Figure 1. Schematic representation of the diversity of sugar dissimilation by micro-organisms. Multiple routes exist for transport and sugar phosphorylation (I), conversion of sugar phosphates to pyruvate (II) and conversion of pyruvate to end products of dissimilation (III). Sugars may be transported by facilitated diffusion, active transport or group translocation; oligosaccharides can either be transported and then hydrolysed intracellularly or, alternatively be hydrolysed extracellularly and then transported as monosaccharides. Cleavage and oxidation of sugar phosphates may occur via the Embden-Meyerhof pathway (1), the hexose-monophosphate pathway (2) and the Entner-Doudoroff pathway (3). Under some conditions, triose phosphates may also be converted to pyruvate via the methylglyoxal by-pass. Virtually all possible combinations of the variants of I, II and III are encountered in nature.

illustrated by its central position on many metabolic pathway maps, pyruvate is located right at the heart of heterotrophic carbon metabolism. Neuberg first pointed to the importance of pyruvate as an intermediate in the fermentative metabolism of sugars by yeasts.⁹⁵ Kluyver, who was later to become a pioneer in the taxonomy, biochemistry and physiology of yeasts, was not immediately convinced. One of the (obligatory) statements accompanying his PhD thesis read: 'Pyruvate is not, as postulated by Neuberg, an intermediate in the alcoholic fermentation'.⁷¹ This youthful transgression did not keep Kluyver from becoming a full professor in our department (1922–1956). We hope that readers will extend a similar clemency to the inevitable errors and omissions in this review.

Modes of carbohydrate metabolism in yeasts and other microorganisms

Microorganisms use different routes for the metabolism of sugars. As illustrated in Figure 1, this metabolic diversity is apparent at three levels: (i) transport and, in the case of oligosaccharides, hydrolysis; (ii) conversion of sugar phosphates to pyruvate by cleavage and oxidation; and (iii) further metabolism of pyruvate. Figure 1 represents a simplified scheme to which many exceptions exist. For example, some bacteria (including various pseudomonads) first carry out one or more oxidation steps outside the cell membrane, after which transport of the resulting organic acid, phosphorylation and cleavage occur.⁸²

A common motif in virtually all sugar-metabolizing microorganisms is that the lower part

of the Embden-Meyerhof pathway is involved in the conversion of triose phosphates to pyruvate. In some cases, this reaction sequence can be by-passed by a route involving methylglyoxal and D-lactate as intermediates.²⁷ The methylglyoxal by-pass, which is not coupled to substrate-level phosphorylation, probably functions mainly during 'overflow metabolism'.¹³⁹

The diversity of microbial sugar metabolism is especially evident in the further metabolism of pyruvate (Figure 1). During fermentative growth, pyruvate may be converted into a multitude of compounds, including molecular hydrogen, carbon dioxide and many organic metabolites. Alternatively, respiratory dissimilation of pyruvate via the tricarboxylic acid (TCA) cycle leads to its complete oxidation to carbon dioxide and water.

In view of the staggering diversity of sugar metabolism in the microbial world, a surprising unity exists among yeasts. Of the ca. 700 yeast species that are currently recognized, all strains investigated seem to predominantly use the Embden-Meyerhof pathway for conversion of hexose phosphates to pyruvate. In order not to make yeasts appear overtly boring in this respect, it should be mentioned that important differences occur in the initial steps leading from extracellular sugar to intracellular hexose phosphates (Figure 2).⁴ Furthermore, depending on growth conditions and yeast species, the hexose monophosphate pathway may make an important contribution to sugar metabolism.^{20,21,24}

The unity in the carbohydrate metabolism of yeasts becomes most evident from their fermentative sugar metabolism. Whenever yeast species exhibit a fermentative sugar metabolism, ethanol and carbon dioxide are the predominant fermentation products.³³ The glycerol that is also frequently found does not primarily result from sugar dissimilation. Rather, glycerol formation enables the reoxidation of NADH that is generated during the conversion of sugar into biomass.^{102,146,147} Under aerobic conditions, this assimilatory NADH can easily be disposed of by respiration. When, under conditions of extreme oxygen limitation or anaerobiosis, this becomes impossible, glycerol formation acts as an essential redox valve.¹⁵⁸

In addition to ethanol and glycerol, fermenting yeast cultures often excrete small amounts of other fermentation products, in particular organic acids (e.g. acetate and succinate^{102,151}). Under some

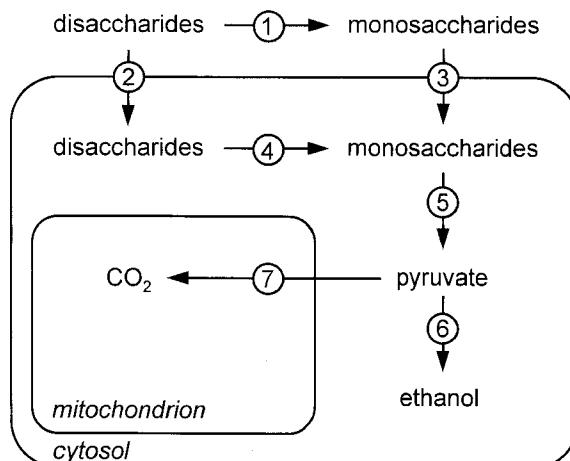


Figure 2. Schematic representation of carbohydrate dissimilation by yeasts. Hydrolysis of disaccharides may occur by extracellular enzymes with a low pH optimum (1) or intracellularly (4) by enzymes with a near-neutral pH optimum. Disaccharide transport (2) appears to occur exclusively via proton symport, whereas monosaccharide transport (3) may involve either facilitated diffusion or proton symport, depending on yeast species and environmental conditions. In the case of hexoses, the Embden-Meyerhof pathway is the main route of sugar dissimilation (5) in yeasts. Pyruvate, the end-product of glycolysis, is either converted to ethanol and carbon dioxide (6) or respiration to carbon dioxide and water (7) in the mitochondria.

conditions, D-lactate is produced in small amounts by cultures of *Saccharomyces cerevisiae* and *Candida utilis* (Luttik *et al.*, unpublished),⁶⁶ suggesting involvement of the methylglyoxal by-pass, albeit at very low rates. Other metabolites excreted by yeasts include higher alcohols, esters and aldehydes. Although specific rates of formation of these compounds are often orders of magnitude lower than the rates of ethanol formation, some play an important role as (off-)flavours in alcoholic beverages.

Regulation of sugar metabolism in yeasts

The range of sugars that support growth differs greatly among yeast species. Nevertheless, all wild-type yeast strains that have so far been tested can utilize glucose as a carbon source.⁵ However, not all yeast strains are able to ferment glucose to ethanol. At the time of writing this review, the total number of strains deposited in the collection of the Centraalbureau voor Schimmelcultures was 4738. Of these, 4180 strains had been tested for their fermentative capacity, using the standard taxonomic test method (i.e. measuring gas

Table 1. Regulation of fermentative sugar metabolism in yeasts.

Regulatory phenomenon	Definition and environmental conditions	Proposed mechanism	References
Crabtree effect (long-term)	Aerobic alcoholic fermentation at high growth rates, irrespective of the mode of cultivation (growth under sugar limitation or growth with excess sugar)	Insufficient capacity of respiratory routes of pyruvate dissimilation	107,109,114
Crabtree effect (short-term)	Instantaneous aerobic alcoholic fermentation after transition of sugar-limited cultures to sugar excess	Respiratory metabolism becomes saturated, causing overflow at the level of pyruvate	114,140,144
Pasteur effect	Suppression of alcoholic fermentation in the presence of oxygen. Observed in all facultatively fermentative yeasts that do not exhibit a Crabtree effect. In <i>S. cerevisiae</i> only evident at low glycolytic fluxes (e.g. in slowly growing cells)	The affinity (V_{max}/K_m) of the respiratory system for pyruvate, acetaldehyde and/or NADH is higher than that of the fermentative route	78
Kluyver effect	Absence of alcoholic fermentation during oxygen-limited growth on a sugar (often a disaccharide), even though glucose is readily fermented. Widespread among yeasts, does not occur in <i>S. cerevisiae</i>	Control of the synthesis and/or activity of the sugar carrier. When the effect is observed for disaccharides, these are hydrolysed intracellularly	4,66,133,158
Custers effect	Oxygen requirement for alcoholic fermentation, evident upon transfer from oxygen-limited to anaerobic conditions. Observed with glucose (e.g. in <i>Brettanomyces</i> spp.) as well as with other sugars (e.g. xylose in <i>Candida utilis</i>)	Redox imbalance, either due to an inability to form glycerol or other reduced metabolites (<i>Brettanomyces</i>), or to different cofactor specificity of reduction and oxidation reactions (xylose fermentation)	22,23,33,125

production in Durham tubes). According to this test, 1555 strains (or 37% of the total) were unable to perform alcoholic fermentation.

As the Durham test is rather insensitive, and therefore prone to false-negative results,³⁴ the true percentage of non-fermentative yeasts is probably lower than the taxonomic tests suggest. Since the group of strictly-fermentative yeasts (i.e. yeasts that are unable to respire sugars) is also relatively small, the large majority of yeast strains can either respire sugars or ferment them to ethanol and carbon dioxide. All these facultatively fermentative yeasts exhibit alcoholic fermentation under oxygen-limited growth conditions.^{33,34} This is where uniformity ends, as in many yeasts the oxygen concentration is not the sole factor determining the contribution of respiration and fermentation to the overall rate of sugar metabolism.

The diversity among facultatively fermentative yeasts with respect to the regulation of alcoholic fermentation is evident from phenomena indicated by such terms as 'Pasteur effect', 'Crabtree effect', 'Kluyver effect' and 'Custers effect'. All these 'effects' represent regulatory mechanisms that affect the balance between fermentation and respiration. Since it would be beyond the scope of this review to discuss these phenomena in detail, only a brief summary of the phenomenology of these effects and their proposed mechanistic explanations are given in Table 1. The Crabtree effect (occurrence of alcoholic fermentation under aerobic conditions) is one of the most important metabolic phenomena in biomass-directed industrial applications of *S. cerevisiae* and, as will be discussed below, a major incentive for studying pyruvate metabolism in this yeast.

S. cerevisiae: laboratory model and cell factory

It is not necessary to reiterate the etymology of the word 'enzyme' to illustrate the unique role of *S. cerevisiae* in the development of biochemistry and physiology. While continuing to be an important model organism for studies in these fields (as will hopefully be illustrated in this review), *S. cerevisiae* has also become a key model organism for studying eukaryotic genetics and cell biology. This role will only become more important now that this yeast has become the first eukaryote to have its complete genome sequenced. The functional analysis of the many open reading frames to which no discrete physiological function has yet been attributed, is one of the major challenges in yeast biology.⁹⁹

Developments with respect to the industrial applications of *S. cerevisiae* are in many aspects similar to those in fundamental research. Areas of yeast biology that have already existed for decades or even centuries, including the production of alcoholic beverages and bakers' yeast, continue to merit research. This is partly due to the need for improved process control, which necessitates a quantitative description of the metabolic fluxes within the cells. Furthermore, in spite of the long history of *S. cerevisiae* as an industrial micro-organism, some inherent problems are still not completely understood or solved. This is perhaps best illustrated by the classical bakers' yeast production process.

During the production of bakers' yeast, the costs of the carbohydrate feedstock are a major factor in the overall economy of the process. Thus, a high biomass yield on the sugar feedstock (usually molasses) is a major optimization criterion. This implies that fermentative sugar metabolism (which leads to a much lower biomass yield than respiratory metabolism; for a review see ref. 148) should be avoided during the production phase. On the other hand, commercial bakers' yeast should have a high fermentative capacity in the dough application. During the industrial production of bakers' yeast, the strong inclination of *S. cerevisiae* to perform alcoholic fermentation is largely overcome by careful manipulation of the rate of sugar supply and by controlling other environmental conditions.¹² So far, it has not been possible to use metabolic engineering to control the tendency of *S. cerevisiae* towards aerobic fermentation, while, at the same time, maintaining a high fermentative capacity in the dough environment.

While classical applications of *S. cerevisiae* continue to present challenges, novel processes are rapidly gaining ground. The most important example of this is the use of *S. cerevisiae* as a host for the expression of heterologous proteins.^{52,117} In addition to problems dealing with expression of heterologous genes and the excretion and modification of the products, a number of physiological properties of *S. cerevisiae* need to be taken into consideration for this application. To facilitate downstream processing, high product concentrations are desirable. Therefore, large-scale heterologous protein production is performed at high biomass densities. At high biomass densities, even low specific rates of by-product formation will rapidly lead to the build-up of toxic metabolite levels, with detrimental effects on productivity.^{51,148}

Minimization of by-product formation is not the only way in which physiological research may contribute to heterologous protein production. At present, only a small fraction of the carbon and nitrogen substrates fed to heterologous-protein-producing cultures is converted into the product of interest. Future research will therefore also have to address the question how the fluxes leading to heterologous protein can be maximized while at the same time keeping the yeast cell viable under the conditions used for industrial production.

Aim and scope of this review

Further optimization of *S. cerevisiae* strains as dedicated 'cell factories' will to a large extent depend on the rerouting of metabolism by metabolic pathway engineering. Rational strategies for modifying the distribution of fluxes at key branch-points in the metabolic network depend on detailed knowledge of the physiological role of the different branches and of the regulatory mechanisms that operate in wild-type cells.

The aim of this paper is to provide an overview of the literature on the junction in the *S. cerevisiae* metabolic network where fermentative and respiratory sugar metabolism diverge: the pyruvate branch-point. We have focussed on the biochemistry and physiology of pyruvate metabolism and, in particular, on the use of molecular genetic techniques to study the role and regulation of key enzymes active at this branch-point. Regulation of enzyme synthesis is discussed in terms of enzyme activity and (in some cases) mRNA levels, but we

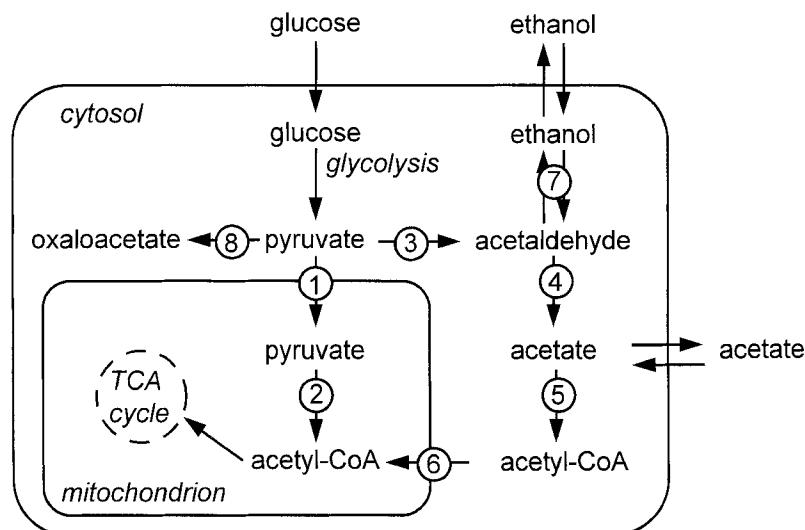


Figure 3. Key enzymic reactions at the pyruvate branch-point in *S. cerevisiae*. Numbered reactions are catalysed by the following enzymes: 1, mitochondrial pyruvate carrier; 2, pyruvate dehydrogenase complex; 3, pyruvate decarboxylase; 4, acetaldehyde dehydrogenase; 5, acetyl-CoA synthetase; 6, carnitine shuttle; 7, alcohol dehydrogenase; 8, pyruvate carboxylase. In addition to a cytosolic acetaldehyde dehydrogenase, *S. cerevisiae* also contains a mitochondrial isoenzyme.⁶³ The pyruvate dehydrogenase by-pass consists of enzymes 3, 4 and 5. Formation of acetyl-CoA from acetate requires two ATP equivalents, since acetyl-CoA synthetase hydrolyses ATP to AMP and pyrophosphate.

have chosen not to discuss signal transduction mechanisms.

A further restriction is that we will focus on three major conversions of pyruvate that occur during growth on sugars: oxidative decarboxylation to acetyl-CoA, decarboxylation to acetaldehyde and carboxylation to oxaloacetate.

This paper deals with a single yeast species, *S. cerevisiae*. Although 'yeast' and '*S. cerevisiae*' are often used as synonyms in the literature, it should be realized that in many physiological aspects, *S. cerevisiae* is an exceptional yeast. For example, *S. cerevisiae* is one of very few yeasts capable of growth under strictly anaerobic conditions.¹⁵⁰ The regulation of pyruvate metabolism in *S. cerevisiae*, with its strong tendency towards alcoholic fermentation, is also clearly different from other yeasts used as laboratory model organisms and/or industrial microorganisms (e.g. *C. utilis*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia stipitis*, *Yarrowia lipolytica*, etc.). Therefore, the data discussed in this paper cannot necessarily be extrapolated to other yeast species.

MAJOR ROUTES OF PYRUVATE METABOLISM: ENZYMES AND GENES

The three major metabolic pathways in *S. cerevisiae* that originate from pyruvate are schematically shown in Figure 3. Of these pathways, only the conversion of pyruvate into ethanol has an exclusively dissimilatory function: ATP production from substrate-level phosphorylation reactions in glycolysis can only occur when the NADH produced in this pathway is reoxidized. Acetaldehyde, the electron acceptor used for NADH reoxidation during fermentative growth, is formed from pyruvate by pyruvate decarboxylase (EC 4.1.1.1).

The energetically more favourable, respiratory dissimilation of carbohydrates requires coupling of glycolysis to the TCA cycle. Acetyl-CoA, the fuel of the cycle, can be synthesized from pyruvate by a direct oxidative decarboxylation, catalysed by the pyruvate-dehydrogenase complex.^{60,75,76} In contrast to the enzymes of glycolysis, which are all located in the cytosol, the pyruvate-dehydrogenase complex is located in the

Table 2. Structural genes of enzymes involved in pyruvate metabolism by *S. cerevisiae*, predicted size of the encoded peptides and chromosomal localization. Nomenclature of genes is according to the Yeast Protein Database.

Enzyme	Structural gene	Peptide size (kDa)	Chromosome	Reference
<i>Pyruvate dehydrogenase</i> heteromultimer, 8–9 MD				
E1 α subunit	<i>PDA1</i>	45	V	7,136
E1 β subunit	<i>PDB1</i>	36	II	90
E2 subunit	<i>LAT1</i>	49	XIV	98
E3 subunit	<i>LPDI</i>	52	VI	118,120
Protein X	<i>PDX1</i>	42	VII	8
<i>Pyruvate decarboxylase</i> tetramer, 248 kDa				
	<i>PDC1</i>	62	XII	69,128
	<i>PDC5</i>	62	XII	54,131
	<i>PDC6</i>	62	VII	55
<i>Pyruvate carboxylase</i> tetramer, 520 kDa				
	<i>PYCI</i>	130	VII	92
	<i>PYC2</i>	130	II	137,153

mitochondrial matrix.⁶⁰ Therefore, pyruvate oxidation by the complex requires transport of the substrate across the mitochondrial membranes.

In addition to the pyruvate dehydrogenase reaction, conversion of pyruvate to acetyl-CoA can occur via an indirect route (Figure 3). This 'pyruvate dehydrogenase by-pass' involves the enzymes pyruvate decarboxylase (which is also a key enzyme in alcoholic fermentation), acetaldehyde dehydrogenase and acetyl-CoA synthetase.⁶⁰ The by-pass route is the source of the acetate that accumulates in sugar-grown *S. cerevisiae* cultures under some growth conditions (e.g. during transfer from glucose limitation to glucose excess).¹⁴⁴

Conversion of pyruvate into acetyl-CoA is not only a dissimilatory reaction: acetyl-CoA, as well as some TCA-cycle intermediates synthesized from it, is an essential biosynthetic building block. This assimilatory function of the TCA cycle is, in principle, incompatible with its role in dissimilation, since complete dissimilation of acetyl-CoA requires that oxaloacetate be regenerated at each turn of the cycle. During growth of *S. cerevisiae* on carbohydrates, the withdrawal of TCA-cycle intermediates for biosynthesis is compensated for by the third major pathway originating from pyruvate, i.e. the carboxylation of pyruvate to oxaloacetate. This vital assimilatory reaction is catalysed by pyruvate carboxylase (EC 6.4.1.1).

Some relevant enzymological and regulatory characteristics of the three enzymes that compete

for the common intermediate pyruvate, as well as some characteristics of their structural genes, are discussed below. In addition, the scarce information on transport of pyruvate into *S. cerevisiae* mitochondria is summarized.

The pyruvate dehydrogenase complex

As in other organisms, the *S. cerevisiae* pyruvate dehydrogenase complex is a large multi-component enzyme complex ($M_r = 8-9 \times 10^6$).^{75,76} It belongs to the family of α -oxoacid-dehydrogenase complexes, which catalyse the lipoamide-mediated oxidative decarboxylation of α -oxoacids.^{112,161} These complexes consist of three major catalytic components called E1, E2 and E3.¹⁶¹ In contrast to the E1 and E2 subunits, which are complex-specific, the E3 subunit of the pyruvate dehydrogenase complex is also part of 2-oxoglutarate dehydrogenase and branched-chain 2-oxoacid dehydrogenase, the other α -oxoacid-dehydrogenase complexes in *S. cerevisiae*,^{29,30} and of glycine decarboxylase.¹³⁴ A fourth component, X, does not appear to have a catalytic function but is probably involved in assembly of the complex.⁸⁰ The structural genes encoding the subunits of the *S. cerevisiae* pyruvate dehydrogenase complex have all been cloned (Table 2).

Conversion of pyruvate into acetyl-CoA is catalysed by the concerted action of the catalytic subunits.^{112,113} In the first step, pyruvate is covalently linked to thiamine pyrophosphate (TPP), the cofactor of the E1 subunit (pyruvate

dehydrogenase, EC 1.2.4.1). This reaction yields 2- α -hydroxy-ethyl-TPP ('active aldehyde').⁶¹ In *S. cerevisiae*, as in most other organisms, the E1 moiety consists of two subunits, E1 α and E1 β .⁷⁶ The α -hydroxy group of active aldehyde is oxidized and the resulting acetyl moiety is coupled to the lipoamide cofactor of the E2 subunit (dihydrolipoamide acetyl transferase, EC 2.3.1.12), which subsequently transfers it to coenzyme A. During the oxidation of active aldehyde, an E2 lipoamide group is reduced to dihydrolipoamide. Its reoxidation is catalysed by the E3 subunit (dihydrolipoamide dehydrogenase, EC 1.6.4.3) that uses NAD $^+$ as the electron acceptor. Thus, the overall reaction catalysed by the complex is:



Kinetic analysis with the purified pyruvate dehydrogenase complex from *S. cerevisiae* yielded K_m values for pyruvate, NAD $^+$ and coenzyme A of 625 μM , 23 μM and 18 μM , respectively.⁷⁵ These K_m values were measured at pH 8.1; the K_m for pyruvate decreases to ca. 200 μM when the pH is lowered to 6.5.^{67,75} Cooperativity with respect to pyruvate, as occurs with prokaryotic pyruvate dehydrogenase complexes, was not observed with the *S. cerevisiae* complex.^{67,75} The products NADH and acetyl-CoA exhibited competitive inhibition with respect to NAD $^+$ and coenzyme A (K_i=23 μM and K_i=18 μM , respectively).

Activity of the pyruvate dehydrogenase complex is difficult to assay in crude cell extracts due to proteolytic degradation and interference by other enzymes, in particular pyruvate decarboxylase.^{75,136} However, evidence from measurements on crude mitochondrial fractions indicates that pyruvate dehydrogenase activity is expressed under conditions which do not require its dissimilatory function. For example, activity has been measured in cells from anaerobic cultures and in cultures grown aerobically on ethanol.¹⁵⁵ This may be indicative of a role of the pyruvate dehydrogenase complex in mitochondrial assimilatory processes requiring active aldehyde or acetyl-CoA. An observation that may be relevant to this hypothesis is that, with the exception of *LAT1*, all genes encoding subunits of the complex contain putative GCN4 boxes.¹⁵⁵ Gcn4p is a general regulator of amino acid metabolism.⁵³ Although it is as yet unclear to what extent these sequences are functional, their presence is consistent with a role of the pyruvate dehydrogenase complex in amino acid

synthesis. This is further supported by a partial leucine requirement of mutants lacking pyruvate dehydrogenase activity.¹⁵⁴

Transcriptional regulation has been studied for two of the structural genes encoding subunits of the complex. Levels of the *PDA1* transcript, which encodes the E1 α subunit, were essentially constant under all conditions tested, including aerobic growth on ethanol and anaerobic growth in glucose-limited chemostat cultures.¹⁵⁵ In fact, due to its constant level and stability, it has been proposed that the *PDA1* transcript is a suitable loading standard for quantitative mRNA assays.¹⁵⁷ In contrast, transcription of the *LPD1* gene (encoding the E3 subunit, which is also part of the two other α -oxoacid-dehydrogenase complexes) is subject to glucose catabolite repression, although significant transcription levels are still observed in the presence of glucose.¹²⁰ Full derepression of *LPD1* requires a HAP2/3/4 binding site in its promoter.¹⁷

The possible involvement of phosphorylation of the pyruvate dehydrogenase complex in the regulation of its activity will be discussed in a separate paragraph.

Pyruvate decarboxylase

Pyruvate decarboxylase (EC 4.1.1.1) catalyses the TPP- and magnesium-dependent decarboxylation of pyruvate to acetaldehyde and carbon dioxide.^{47,84,96} The native *S. cerevisiae* enzyme, which occurs in the cytosol,¹⁴³ is a tetramer, composed of four identical or highly related subunits of ca. 62 kDa.^{46,77}

Pyruvate decarboxylase exhibits cooperativity with respect to pyruvate, an effect which is enhanced by phosphate.^{14,62} At the same time, phosphate is a competitive inhibitor of the enzyme: the K_m for pyruvate (1–3 mM in the absence of phosphate) increases about fourfold in the presence of 25 mM phosphate.^{14,143} Normal intracellular concentrations of phosphate in *S. cerevisiae* (5–15 mM^{58,143}) are higher than the K_i for phosphate (ca. 1 mM), indicating that phosphate is a physiologically relevant effector of *in vivo* pyruvate decarboxylase activity.

Studies on pyruvate decarboxylase are complicated by the presence of three structural genes (Table 2) that each potentially encode an active enzyme. The *PDC1* gene was isolated by complementation of mutants with low pyruvate decarboxylase levels.^{69,128} The mutants used for these

complementation studies were obtained by mutagenesis with ethyl methane sulfonate.¹²⁷ Surprisingly, a null mutation of the *PDC1* gene resulted in a strain with a much higher pyruvate decarboxylase activity than that of the previously isolated point mutants.¹²³ This activity was subsequently shown to be encoded by a second, highly homologous structural gene called *PDC5*.^{54,131} In glucose-grown shake-flask cultures, *PDC5* was expressed to a much higher level in the *pdc1* deletion mutant than in strains containing point-mutation alleles of *PDC1*. This strongly suggests that expression of *PDC* genes is subject to autoregulation.⁵⁴

Inactivation of both *PDC1* and *PDC5* yields strains which, during growth in complex medium with glucose, do not express detectable levels of pyruvate decarboxylase. A third *PDC* gene, *PDC6*, was isolated by low-stringency hybridization of a genomic library with a *PDC1* probe.⁵⁵ Although the *PDC6* sequence had a high similarity with *PDC1* and *PDC5*, its disruption did not cause significant changes of pyruvate decarboxylase activity.^{40,55} However, a number of spontaneous revertants of *pdc1 pdc5* double mutants have been isolated in which a recombination event had caused a fusion of the *PDC6* open reading frame with the *PDC1* promoter.⁵⁶ So far, it is unclear whether growth conditions exist under which the native *PDC6* gene contributes significantly to pyruvate decarboxylase activity in wild-type *S. cerevisiae*.

Full expression of *PDC1* and *PDC5* requires the presence of a functional *PDC2* gene, which encodes a positive transcription regulator.⁵⁷ The role of two other genes that are required for optimal expression of pyruvate decarboxylase genes, *PDC3* and *PDC4*¹⁶⁰ (Seehaus, cited in ref. 57) remains unclear.

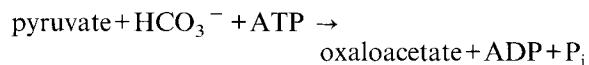
Regulation of pyruvate decarboxylase expression in *S. cerevisiae* has been studied almost exclusively in shake-flask cultures. In such experiments, a strong increase of pyruvate decarboxylase activity is invariably observed when cultures are switched from a non-fermentable carbon source (e.g. ethanol) to a glucose-containing medium (see e.g. refs 15,16,40,87). In mutants affected in the expression of various glycolytic enzymes, induction of pyruvate decarboxylase is correlated with the levels of metabolites originating from the lower part of glycolysis.^{15,16} While these experiments demonstrate that the activity of pyruvate decarboxylase in *S. cerevisiae* varies as a function of growth conditions, shake-flask experiments do not

allow discrimination between the effects of individual growth parameters. Changes of the carbon source will have a profound impact on many growth parameters, including pH, growth rate, viability, metabolite concentrations and dissolved-oxygen concentration.

In particular, the dissolved-oxygen concentration has been shown to have a strong effect on the regulation of pyruvate decarboxylase in yeast species.^{41,135,145,158} This parameter cannot be adequately controlled in batch cultures which, especially during rapid growth on sugars, become oxygen limited.³⁴ In aerobic, carbon-limited chemostat cultures of *S. cerevisiae* grown on ethanol or glucose at a dilution rate of 0·10 h⁻¹, no significant differences in pyruvate decarboxylase activities were observed.¹¹⁰ In aerobic, sugar-limited cultures, an increase of the pyruvate decarboxylase activity occurs above the critical growth rate at which alcoholic fermentation is triggered.^{88,109} So far, no experiments have been performed to study the differential expression of the three structural *PDC* genes under carefully controlled growth conditions (e.g. as a function of the oxygen supply in cultures grown at a fixed rate or as a function of growth rate in aerobic and anaerobic cultures).

Pyruvate carboxylase

In *S. cerevisiae*, the anaplerotic synthesis of oxaloacetate from pyruvate is catalysed by pyruvate carboxylase (EC 6.4.1.1).^{42,86} The enzyme catalyses the magnesium- and ATP-dependent carboxylation of pyruvate to oxaloacetate:¹²¹



In contrast to many higher organisms, in which pyruvate carboxylase is a mitochondrial enzyme, its location in *S. cerevisiae* is exclusively cytosolic.^{49,116,143,153}

The native *S. cerevisiae* enzyme is a tetramer consisting of identical or highly related 130 kDa subunits.^{3,26,115} Each subunit contains a covalently linked biotin cofactor that is attached to the inactive apoenzyme by a specific, ATP-dependent holoenzyme synthetase.¹³⁸

The K_m values of the *S. cerevisiae* pyruvate carboxylase for its substrates pyruvate, bicarbonate and ATP have been estimated at 0·8 mm, 2·7 mm and 0·24 mm, respectively.¹²¹ In addition to the concentrations of its substrates, activity of

pyruvate carboxylase can be modulated by a number of other metabolites, including acetyl-CoA, palmitoyl-CoA and aspartate. These metabolites not only affect the maximum specific activity of pyruvate carboxylase, but also the affinity for its three substrates.^{6,130}

In contrast to pyruvate carboxylase from a variety of other sources, the yeast enzyme exhibits activity in the absence of acetyl-CoA. This basal activity is dependent on the presence of potassium or other monovalent cations⁹³. At saturating concentrations of acetyl-CoA ($K_a = 6.6 \mu\text{M}$;⁹³), maximum specific activities are three- to four-fold higher than in its absence^{93,121}. The positive effect of acetyl-CoA, the fuel of the TCA cycle, is in line with the anaplerotic role of pyruvate carboxylase: accumulation of acetyl-CoA will increase replenishment of the acceptor molecule oxaloacetate. The activation by acetyl-CoA has been reported to be competitively inhibited by NADH which, however, did not affect the basal acetyl-CoA-independent activity²⁵.

In addition to acetyl-CoA, long-chain acyl-CoA esters are potent activators of the enzyme. In fact, the K_a for palmitoyl-CoA ($0.04 \mu\text{M}$) is two orders of magnitude lower than that for acetyl-CoA.⁹³ The maximum activity of *S. cerevisiae* pyruvate carboxylase also depends on the relative concentrations of adenine nucleotides: activity increases with increasing adenylate energy charge.⁸⁹

Aspartate is a non-competitive inhibitor of the enzyme ($K_i = 1.9 \text{ mM}$), whereas the product oxaloacetate exhibits competitive inhibition with respect to pyruvate ($K_i = 0.22 \text{ mM}$). Inhibition by aspartate, a biosynthetic building block directly derived from oxaloacetate, appears to be a specific feed-back control mechanism since most other dicarboxylic acids do not cause substantial inhibition.¹⁰⁴ Inhibition by α -oxoglutarate⁸³ is unlikely to be relevant under physiological conditions in view of its high K_i (ca. 18 mM).

In contrast to the extensive regulation of pyruvate carboxylase at the enzyme activity level, synthesis of the enzyme seems to be largely constitutive. In aerobic batch and chemostat cultures grown on a number of substrates, the pyruvate carboxylase activity in cell extracts varied by no more than two-fold.^{36,48,65,103} In comparison with aerobic, glucose-limited chemostat cultures grown at the same dilution rate, activities in anaerobic cultures were about two-fold higher.^{48,103} Addition of aspartate to growth media led to a decrease of pyruvate carboxylase activity in dialysed cell

extracts of ca. 50%.⁴⁸ However, even in aspartate-containing media, carbon dioxide fixation via pyruvate carboxylase continued.¹⁰³

S. cerevisiae contains two structural genes for pyruvate carboxylase (Table 2), each encoding an apoenzyme that can be activated by binding of biotin. The *PYC1* gene was cloned using an oligonucleotide probe based on a biotin-attachment consensus sequence.⁹² The highly homologous *PYC2* gene was subsequently isolated by hybridization of genomic libraries with a *PYC1* probe.^{137,153} Kinetic studies with the two isoenzymes, partially purified from disruption mutants, yielded similar K_m values for pyruvate and ATP, while aspartate inhibition kinetics were also the same.¹³⁷

Both *PYC* genes are transcribed in wild-type *S. cerevisiae* grown on glucose or ethanol in shake-flask cultures, although transcript levels were dependent on the growth phase.¹⁸ Peculiarly, levels of the *PYC1* transcript were higher in ethanol-grown cultures than in cultures grown on glucose. This is unexpected, since the glyoxylate cycle is generally assumed to account for replenishment of oxaloacetate during growth on C₂-compounds. However, since transcript levels were related to total RNA content of the samples, the observed difference might, at least in part, be caused by different rRNA contents of glucose- and ethanol-grown cells.

Transport of pyruvate into the mitochondrion

As discussed above, the *S. cerevisiae* pyruvate dehydrogenase complex is located inside the mitochondria. The flux through this enzyme might therefore not only be affected by synthesis of its subunits and by the intramitochondrial concentrations of its substrates and effectors, but also by regulation of pyruvate transport into the mitochondrial matrix. The kinetics of pyruvate transport into the mitochondria are also likely to have an impact on the competition of mitochondrial pyruvate oxidation with the cytosolic enzymes pyruvate decarboxylase and pyruvate carboxylase.⁵⁹ Of the key reactions at the pyruvate branch-point in *S. cerevisiae*, pyruvate transport into the mitochondria is by far the least studied and characterized.

Being a small molecule, pyruvate can readily cross the outer mitochondrial membrane via pores.^{9,31} Free diffusion of the non-dissociated acid across the phospholipid bilayer may contrib-

ute to transport across the inner mitochondrial membrane.² Involvement of a transporter was demonstrated by the observation that ΔpH -dependent uptake of pyruvate by *S. cerevisiae* mitochondria is competitively inhibited by α -cyano-3-hydroxycinnamate,¹⁹ a well-known inhibitor of mammalian mitochondrial pyruvate transporters.

Two peptides of 26 and 50 kDa, isolated by affinity-chromatography on immobilized 4-hydroxy-cyanocinnamate, catalysed pyruvate transport upon reconstitution in proteoliposomes.⁹⁴ In addition to pyruvate/pyruvate exchange, the reconstituted proteoliposomes also catalysed exchange of acetoacetate and branched-chain oxoacids. When exchange with acetoacetate was measured, the K_m for pyruvate was 0.8 mM. This is close to the K_m for pyruvate oxidation by intact mitochondria (0.3 mM)¹⁴³ and the K_m of the pyruvate dehydrogenase complex (0.2–0.6 mM),⁷⁵ suggesting that the overall K_m of mitochondria for pyruvate is of the order of 0.2 to 1 mM.

Although the systematic sequencing of the yeast genome has yielded a number of sequences that encode putative mitochondrial transporters, none of these has been linked conclusively to a pyruvate carrier. It therefore remains unclear which genes are involved in the uptake of pyruvate into the mitochondrial compartment of *S. cerevisiae* and how this important process is regulated at the level of carrier synthesis and/or transport activity.

GENE DISRUPTIONS

Inactivation of structural and regulatory genes by disruption or replacement, using the one-step gene disruption procedure^{100,119} has become an indispensable tool for physiological studies. By comparing null mutants with the isogenic wild type, important information can be obtained about the physiological role of the gene involved. Many marker genes that are available to disrupt or replace *S. cerevisiae* genes complement auxotrophic requirements and can therefore only be applied in auxotrophic laboratory strains. As will be briefly discussed below, such strains are poorly suited for quantitative studies on intermediary carbon metabolism.

The substrates (normally amino acids or bases) for which a yeast is auxotrophic have to be added to its growth medium. In a physiological sense, this is not equivalent to the situation in the complemented disruptant, which is able to synthesize the

amino acid or base itself. For example, the kinetics of amino-acid uptake from the medium may be different from those of intracellular amino-acid synthesis. This will directly affect the specific growth rate and intracellular metabolite pools and thus have regulatory effects that extend beyond amino acid metabolism. Furthermore, although amino-acid synthesis is not a major energy-requiring step in the synthesis of yeast biomass,¹⁴⁹ small effects on growth energetics cannot be excluded. Also energy requirements for active uptake of amino acids may affect bioenergetics.

Synthesis of amino acids is an integral part of intermediary carbon metabolism. Effects of gene disruption may therefore easily be obscured when amino acids have to be added to the growth medium. An example of this will be given below when the disruption of the *PDA1* gene, encoding the E1 α subunit of the pyruvate dehydrogenase complex,¹⁵⁴ is discussed.

A problem that is not inherent to the use of auxotrophic markers, but nevertheless worth mentioning, is that the concentrations of amino acids or nucleotides in growth media for auxotrophic strains used in the literature are often too low. For example, L-leucine is often added at a fixed concentration of 20 mg l⁻¹ to media containing 10 g l⁻¹ or more of glucose. As will be illustrated by the following calculation, this is inadequate. The protein content of *S. cerevisiae* is about 45% and the biomass yield on glucose in (respirofermentative) batch cultures is about 0.4 g g glucose⁻¹ at the time of ethanol exhaustion. Approximately 10% of *S. cerevisiae* protein consists of leucine.¹⁰¹ This means that, to achieve a biomass concentration of 4 g l⁻¹, at least 160 mg l⁻¹ leucine should be added to the medium of a leucine auxotroph. At lower leucine concentrations, growth will be limited by the amino acid long before the carbon source is exhausted.

The disadvantages of auxotrophic markers do not hold for dominant selectable marker genes (encoding, for example, antibiotic resistance), which can be used in prototrophic *S. cerevisiae* strains. However, in this case it is still important to rule out effects of the marker gene. Many antibiotic resistance cassettes use strong constitutive promoters, which may, at least in theory, lead to the production of substantial amounts of the encoded proteins. Preferably, control experiments should be performed under growth conditions where no effect of the gene disruption is expected, to rule out interference of the marker-gene-

encoded proteins with cellular metabolism. The best available system for gene inactivation probably consists of a two-step approach, in which the expression cassette that has been used to replace a gene is itself removed from the genome by a recombination of two direct repeats flanking the marker gene.^{1,119,152}

Special care is required when disruption mutants are grown in chemostat cultures. This holds in particular for disruptions in genes of which 'sleeping' isogenes or pseudogenes are present in the genome. The selective pressure in chemostats (or even in batch cultures as in the case of *PDC6*⁵⁶) may confer a strong selective advantage to revertants in which these genes have been 'awoken' by recombination events or other mutations. It may therefore be necessary to also disrupt pseudogenes to construct stable null mutants for physiological studies.

Below, the physiological effects of disruptions in structural genes encoding pyruvate decarboxylase, pyruvate carboxylase and components of the pyruvate dehydrogenase complex will be discussed.

Disruption of genes encoding subunits of the pyruvate dehydrogenase complex

Gene disruptions have been introduced in all four structural genes encoding subunits that are unique to the pyruvate dehydrogenase complex, but no disruption mutants have been described for the *LPD1* gene. Null mutations in the genes encoding the E1 α , E1 β , E2 and X subunits of the complex all result in complete loss of pyruvate dehydrogenase activity.^{80,81,90,154} Nevertheless, these pyruvate dehydrogenase-negative (*Pdh*⁻) strains were all viable in complex and defined media containing either glucose or non-fermentable carbon sources.

For the *lat1* and *pdx1* null mutants (lacking the E2 and X subunits, respectively), growth rates on glucose and other carbon sources were reported not to differ significantly from the isogenic wild-type strains. For the *pdhβ1* null mutant, the only phenotype reported was slightly retarded growth on glycerol plates.⁹⁰ Physiological effects of a null mutation in *PDA1* have been studied in most detail.¹⁵⁴ A null mutant constructed by replacement of the *PDA1* gene with the antibiotic resistance gene *Tn5ble* exhibited a growth rate on a glucose-containing mineral medium that was less than half that of the wild type. Peculiarly, when the same mutation was introduced in the auxo-

trophic strain M5, only a small difference in growth rate with the wild type was found. This difference was shown to be due to a partial leucine requirement of the *Pdh*⁻ strains: when leucine was added to the growth medium of the prototrophic null mutant, its growth rate increased to a value close to the wild-type rate.¹⁵⁴ In the other mutant, this effect was masked by the *leu2* allele of the auxotrophic M5 strain. A partial leucine requirement has not been reported for strains carrying null mutations in *PDHβ1*, *LAT1* or *PDX1*. The possibility therefore cannot be excluded that the partial leucine requirement in the *pda1* null mutant is caused by the absence of an active E1 α subunit, rather than by the absence of a functional pyruvate dehydrogenase complex. However, it seems more likely that this phenotype has been overlooked because the other genes were disrupted in auxotrophic strains. This clearly illustrates the pitfalls of using auxotrophic yeast strains for physiological studies.

An explanation for the partial leucine auxotrophy of *pda1* null mutants was initially sought in production by the E1 subunit of active aldehyde, which is an early intermediate in the synthesis of valine, isoleucine and leucine (Figure 4). The fact that slow growth occurred in the absence of leucine already indicates that the pyruvate dehydrogenase complex is not the only source of active aldehyde in *S. cerevisiae*. Furthermore, the growth rate of the null mutants was not increased by addition of valine or isoleucine.¹⁵⁴ Synthesis of leucine branches off from that of the other branched-chain amino acids at the level of 2-oxoisovalerate (Figure 4). The first enzyme in the branch to leucine, the mitochondrial enzyme 2-isopropyl malate synthase (EC 4.1.3.12)¹²² is inhibited at high CoA/acetyl-CoA ratios.⁵⁰ It has therefore been proposed that the partial leucine requirement of *Pdh*⁻ *S. cerevisiae* may be due to an altered intramitochondrial CoA/acetyl-CoA ratio.¹⁵⁴

A second unexpected phenotype of *pda1* null mutants was an increased frequency of respiratory-deficient mutants during batch cultivation on glucose-containing media.¹⁵⁴ This effect was not observed in glucose-limited chemostat cultures.¹¹⁰ Loss of respiratory capacity was accompanied by loss of mitochondrial DNA (ρ o⁰) and could be prevented by complementation with an intact copy of the *PDA1* gene.¹⁵⁴ The mechanism by which the *pda1* null mutation causes instability of the mitochondrial genome is unknown. *ILV5*, the structural gene encoding acetohydroxy-acid

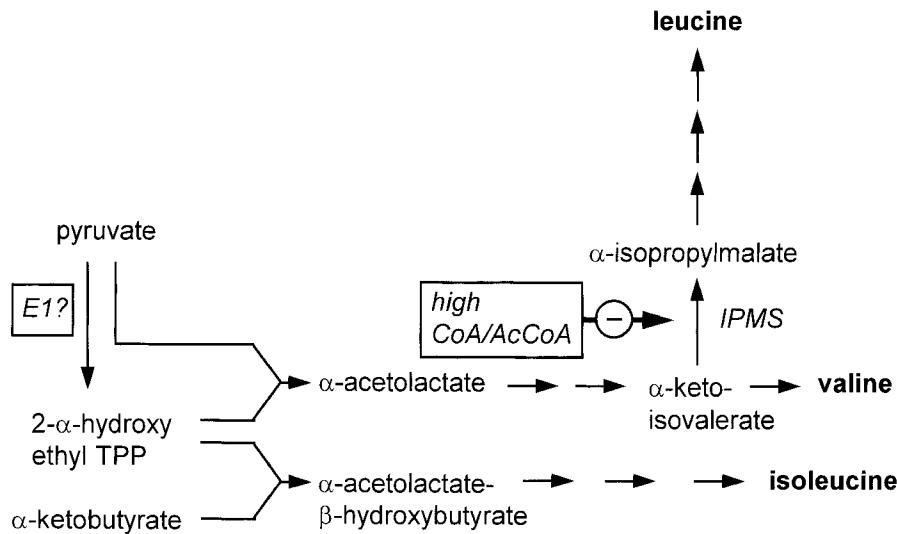


Figure 4. Possible explanations for the partial leucine requirement of *pda1* null mutants of *S. cerevisiae*. If the leucine requirement is caused by involvement of the E1 subunit of the pyruvate dehydrogenase complex in the synthesis of the intermediate 2- α -hydroxy-ethyl-thiamine pyrophosphate (2- α -hydroxy-ethyl-TPP; 'active aldehyde'), this would be expected to also affect synthesis of valine and isoleucine. Since no requirement of *pda1* null mutants for these amino acids was found, it has been proposed that absence of an active pyruvate dehydrogenase complex causes an increased intramitochondrial CoA/acetyl-CoA ratio.¹⁵⁴ This inhibits α -isopropyl malate synthase (IPMS), the first enzyme in the branch to leucine.

reducto-isomerase, a mitochondrial enzyme involved in branched-chain amino-acid biosynthesis,¹⁰⁶ acts as a multi-copy suppressor of mitochondrial DNA instability in cells lacking a functional *ABF2* gene.¹⁶² This observation indicates that a link between branched-chain amino acid synthesis and stability of the mitochondrial genome is not unique to the *PDA1* gene.

The ability of pyruvate dehydrogenase-negative mutants to grow on glucose indicates that, in the absence of pyruvate dehydrogenase activity, the pyruvate decarboxylase by-pass (Figure 3) can meet the cellular demand for acetyl-CoA. An important difference between these two paths from pyruvate to acetyl-CoA is that the by-pass sequence, but not the pyruvate dehydrogenase reaction, involves hydrolysis of ATP (in the acetyl-CoA synthetase reaction). It was therefore anticipated that respiratory growth of a *Pdh*⁻ strain should result in a lower biomass yield than in wild-type *S. cerevisiae*. Indeed, the biomass yield of a *pda1* null mutant in aerobic, glucose-limited chemostat cultures was substantially lower than that of the isogenic wild type (Table 3). The magnitude of the difference in biomass yield indicated that in wild-type *S. cerevisiae* grown

aerobically under glucose limitation, conversion of pyruvate into acetyl-CoA occurs predominantly via the pyruvate dehydrogenase complex.¹¹⁰

Disruption of structural genes encoding pyruvate decarboxylase

An *S. cerevisiae* strain in which all three structural *PDC* genes were disrupted, and which was consequently devoid of pyruvate decarboxylase activity, was first constructed in the auxotrophic strain M5. In complex media, its growth rate on glucose and galactose was about 20% of that of the wild type, whereas only a minor effect on growth rate was found in ethanol-grown cultures.⁵⁵ A reduced growth rate on complex media with glucose has also been reported for point mutants virtually devoid of pyruvate decarboxylase activity^{79,127} and for a prototrophic strain in which the structural *PDC* genes had been replaced by dominant marker genes.⁴⁰

In pyruvate decarboxylase-negative (*Pdc*⁻) yeast, the NADH formed in glycolysis can no longer be regenerated by alcoholic fermentation, so sugar metabolism becomes critically dependent on respiration. Indeed, growth of *Pdc*⁻ mutants

Table 3. Growth yields and protein contents of the wild-type *S. cerevisiae* strain T2-3D and the isogenic pyruvate dehydrogenase-negative mutant T2-3C (*pda1::Tn5ble*).

Growth substrate	<i>S. cerevisiae</i> T2-3D (wild type)		<i>S. cerevisiae</i> T2-3C (<i>Pdh</i> ⁻)	
	Biomass yield (g biomass g ⁻¹)	Protein content (g [g biomass] ⁻¹)	Biomass yield (g biomass g ⁻¹)	Protein content (g [g biomass] ⁻¹)
Ethanol	0.59 ± 0.02	0.41 ± 0.01	0.59 ± 0.01	0.41 ± 0.02
Glucose	0.52 ± 0.01	0.40 ± 0.01	0.44 ± 0.01	0.40 ± 0.02

Cells were grown in aerobic, glucose-limited chemostat cultures ($D=0.10 \text{ h}^{-1}$). Ethanol-limited chemostat cultures were included as a control. All cultures exhibited completely respiratory metabolism without significant excretion of metabolites.¹¹⁰ Note that the decreased biomass yield of the *Pdh*⁻ strain is not due to an altered biomass composition: the protein content of the biomass is essentially the same in all cultures.

on complex medium with glucose was arrested by the respiratory inhibitor antimycin A.⁵⁵ In *S. cerevisiae*, the synthesis of many respiratory enzymes is subject to glucose repression.^{38,44,45,159} This offers a plausible explanation for the reduced growth rate of *Pdc*⁻ mutants in complex, glucose-containing media. Repression appeared to be even stronger in defined mineral media containing glucose as the sole carbon source: the growth rate of a *Pdc*⁻ strain in such a medium was negligible, although growth on ethanol was normal.⁴⁰

In glucose-limited chemostat cultures, the low residual substrate concentrations alleviate glucose repression.^{37,65,132} Therefore, if glucose repression of respiratory enzymes were the sole cause of the inability of a *Pdc*⁻ strain to grow on glucose in defined mineral media, growth in glucose-limited chemostat cultures should be possible. However, when ethanol-limited chemostat cultures of a *Pdc*⁻ strain were shifted to a feed containing glucose as the growth-limiting carbon source, growth came to a stand-still and the culture washed out. When ethanol-limited chemostat cultures were instead shifted to a feed containing a mixture of glucose and ethanol, glucose was completely consumed and steady-state cultures were obtained. These mixed-substrate cultures exhibited the same growth efficiency as wild-type *S. cerevisiae* grown under identical conditions.

It subsequently appeared that growth of a *Pdc*⁻ strain in glucose-limited chemostat cultures was only possible when small amounts of acetate or ethanol (3–5% of the total carbon supplied) were also added to the reservoir media (Flikweert *et al.*, unpublished).⁴⁰ Omission of these C₂-compounds from the medium invariably resulted in complete cessation of growth (Figure 5). Glucose-acetate

mixtures that supported growth under carbon-limited conditions did not do so in batch cultures.⁴⁰ These observations show that two factors contribute to the inability of *Pdc*⁻ *S. cerevisiae* to grow on glucose in batch cultures: glucose repression of the synthesis of respiratory enzymes prevents energy transduction and, secondly, absence of pyruvate decarboxylase causes a requirement for C₂-compounds. A *Pdc*⁻ mutant grew in batch cultures on galactose-acetate mixtures, albeit slowly, whereas growth on galactose alone was

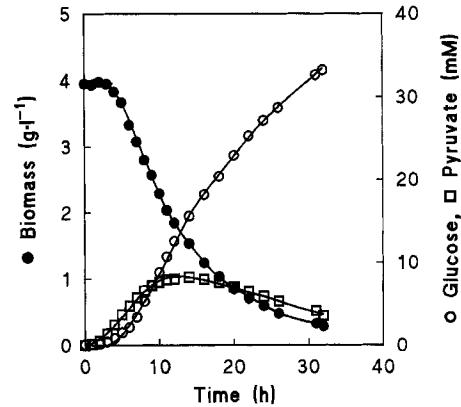


Figure 5. C₂-requirement of a pyruvate decarboxylase-negative mutant of *S. cerevisiae*. Concentrations of biomass, glucose and pyruvate after switching a steady-state chemostat culture ($D=0.10 \text{ h}^{-1}$) of a *Pdc*⁻ *S. cerevisiae* strain to a medium feed with glucose as the sole carbon source. The culture was pre-grown on a mixture of glucose and acetate, with acetate accounting for 5% of the total carbon in the feed. Note that, initially, growth appears normal as the biomass concentration remains approximately constant. Cessation of growth and wash-out occur after ca. 5 h, probably as a result of depletion of endogenous lipid reserves (Flikweert *et al.*, unpublished).

negligible (Pronk *et al.*, unpublished). This is consistent with the proposed role of glucose repression in the phenotype of Pdc^- *S. cerevisiae*, since galactose represses respiration to a lesser extent than glucose.^{44,78}

The C_2 -requirement of Pdc^- *S. cerevisiae* indicates that the mitochondrial pyruvate dehydrogenase complex is unable to provide all acetyl-CoA required for growth. Acetyl-CoA is a precursor for a number of biosynthetic processes including lipid synthesis, which, in *S. cerevisiae*, is a cytosolic process.¹¹¹ It has been proposed that the pyruvate dehydrogenase by-pass may be essential for the provision of acetyl-CoA in the cytosolic compartment.⁴⁰ Four observations are consistent with this hypothesis: 1. The minimum amount of acetate required to sustain carbon-limited growth on glucose-acetate mixtures (ca. 2 mmol g biomass⁻¹) is close to the amount of acetyl-CoA required for lipid synthesis (Pronk *et al.*, unpublished). 2. In the pyruvate dehydrogenase by-pass, conversion of acetate into acetyl-CoA is catalysed by acetyl-CoA synthetase (EC 6.2.1.1). *S. cerevisiae* contains two structural genes encoding acetyl-CoA synthetase isoenzymes, *ACSI* and *ACS2*.^{10,28} The *ACSI* gene is subject to glucose repression, whereas *ACS2* is expressed constitutively.^{11,74} Disruption mutants in which the constitutively expressed *ACS2* gene has been inactivated, and which therefore do not synthesize acetyl-CoA synthetase in the presence of glucose, fail to grow on this sugar.¹⁰ 3. The three enzyme activities of the pyruvate dehydrogenase by-pass have all been reported to occur either exclusively or at least partly in the cytosol.^{63,70,143} 4. ATP-citrate lyase (EC 4.1.3.8), a key enzyme in the export of acetyl-CoA units from the mitochondrial matrix to the cytosol in lipid-accumulating yeasts, is absent in *S. cerevisiae*.¹¹¹

An interesting implication of this hypothesis is that the carnitine shuttle, which facilitates the entry of acetyl-CoA units into the mitochondria,^{72,126} is apparently not reversible in growing *S. cerevisiae* cells. We are currently testing this hypothesis by *in vivo* labelling studies using ¹³C nuclear magnetic resonance.

Disruption of structural genes encoding pyruvate carboxylase

The effect of disruption of the two *PYC* genes was first investigated in the multiply auxotrophic strain W303.¹³⁷ In mutants expressing a single

PYC gene, no clear phenotype was observed. However, Gancedo and coworkers found that when both the *PYC1* and *PYC2* genes were disrupted, the resulting strain was devoid of pyruvate-carboxylase activity and unable to grow in a defined medium containing glucose as the sole carbon source. Growth of this pyruvate-carboxylase-negative (*Pyc*⁻) mutant was possible when aspartate instead of ammonium was added, thus by-passing the anaplerotic function of pyruvate carboxylase.¹³⁷ In a separate study, a similar phenotype was found for a *pyc1 pyc2* double null mutant constructed in the auxotrophic strain DBY746.¹⁸ In this background, a partial aspartate requirement was also observed for a strain in which only *PYC1* had been disrupted. This suggests that the relative contribution of isoenzymes to the overall pyruvate-carboxylase activity may be a strain-dependent property.

An observation difficult to interpret at present is the report by Brewster *et al.*¹⁸ that their *Pyc*⁻ strain requires aspartate for growth on ethanol. The glyoxylate cycle is generally assumed to be the exclusive source of oxaloacetate during growth on C_2 -compounds, since an *S. cerevisiae* strain in which *ICL1*, the structural gene encoding isocitrate lyase, had been inactivated was unable to grow on ethanol.¹²⁹ Even from a theoretical point of view, pyruvate carboxylase cannot meet the requirement for oxaloacetate during growth on ethanol: pyruvate, its substrate, has itself to be synthesized via oxaloacetate during growth on C_2 -compounds. Also the aspartate requirement of *Pyc*⁻ mutants during growth on ethanol appears to be strain-specific, as it has been reported that the *Pyc*⁻ strain constructed by Stucka *et al.*¹³⁷ does not require aspartate during gluconeogenic growth.¹³ Further research is required to investigate a possible role of pyruvate carboxylase during growth of *S. cerevisiae* on ethanol (e.g. by providing 'sparking' amounts of oxaloacetate to allow proper induction of glyoxylate-cycle enzymes during the initial stages of batch growth).

The inability of *Pyc*⁻ strains to grow on glucose^{18,137} confirms that the glyoxylate cycle cannot by-pass pyruvate carboxylase under these conditions. This is in line with the fact that synthesis of the glyoxylate-cycle enzymes is repressed by glucose and that isocitrate lyase is even inactivated in the presence of glucose.⁸⁵ Interestingly, a dominant suppressor mutation has been isolated which allows *Pyc*⁻ *S. cerevisiae* to grow on glucose in batch cultures.¹³ The suppressor mutation was

shown to be allelic to a previously isolated mutation called *DGT1*, which causes a reduced uptake of glucose and thus alleviates glucose catabolite repression.⁴³

Pyc⁻ mutants containing the suppressor mutation exhibited significant levels of isocitrate lyase, indicating that the glyoxylate cycle had taken over the role of pyruvate carboxylase.¹³ This should have substantial implications for glucose metabolism, not only because of changed metabolic fluxes, but also because the glyoxylate cycle occurs in a separate metabolic compartment (the glyoxysome). In theory, it should be possible to alleviate glucose repression in *Pyc⁻* mutants lacking the suppressor mutation by growing them in glucose-limited chemostat cultures. This may be a useful method to study the consequences of this drastic re-routing of carbon metabolism on growth efficiency and metabolic compartmentation.

REGULATION OF METABOLIC FLUXES AT THE PYRUVATE BRANCH-POINT

Competition of key enzymes for pyruvate

The contribution of any reaction in a metabolic network can be controlled at three levels: (i) synthesis of relevant enzymes, (ii) (covalent) modification of these enzymes, and (iii) the intracellular concentration of substrates and effectors. All three mechanisms can, in principle, affect pyruvate metabolism in *S. cerevisiae*. Nevertheless, it is logical that the concentration of the common substrate of the enzymes at the pyruvate branch-point has received special attention.

Holzer⁵⁹ first proposed that the intracellular concentration of pyruvate might be an important parameter in the regulation of its fermentative and respiratory dissimilation. This was based on the observation, later confirmed by others,^{14,75} that the K_m of the pyruvate dehydrogenase complex is an order of magnitude lower than that of pyruvate decarboxylase. As already indicated by Holzer,⁵⁹ the pyruvate dehydrogenase complex and pyruvate decarboxylase occur in different subcellular compartments and therefore cannot directly compete for pyruvate. The K_m of isolated mitochondria for pyruvate is, however, similar to that of the pyruvate dehydrogenase complex.¹⁴³ According to Holzer's model, pyruvate decarboxylase is largely bypassed at low intracellular pyruvate concentrations, thus enabling respiratory dissimilation of pyruvate via the pyruvate dehydrogenase complex.

In contrast, high intracellular concentrations of pyruvate will involve pyruvate decarboxylase in its dissimilation and thus trigger alcoholic fermentation. Indeed, in aerobic, glucose-limited chemostat cultures, the onset of respirofermentative metabolism ('long-term Crabtree effect'; Table 1) coincides with an increase of the extracellular pyruvate concentration (taken as an indicator for the intracellular concentration).¹⁰⁹

In discussions on competition of enzymes for a common substrate it should be considered that affinity is not solely determined by K_m . From the Michaelis-Menten equation ($v = V_{max} s / (K_m + s)$) it follows that, at low substrate concentrations ($s \ll K_m$), the relation between reaction rate and substrate concentration can be approximated by the first-order equation $v = (V_{max}/K_m) s$. Since the amount of an enzyme in the cell also determines its contribution to metabolism at limiting substrate concentrations, affinity is equivalent to V_{max}/K_m rather than $1/K_m$. As will be discussed below, this seems relevant for the competition between pyruvate decarboxylase and the pyruvate dehydrogenase complex.

In *S. cerevisiae*, pyruvate decarboxylase is present at high levels, even during glucose-limited, respiratory growth.^{109,110} It is not possible to estimate the *in vivo* capacity of mitochondrial pyruvate oxidation from studies on isolated organelles. However, an indication can be obtained from experiments in which the respiratory capacity of *S. cerevisiae* is saturated by adding excess glucose to respiring, glucose-limited chemostat cultures. In such experiments, performed with either wild-type or pyruvate decarboxylase-negative strains, only a relatively small increase of the respiration rate is observed directly after a glucose pulse.^{107,144} This indicates that the capacity of mitochondrial pyruvate oxidation is close to the *in vivo* rate of pyruvate oxidation in these cultures. This capacity is at least ten-fold lower than the *in vitro* pyruvate decarboxylase capacity, as calculated from activity measurements with cell extracts (Flikweert *et al.*, unpublished).

Based on the above, the high capacity (V_{max}) of pyruvate decarboxylase would be expected to compensate for its high K_m and to allow the enzyme to compete efficiently with mitochondrial pyruvate oxidation. Nevertheless, the pyruvate dehydrogenase complex is predominantly responsible for pyruvate dissimilation in aerobic, glucose-limited chemostat cultures grown at a low dilution rate.¹¹⁰ The cooperativity of pyruvate decarboxylase with

respect to pyruvate^{14,62} may well be a crucial factor in preventing a major involvement of pyruvate decarboxylase under these conditions; at low pyruvate concentrations, cooperativity causes a lower conversion rate than would be predicted on the basis of Michaelis-Menten kinetics.

As discussed above, even under respiratory growth conditions, pyruvate decarboxylase plays an essential role by providing cytosolic acetyl-CoA. It is as yet unclear how, in respiring, glucose-limited cultures of *S. cerevisiae*, sufficient pyruvate is diverted via pyruvate decarboxylase to meet this assimilatory requirement, while at the same time avoiding the occurrence of alcoholic fermentation.

In the literature, discussion on competition for pyruvate by enzyme systems in *S. cerevisiae* has consistently focussed on pyruvate decarboxylase and the pyruvate dehydrogenase complex. Peculiarly, the third key enzyme at the branch-point, pyruvate carboxylase, has escaped attention. As discussed in one of the preceding sections, the *in vivo* activity of this enzyme appears to be predominantly regulated by the concentrations of substrate and effectors, rather than by changes in enzyme synthesis, for instance as a function of growth rate.

As with all assimilatory reactions, the rate of oxaloacetate formation has to increase linearly with the growth rate. One way to meet this objective is an increase of the cytosolic pyruvate concentration with increasing growth rate. In view of the K_m values of pyruvate carboxylase (ca. 0.8 mM)¹²¹ and pyruvate decarboxylase (ca. 5 mM),¹⁰⁹ this will inevitably lead to an increased flux through pyruvate decarboxylase. Thus, increased *in vivo* fluxes through pyruvate decarboxylase might be a direct consequence of the requirement for an increased rate of oxaloacetate formation via pyruvate carboxylase. This may play a role in the switch to respirofermentative metabolism at high growth rates in sugar-limited, aerobic chemostat cultures of *S. cerevisiae*.

Pyruvate dissimilation via pyruvate decarboxylase is not tantamount to alcoholic fermentation: acetaldehyde can also be dissimilated by respiration (Figure 3). Also, at this level, kinetic properties of key enzymes seem to favour respiratory dissimilation; the K_m of acetaldehyde dehydrogenase for acetaldehyde is two orders of magnitude lower than that of alcohol dehydrogenase.¹⁰⁹

At neither the pyruvate branch-point nor the acetaldehyde branch-point, is affinity for the carbon substrate the sole factor affecting its

metabolic fate. In addition to the concentration of cofactors and effectors, regulation of the synthesis of key enzymes may be involved. For example, it has been proposed that the switch to respiro-fermentative metabolism, observed at high growth rates in glucose-limited cultures, is at least partly due to a limited synthesis of acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase. This reduces the capacity of the pyruvate dehydrogenase by-pass, thereby limiting the ability of the cells to respire pyruvate once it has been diverted via pyruvate decarboxylase.¹⁰⁹

Pyruvate decarboxylase: a glycolytic pacemaker?

In glycolysis, conversion of glucose to pyruvate is coupled to reduction of NAD⁺ and to phosphorylation of ADP. Since only small amounts of these two coenzymes are available in the cytosol, prolonged glycolytic activity requires their efficient regeneration. Consequently, the rate of glycolysis can be controlled by the following three processes: (i) the linear reaction pathway leading from extracellular glucose to intracellular pyruvate (this encompasses synthesis and activity modulation of sugar carriers and enzymes of the glycolytic pathway); (ii) hydrolysis of the ATP generated in glycolysis, either by growth-associated events, futile cycles, or maintenance processes; and (iii) the reoxidation of the NADH formed in glycolysis, either by respiration or by fermentation.

It is now generally accepted that the flux through a metabolic pathway can hardly ever be described in terms of a single enzyme that limits the overall rate (so-called 'bottleneck' or 'pacemaker' reactions). Instead, it has emerged that control of metabolic flux may be distributed over more than one reaction in a pathway (for a review see ref. 68). A practical implication of this is that, in order to optimize flux capacity, it may be necessary to simultaneously increase the levels of more than one enzyme in a pathway.⁹⁷

In *S. cerevisiae*, high glycolytic fluxes are invariably accompanied by alcoholic fermentation.³⁵ This implies that, particularly at high glycolytic fluxes, pyruvate decarboxylase becomes a pivotal enzyme in NADH reoxidation. It has therefore been investigated whether, and to what extent, this enzyme exerts control over the magnitude of the glycolytic flux in growing *S. cerevisiae* cells. Schmitt and Zimmermann¹²⁷ grew a series of *pdc* mutants with different levels of pyruvate decarboxylase in shake-flask cultures on a mineral

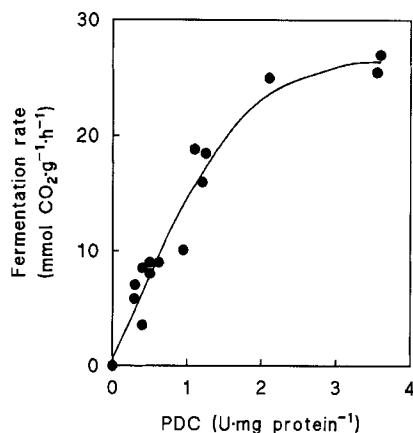


Figure 6. Correlation between pyruvate decarboxylase activity and fermentation rate. Pyruvate decarboxylase activities determined in cell extracts (U mg protein^{-1}) and fermentation rates ($\text{mmol ethanol g}^{-1} \text{ per h}$) in glucose-grown shake-flask cultures of various *S. cerevisiae* strains (wild-type and *pdc* mutants). Data from Schmitt and Zimmermann.¹²⁷

medium with glucose. Over a wide range of pyruvate decarboxylase activities, fermentation rates exhibited a linear correlation with the enzyme activity measured in cell extracts (Figure 6). However, saturation set in below the wild-type pyruvate decarboxylase level, suggesting that (under the experimental conditions) pyruvate decarboxylase was not a major factor in controlling glycolytic flux in growing cells of wild-type *S. cerevisiae*. Indeed, in a now classical study on fermentation and growth rates in a set of *S. cerevisiae* strains overexpressing individual glycolytic enzymes, a four-fold overexpression of pyruvate decarboxylase did not enhance alcoholic fermentation or growth rate.¹²⁴

The experiments discussed above seem to argue against a significant role of fermentative NADH reoxidation in controlling fermentative capacity of *S. cerevisiae*. However, the extent to which a single reaction exerts control over the flux through a pathway depends on external conditions and the overall make-up of the metabolic network. In this respect, it is important to note that the experiments of Schmitt and Zimmermann¹²⁷ and Schaaff *et al.*¹²⁴ were performed in growing shake-flask cultures. These systems are characterized by a very high glycolytic activity of the cells and by a coupling of fermentative dissimilation and biomass formation.

One of the goals of applied research on glycolytic flux in *S. cerevisiae* is improvement of the fermentative capacity of bakers' yeast. In the bakers' yeast process, biomass is grown in fed-batch cultures that are essentially aerobic and carbon-limited.¹² Subsequently, the yeast is transferred to an anaerobic dough environment, where sugar is present in excess. During the initial phase of the dough fermentation, the fermentative capacity will to a large extent depend on the conditions in the fed-batch reactor which, obviously, differ substantially from those in shake-flask cultures. Furthermore, the dough environment is probably far from optimal for balanced glycolytic activity and growth. Therefore, to identify rate-controlling steps during the initial stages of a dough fermentation, glycolytic flux measurements should be performed under conditions that resemble the dough environment, with cells pre-grown under conditions that mimic those extant during the industrial production of bakers' yeast. Published data on the regulation of fermentative capacity of *S. cerevisiae* during aerobic, sugar-limited growth are few. In glucose-limited, aerobic chemostat cultures the fermentative capacity, determined under anaerobic conditions, has been reported to increase sharply above the critical dilution rate at which alcoholic fermentation sets in. This increase of fermentation capacity was paralleled by an increase of the pyruvate decarboxylase activity.³² Although not substantiated by experiments in which pyruvate decarboxylase was overexpressed, these results indicated that pyruvate decarboxylase should not yet be eliminated as a potential target for attempts at increasing the fermentative capacity of bakers' yeast in dough.

*Does phosphorylation of the pyruvate dehydrogenase complex occur in *S. cerevisiae*?*

In mammalian cells, activity of the pyruvate dehydrogenase complex is modulated by a reversible phosphorylation of the E1 α subunit. Phosphorylation and dephosphorylation are catalysed by a pyruvate dehydrogenase-specific kinase and phosphatase, respectively, which act on three serine residues in the E1 α peptide. Phosphorylation of all three serines leads to complete inactivation, whereas phosphorylation of one or two serines merely causes a decrease of enzyme activity. The activity of the mammalian pyruvate dehydrogenase kinase and phosphatase is regulated by a

number of physiological parameters, including the intramitochondrial NAD⁺/NADH, CoA/acetyl-CoA and ADP/ATP ratios (for reviews see refs 113,161). This reversible modulation of the activity of the pyruvate dehydrogenase complex provides a mechanism to adapt the flux through this key enzyme to the carbon, redox and energy status of mammalian cells.

If the pyruvate dehydrogenase complex in *S. cerevisiae* were subject to a similar rapid activation-inactivation mechanism, this could (in principle) be highly relevant during transient growth conditions. For example, if the pyruvate dehydrogenase complex were inactivated after exposure of respiring cells to excess glucose, this might contribute to the redirection of pyruvate metabolism via pyruvate decarboxylase that is seen during the short-term Crabtree effect (Table 1).

During the original studies of Kresze and Ronft^{75,76} on the isolation and characterization of the pyruvate dehydrogenase complex from *S. cerevisiae*, no indications were obtained for the existence of a specific kinase in this yeast. However, it has been demonstrated that the *S. cerevisiae* E1 α subunit can be phosphorylated and inactivated by pyruvate dehydrogenase kinases from bovine kidney and rat liver.^{91,142} Although these studies did not show the presence of a pyruvate dehydrogenase kinase in *S. cerevisiae*, it was demonstrated that the sequence around the phosphorylated serine residue (at position 313 from the first methionine)¹³⁶ was highly homologous to that of bovine and porcine E1 α phosphorylation sites.¹⁴² Replacement of ser313 by a his residue abolished pyruvate dehydrogenase activity (Wenzel and Steensma, unpublished).

Upon incubation of isolated *S. cerevisiae* mitochondria with radiolabelled ATP or pyrophosphate, phosphorylation of a 40 kDa mitochondrial peptide was observed, which was tentatively identified as the pyruvate dehydrogenase E1 α subunit.¹⁰⁵ This result should be interpreted carefully, since the estimated size of the labelled peptide is smaller than that predicted from the nucleotide sequence of the *PDA1* gene.^{7,136} More convincing evidence that a pyruvate dehydrogenase kinase is present in *S. cerevisiae* was obtained by James *et al.*⁶⁴ After incubation of isolated mitochondria with radioactive phosphate, a labelled 45 kDa peptide could be immunoprecipitated with a specific antiserum against the *S. cerevisiae* E1 α subunit. Moreover, it was

demonstrated that pyruvate dehydrogenase activity of isolated mitochondria could be reversibly inhibited by ATP. A peculiar observation in this study was that phosphorylation could only be reproducibly observed in exponentially growing cells when galactose was the carbon source.⁶⁴ This seems difficult to reconcile with the fact that respiration rates during growth on galactose are higher than during growth on glucose.⁷⁸

It is clear that the mechanisms involved in short-term regulation of pyruvate dehydrogenase activity require further study. In this respect, it is interesting to note that the systematic sequencing of the *S. cerevisiae* genome has revealed an open reading frame that exhibits 28% homology with rat pyruvate dehydrogenase kinase (YIL042C; Barrell and Rajandream, GenBank Z46861). It will be interesting to study whether the *in vitro* phosphorylation phenomena discussed above are abolished when this open reading frame is disrupted.

In wild-type *S. cerevisiae*, inactivation of the pyruvate dehydrogenase complex does not necessarily preclude respiratory dissimilation of pyruvate, as this may still occur via the pyruvate dehydrogenase by-pass (Figure 3). In a pyruvate decarboxylase-negative strain, only the pyruvate dehydrogenase complex can act as an interface between glycolysis and TCA cycle. This makes Pdc⁻ *S. cerevisiae* strains excellently suited to study the *in vivo* regulation of pyruvate dehydrogenase activity. We have therefore used a Pdc⁻ strain to investigate if phosphorylation of E1 α is a relevant mechanism during the exposure of respiring *S. cerevisiae* cultures to excess glucose (Flikweert *et al.*, unpublished). In a Pdc⁻ strain, inactivation of the pyruvate dehydrogenase complex should largely abolish respiration of glucose. However, such a decrease of the respiration rate was not observed after a glucose pulse to carbon-limited, respiring chemostat cultures (Figure 7).

An additional advantage of Pdc⁻ *S. cerevisiae* is that pyruvate decarboxylase does not interfere with assays of pyruvate dehydrogenase activity in cell extracts. As a result, activity of the complex could easily be measured as pyruvate- and CoA-dependent reduction of NAD⁺ by cell extracts (Flikweert *et al.*, unpublished). After a glucose pulse, no strong decrease of pyruvate dehydrogenase activity was observed (Figure 7), thus confirming that, at least under these conditions, phosphorylation of E1 α is not a relevant regulatory mechanism.

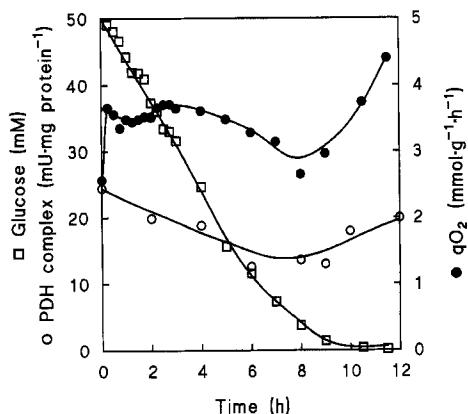


Figure 7. The pyruvate dehydrogenase complex is not inactivated after a glucose pulse to a respiring chemostat culture. At time zero, 50 mM-glucose was added to a carbon-limited chemostat culture ($D=0.1 \text{ h}^{-1}$) of a pyruvate decarboxylase-negative *S. cerevisiae* strain, growing on a mixture of glucose and acetate (see Figure 5). Oxygen uptake by the culture was determined on-line, pyruvate-dehydrogenase activities were determined in crude cell extracts (Flikweert *et al.*, unpublished).

CONCLUSIONS

Methodology

Introduction of defined mutations in structural genes is an indispensable tool for studies on function and regulation of branched metabolic pathways. However, functional or regulatory effects of such mutations on amino acid or nucleotide synthesis may easily be overlooked in auxotrophic strains (as illustrated by the leucine requirement of *pda1* null mutants). The yeast strains used in this type of study should therefore lack auxotrophic lesions. The multitude of nutrients and intermediates present in complex media may obscure effects of mutations on intermediary carbon metabolism (for example, the aspartate requirement of *Pyc*⁻ mutants might easily have been overlooked in media containing peptone). Therefore, defined mineral media should be used for growth studies. Shake-flask cultures, however useful for the initial phenotypic characterization of yeast strains, have major drawbacks for physiological research, especially with *S. cerevisiae*. Chemostat cultivation, although more laborious, allows independent manipulation of key culture parameters and enables studies on sugar metabolism without the major interference of glucose repression (as illustrated by the analysis of the phenotype of *Pdc*⁻ mutants).

Physiological role of key reactions at the pyruvate branch-point

The pyruvate dehydrogenase complex is predominantly responsible for the conversion of pyruvate to acetyl-CoA during glucose-limited, respiratory growth. This enzyme complex is to some extent redundant: in *Pdh*⁻ mutants, this role can be taken over by the pyruvate dehydrogenase bypass. However, the latter route involves ATP hydrolysis and therefore leads to lower biomass yields in respiring, glucose-limited cultures. Additional phenotypic characteristics of *pda1* null mutants apparent at high growth rates in batch cultures (a requirement for leucine and an increased frequency of respiratory-deficient mutants) indicate that the role of the pyruvate dehydrogenase complex is not limited to respiratory pyruvate dissimilation.

The bypass route is indispensable for growth of *S. cerevisiae* on glucose. Two factors contribute to the inability of *Pdc*⁻ mutants to grow on glucose in batch cultures: glucose repression of respiration and a requirement for C₂-compounds. The latter requirement is probably caused by an inability of the mitochondrial pyruvate dehydrogenase complex to provide the cytosolic acetyl-CoA needed for lipid synthesis.

The inability of *Pyc*⁻ mutants to grow on glucose in batch cultures indicates that, in wild-type *S. cerevisiae*, the glyoxylate cycle cannot bypass the anaplerotic pyruvate carboxylase reaction. Isolation of a suppressor mutant of a *Pyc*⁻ strain, in which glucose repression of glyoxylate-cycle enzymes is alleviated, shows that this is due to regulatory rather than physiological constraints.

Regulation of fluxes at the pyruvate branch-point

Although their levels are not constant, the key enzymes at the pyruvate branch-point are expressed under all growth conditions investigated. Different isoenzymes contribute to the overall activity of pyruvate decarboxylase (*PDC1*, *PDC5* and, possibly, *PDC6*) and pyruvate carboxylase (*PYCI* and *PYC2*). Although the regulation of the structural genes encoding these isoenzymes may differ, there is no conclusive evidence that they have distinct physiological functions.

High intracellular concentrations of pyruvate favour its dissimilation via pyruvate decarboxylase, which has a high K_m for pyruvate and a high capacity. Since the capacity of the further reactions

of the pyruvate dehydrogenase by-pass is limited, diversion of pyruvate metabolism via pyruvate decarboxylase will ultimately lead to alcoholic fermentation in aerobic cultures. The assimilatory enzyme pyruvate carboxylase may play a key role in this diversion of pyruvate dissimilation if its *in vivo* activity is controlled by the intracellular pyruvate concentration.

In vitro experiments have indicated that the *S. cerevisiae* pyruvate dehydrogenase complex can be inactivated by phosphorylation. The physiological function of this short-term regulatory mechanism is, as yet, unclear. Inactivation of the complex does not occur after exposure of respiring cells to excess glucose.

OUTLOOK

Research on pyruvate metabolism has come a long way since Neuberg's work in the early years of this century. Integration of molecular genetics and quantitative physiology continues to generate new and exciting results, which pay off the time investments required for construction of suitable yeast strains and implementation of techniques for controlled cultivation. Recent studies illustrate the intricacy of the *S. cerevisiae* metabolic network: absence of pyruvate-metabolizing enzymes affects processes as diverse as amino-acid biosynthesis, stability of the mitochondrial genome and lipid synthesis.

In future research, genetic modification of pyruvate metabolism should be extended from straightforward elimination or overexpression of key enzymes to more subtle changes of their catalytic and regulatory properties. Quantitative analysis of the physiology of *S. cerevisiae* strains in which native genes are replaced by heterologous analogs (e.g. replacement of pyruvate decarboxylase by a mammalian lactate dehydrogenase)¹⁰⁸ will yield further interesting results. Techniques for accurate determination of metabolite concentrations in growing yeast cultures^{73,140} have to be implemented to translate *in vitro* enzyme characteristics to the regulation of *in vivo* fluxes. Wherever possible, such measurements should take into account subcellular compartmentation of pyruvate metabolism. Finally, a deeper understanding of the regulation of fluxes at the pyruvate branch-point will have to encompass the signalling pathways involved in modulation of enzyme synthesis and activity in *S. cerevisiae* (for a review, see ref. 141).

Achievement of the ultimate (and ambitious) aim of research in this area, a complete and quantitative description of the regulation of metabolic fluxes at the pyruvate branch-point in *S. cerevisiae*, will enable a better control of product and by-product formation in the industrial applications of this yeast. Furthermore, unravelling of the function and regulation of this comparatively well-known area in the metabolic network of *S. cerevisiae* may be helpful in the development of research strategies for the functional analysis of other genes and gene products whose functions are, at present, less understood.

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REFERENCES

1. Alani, E., Cao, L. and Kleckner, N. (1987). A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics* **116**, 541–545.
2. Bakker, E. P. and van Dam, K. (1974). The movement of monocarboxylic acids across phospholipid membranes: evidence for an exchange diffusion between pyruvate and other monocarboxylate ions. *Biochim. Biophys. Acta* **339**, 285–289.
3. Barden, R. E., Taylor, B. L., Isohashi, F., et al. (1975). Structural properties of pyruvate carboxylases from chicken liver and other sources. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4308–4312.
4. Barnett, J. A. (1981). The utilization of disaccharides and some other sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **39**, 347–404.
5. Barnett, J. A., Payne, R. W. and Yarrow, D. (1990). *Yeasts: Characteristics and Identification*, 2nd edn. Cambridge University Press, Cambridge, U.K.

6. Barrit, G. J. (1985). Regulation of enzymatic activity. In Keech, D. B. and Wallace, J. C. (Eds), *Pyruvate Carboxylase*. CRC Press, Boca Raton, pp. 141–177.
7. Behal, R. H., Browning, K. S. and Reed, L. J. (1989). Nucleotide and deduced amino acid sequence of the alpha subunit of yeast pyruvate dehydrogenase. *Biochem. Biophys. Res. Commun.* **164**, 941–946.
8. Behal, R. H., Browning, K. S., Hall, T. B. and Reed, L. J. (1989). Cloning and nucleotide sequence of the gene for protein X from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8732–8736.
9. Benz, R. (1985). Porin from bacterial and mitochondrial outer membranes. *CRC Crit. Rev. Biochem.* **19**, 145–190.
10. van den Berg, M. A. and Steensma, H. Y. (1995). ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. *Eur. J. Biochem.* **231**, 704–713.
11. van den Berg, M. A., de Jong-Gubbels, P., Kortland, C. J., van Dijken, J. P., Pronk, J. T. and Steensma, H. Y. (1996). The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J. Biol. Chem.*, in press.
12. Beudeker, R. F., van Dam, H. W., van der Plaat, J. B. and Vellenga, K. (1990). Developments in baker's yeast production. In Verachtert, H. and De Mot, R. (Eds), *Yeast Biotechnology and Biocatalysis*. Marcel Dekker, New York, pp. 103–146.
13. Blásquez, M. A., Gamo, F. J. and Gancedo, C. (1995). A mutation affecting carbon catabolite repression suppresses growth defects in pyruvate carboxylase mutants from *Saccharomyces cerevisiae*. *FEBS Lett.* **377**, 197–200.
14. Boiteux, A. and Hess, B. (1970). Allosteric properties of yeast pyruvate decarboxylase. *FEBS Lett.* **9**, 293–296.
15. Boles, E. and Zimmermann, F. K. (1993). Induction of pyruvate decarboxylase in glycolysis mutants of *Saccharomyces cerevisiae* correlates with the concentrations of 3-carbon glycolytic intermediates. *Arch. Microbiol.* **160**, 324–328.
16. Boles, E., Heinisch, J. and Zimmermann, F. K. (1993). Different signals control the activation of glycolysis in the yeast *Saccharomyces cerevisiae*. *Yeast* **9**, 761–770.
17. Bowman, S. B., Zaman, Z., Collinson, L. P., Brown, A. J. P. and Dawes, I. W. (1992). Positive regulation of the *LPDI* gene of *Saccharomyces cerevisiae* by the HAP2/HAP3/HAP4 activation system. *Mol. Gen. Genet.* **231**, 296–303.
18. Brewster, N. K., Val, D. L., Walker, M. E. and Wallace, J. C. (1994). Regulation of pyruvate carboxylase isoenzyme (*PYCI*, *PYC2*) gene expression in *Saccharomyces cerevisiae* during fermentative and non-fermentative growth. *Arch. Biochem. Biophys.* **311**, 62–71.
19. Briquet, M. (1977). Transport of pyruvate and lactate in yeast mitochondria. *Biochim. Biophys. Acta* **459**, 290–299.
20. Bruinenberg, P. M., van Dijken, J. P. and Scheffers, W. A. (1983). An enzymic analysis of NADPH production and consumption in *Candida utilis*. *J. Gen. Microbiol.* **129**, 965–971.
21. Bruinenberg, P. M., van Dijken, J. P. and Scheffers, W. A. (1983). A theoretical analysis of NADPH production and consumption in yeasts. *J. Gen. Microbiol.* **129**, 953–964.
22. Bruinenberg, P. M., de Bot, P. H. M., van Dijken, J. P. and Scheffers, W. A. (1983). The role of the redox balance in the fermentation of xylose by yeasts. *Eur. J. Appl. Microbiol. Biotechnol.* **18**, 287–292.
23. Bruinenberg, P. M., de Bot, P. H. M., van Dijken, J. P. and Scheffers, W. A. (1984). NADH-linked aldose reductase: the key to alcoholic fermentation of xylose by yeasts. *Appl. Microbiol. Biotechnol.* **19**, 256–269.
24. Bruinenberg, P. M., Waslander, G. W., van Dijken, J. P. and Scheffers, W. A. (1986). A comparative radiorespirometric study of glucose metabolism in yeasts. *Yeast* **2**, 117–121.
25. Cazzulo, J. J. and Stoppani, A. O. M. (1969). Effects of adenosine phosphates and nicotinamide nucleotides on pyruvate carboxylase from baker's yeast. *Biochem. J.* **112**, 755–762.
26. Cohen, N. D., Utter, M. F., Wrigley, N. G. and Barret, A. N. (1979). Quaternary structure of yeast pyruvate carboxylase: biochemical and electron microscope studies. *Biochem.* **18**, 2197–2203.
27. Cooper, R. A. (1984). Metabolism of methylglyoxal in microorganisms. *Ann. Rev. Microbiol.* **38**, 49–68.
28. De Virgilio, C., Burckert, N., Barth, G., Neuhaus, J. M., Boller, T. and Wiemken, A. (1992). Cloning and disruption of a gene required for growth on acetate but not on ethanol: the acetyl-coenzyme A synthetase gene of *Saccharomyces cerevisiae*. *Yeast* **8**, 1043–1051.
29. Dickinson, J. R., Roy, D. J. and Dawes, I. W. (1986). A mutation affecting lipoamide dehydrogenase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **204**, 103–107.
30. Dickinson, J. R. and Dawes, I. W. (1992). The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**, 2029–2033.
31. Dihanic, M., Suda, K. and Schatz, G. (1987). A yeast mutant lacking mitochondrial porin is respiratory-deficient, but can recover respiration

- with simultaneous accumulation of an 86 kd extramitochondrial protein. *EMBO J.* **6**, 723–728.
32. van Dijken, J. P., Jonker, R., Houweling-Tan, G. B., Bruinenberg, P. M., Meijer, J. and Scheffers, W. A. (1983). The Crabtree effect in *Saccharomyces cerevisiae* and its significance for the rising power of baker's yeast. In Houwink, E. H., van der Meer, R. R. and Scheffers, W. A. (Eds), *Proc. Symp. Netherlands Soc. Biotechnol. 1983*, p. 171.
 33. van Dijken, J. P. and Scheffers, W. A. (1986). Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol. Rev.* **32**, 199–224.
 34. van Dijken, J. P., van den Bosch, J. J., Hermans, L., de Miranda, R. and Scheffers, W. A. (1986). Alcoholic fermentation by 'non-fermenting' yeasts. *Yeast* **2**, 123–127.
 35. van Dijken, J. P., Weusthuis, R. A. and Pronk, J. T. (1993). Kinetics of growth and sugar consumption in yeasts. *Antonie van Leeuwenhoek* **63**, 343–352.
 36. Divjak, S. and Mor, J. R. (1973). On the activity of carbon dioxide fixation in growing yeasts. *Arch. Mikrobiol.* **94**, 191–199.
 37. Egli, T., Bosshard, C. and Hamer, G. (1993). Kinetics of microbial growth with mixtures of carbon sources. *Antonie van Leeuwenhoek* **63**, 289–298.
 38. Entian, K. D. (1986). Glucose repression: a complex regulatory system. *Microbiol. Sci.* **3**, 366–371.
 39. Erlenmeyer, E. (1881). Verhalten der Glycerinsäure und der Weinsäure gegen wasserentziehende Substanzen. *Chem. Ber.* **14**, 320–323.
 40. Flikweert, M. T., van der Zanden, L., Janssen, W. M. T. M., Steensma, H. Y., van Dijken, J. P. and Pronk, J. T. (1996). Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. *Yeast* **12**, 247–257.
 41. Franzblau, S. G. and Sinclair, N. A. (1983). Induction of pyruvate decarboxylase in *Candida utilis*. *Mycopathol.* **83**, 29–33.
 42. Gailiusis, J., Rinne, R. W. and Benedict, C. R. (1964). Pyruvate-oxaloacetate exchange reaction in baker's yeast. *Biochim. Biophys. Acta* **92**, 595–601.
 43. Gamo, F. J., Lafuente, M. J. and Gancedo, C. (1994). The mutation *DGT1-1* decreases glucose transport and alleviates carbon catabolite repression in *Saccharomyces cerevisiae*. *J. Bacteriol.* **176**, 7423–7429.
 44. Gancedo, C. and Serrano, R. (1989). Energy-yielding metabolism. In Rose, A. H. and Harrison, J. S. (Eds), *The Yeasts*, 2nd edn, vol. 3. Academic Press, New York, pp. 205–259.
 45. Gancedo, J. M. (1992). Carbon catabolite repression in yeast. *Eur. J. Biochem.* **206**, 297–313.
 46. Gounaris, A. D., Turkenkopf, I., Civerchia, L. L. and Greenlie, J. (1975). Pyruvate decarboxylase. III. Specificity restrictions for thiamine pyro-phosphate in the protein association step, subunit structure. *Biochim. Biophys. Acta* **405**, 492–499.
 47. Green, D. E., Herbert, D. E. and Subramanyan, V. (1941). Carboxylase. *J. Biol. Chem.* **138**, 327–339.
 48. Haarasilta, S. and Oura, E. (1975). Effect of aeration on the activity of gluconeogenic enzymes in *Saccharomyces cerevisiae* growing under glucose limitation. *Arch. Microbiol.* **106**, 271–273.
 49. Haarasilta, S. and Taskinen, L. (1977). Location of three key enzymes of gluconeogenesis in baker's yeast. *Arch. Microbiol.* **113**, 159–161.
 50. Hampsey, D. M. and Kohlhaw, G. B. (1981). Inactivation of yeast α -isopropyl malate synthase by CoA. *J. Biol. Chem.* **256**, 3791–3796.
 51. Hensing, M., Vrouwenfelder, H., Hellinga, C., Baartmans, R. and van Dijken, J. P. (1994). Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluveromyces marxianus*. *Appl. Microbiol. Biotechnol.* **42**, 516–521.
 52. Hensing, M., Rouwenhorst, R., Heijnen, J. J., van Dijken, J. P. and Pronk, J. T. (1995). Physiological and technological aspects of large-scale heterologous-protein production with yeasts. *Antonie van Leeuwenhoek* **67**, 261–279.
 53. Hinnebusch, A. G. (1983). Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 248–273.
 54. Hohmann, S. and Cederberg, H. (1990). Auto-regulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. *Eur. J. Biochem.* **188**, 615–621.
 55. Hohmann, S. (1991). Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *J. Bacteriol.* **173**, 7963–7969.
 56. Hohmann, S. (1991). *PDC6*, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promoter. *Curr. Genet.* **20**, 373–378.
 57. Hohmann, S. (1993). Characterization of *PDC2*, a gene necessary for high level expression of pyruvate decarboxylase structural genes in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **241**, 657–666.
 58. den Hollander, J. A., Ugurbil, K., Brown, T. R. and Shulman, R. G. (1981). Phosphorous-31 NMR nuclear magnetic resonance studies of the effect of oxygen upon glycolysis in yeast. *Biochemistry* **20**, 5871–5880.
 59. Holzer, H. (1961). Regulation of carbohydrate metabolism by enzyme competition. *Cold Spring Harbor Symp. Quant. Biol.* **26**, 277–288.
 60. Holzer, H. and Goedde, W. H. (1957). Zwei Wege von Pyruvat zu Acetyl-Coenzym A in Hefe. *Biochem. Z.* **329**, 175–191.
 61. Holzer, H. and Kohlhaw, G. B. (1961). Enzymatic formation of α -acetolactate from α -hydroxyethyl

- 2-thiamine pyrophosphate ('active aldehyde') and pyruvate. *Biochem. Biophys. Res. Comm.* **5**, 452–458.
62. Hubner, G., Weidhase, R. and Schellenberger, A. (1978). The mechanism of substrate activation of pyruvate decarboxylase, a first approach. *Eur. J. Biochem.* **92**, 175–181.
 63. Jacobson, M. K. and Bernofsky, C. (1974). Mitochondrial acetaldehyde dehydrogenase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **350**, 277–291.
 64. James, A. G., Cook, R. M., West, S. M. and Lindsay, J. G. (1995). The pyruvate dehydrogenase complex of *Saccharomyces cerevisiae* is regulated by phosphorylation. *FEBS Lett.* **373**, 111–114.
 65. de Jong-Gubbels, P., Vanrolleghem, P., Heijnen, J. J., van Dijken, J. P. and Pronk, J. T. (1995). Regulation of carbon metabolism in chemostat cultures of *Saccharomyces cerevisiae* grown on mixtures of glucose and ethanol. *Yeast* **11**, 407–418.
 66. Kaliterna, J., Weusthuis, R. A., Castrillo, J. I., van Dijken, J. P. and Pronk, J. T. (1995). Transient responses of *Candida utilis* to oxygen limitation: regulation of the Kluyver effect for maltose. *Yeast* **11**, 317–325.
 67. Keha, E. E., Ronft, H. and Kresze, G. B. (1982). On the origin of mitochondria: a reexamination of the molecular structure and kinetic properties of pyruvate dehydrogenase complex from brewer's yeast. *FEBS Lett.* **145**, 289–292.
 68. Kell, D. B. and Westerhoff, H. V. (1986). Metabolic control theory: its role in microbiology and biotechnology. *FEMS Microbiol. Rev.* **39**, 305–320.
 69. Kellermann, E., Seeboth, P. G. and Hollenberg, C. P. (1986). Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (*PDC1*) from *Saccharomyces cerevisiae*. *Nucl. Acids Res.* **14**, 8963–8977.
 70. Kispal, G., Cseko, J., Alkonyi, I. and Sandor, A. (1991). Isolation and characterization of carnitine acetyl-transferase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1085**, 217–222.
 71. Kluyver, A. J. (1914). Biochemische suikerbepalingen. PhD Thesis, Delft University of Technology, Delft, The Netherlands.
 72. Kohlhaw, G. B. and Tan-Wilson, A. (1977). Carnitine-acetyltransferase: candidate for the transfer of acetyl groups through the mitochondrial membrane of yeast. *J. Bacteriol.* **129**, 1159–1161.
 73. de Koning, W. and van Dam, K. (1992). A method for the determination of glycolytic intermediates in yeast on a subsecond time scale using extraction at neutral pH. *Anal. Biochem.* **204**, 118–123.
 74. Kratzer, S. and Schuller, H. J. (1995). Carbon source-dependent regulation of the acetyl-coenzyme A synthetase-encoding gene *ACSI* from *Saccharomyces cerevisiae*. *Gene* **161**, 75–79.
 75. Kresze, G. B. and Ronft, H. (1981). Pyruvate dehydrogenase complex from baker's yeast. 1. Properties and some kinetic and regulatory properties. *Eur. J. Biochem.* **119**, 573–579.
 76. Kresze, G. B. and Ronft, H. (1981). Pyruvate dehydrogenase complex from baker's yeast. 2. Molecular structure, dissociation, and implications for the origin of mitochondria. *Eur. J. Biochem.* **119**, 581–587.
 77. Kuo, D. J., Dikdan, G. and Jordan, F. (1986). Resolutions of brewers' yeast decarboxylase into two isoenzymes. *J. Biol. Chem.* **261**, 3316–3319.
 78. Lagunas, R. (1986). Misconceptions about the energy metabolism of *Saccharomyces cerevisiae*. *Yeast* **2**, 221–228.
 79. Lancashire, M., Payton, A., Webber, M. J. and Hartley, B. S. (1981). Petite-negative mutants of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **181**, 409–410.
 80. Lawson, J. E., Behal, R. H. and Reed, L. J. (1991). Disruption and mutagenesis of the *Saccharomyces cerevisiae PDX1* gene encoding the protein X component of the pyruvate dehydrogenase complex. *Biochem.* **30**, 2834–2839.
 81. Lawson, J. E., Niu, X. D. and Reed, L. J. (1991). Functional analysis of the domains of dihydro-lipoamide acetyltransferase from *Saccharomyces cerevisiae*. *Biochemistry* **30**, 11249–11254.
 82. Lessie, T. G. and Phibbs, P. V. (1984). Alternative pathways of carbohydrate utilization in *Pseudomonas*. *Ann. Rev. Microbiol.* **38**, 358–387.
 83. Libor, S. M., Sundaram, T. K. and Scrutton, M. C. (1978). Pyruvate carboxylase from a thermophilic *Bacillus*. *Biochem. J.* **169**, 543–558.
 84. Lohmann, K. and Schuster, P. H. (1937). Untersuchungen über die Cocarboxylase. *Biochem. Z.* **294**, 188–214.
 85. López-Boado, Y. S., Herrero, P. S., Gascón, S. and Moreno, F. (1987). Catabolite inactivation of isocitrate lyase from *Saccharomyces cerevisiae*. *Arch. Microbiol.* **147**, 231–234.
 86. Losada, M., Canovas, J. L. and Ruiz-Amil, M. (1964). Oxaloacetate, citramalate and glutamate formation from pyruvate in baker's yeast. *Biochem. Z.* **340**, 60–74.
 87. Maitra, P. K. and Lobo, Z. (1971). A kinetic study of glycolytic enzyme synthesis in yeast. *J. Biol. Chem.* **246**, 475–488.
 88. von Meyenburg, K. (1969). Katabolit-repression und der Sprossungszyklus von *Saccharomyces cerevisiae*. PhD Thesis, ETH Zurich, Zurich, Switzerland.
 89. Miller, A. L. and Atkinson, D. E. (1972). Response of yeast pyruvate carboxylase to the adenylate energy charge and other regulatory parameters. *Arch. Biochem. Biophys.* **152**, 531–538.

90. Miran, S. G., Lawson, J. E. and Reed, L. J. (1993). Characterization of *PDHβ1*, the structural gene for the pyruvate dehydrogenase β subunit from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1252–1256.
91. Mistry, S. C., Priestman, D. A., Kerbey, A. L. and Randle, P. J. (1991). Evidence that rat liver pyruvate dehydrogenase kinase activator protein is a pyruvate dehydrogenase kinase. *Biochem. J.* **275**, 775–779.
92. Morris, C. P., Lim, F. and Wallace, J. C. (1987). Yeast pyruvate carboxylase: gene isolation. *Biochem. Biophys. Res. Comm.* **145**, 390–396.
93. Myers, D. E., Tolbert, B. and Utter, M. F. (1983). Activation of yeast pyruvate carboxylase: interactions between acyl-Coenzyme A compounds, aspartate, and substrates of the reaction. *Biochem. J.* **22**, 5090–5096.
94. Nalecz, M. J., Nalecz, K. A. and Azzi, A. (1991). Purification and functional characterization of the pyruvate (monocarboxylate) carrier from baker's yeast mitochondria (*Saccharomyces cerevisiae*). *Biochim. Biophys. Acta* **1079**, 87–95.
95. Neuberg, C. and Karczag, L. (1911). Über zuckerfreie Hefegarungen. IV. Carboxylase, ein neues Enzym der Hefe. *Biochem. Z.* **36**, 68–75.
96. Neuberg, C. and Karczag, L. (1911). Über zuckerfreie Hefegarungen. IV. Zur Kenntnis der Carboxylase. *Biochem. Z.* **36**, 76–81.
97. Niederberger, P., Prasad, R., Miozzari, G. and Kacser, H. (1992). A strategy for increasing an *in vivo* flux by genetic manipulations. The tryptophan system of yeast. *Biochem. J.* **287**, 473–479.
98. Niu, X., Browning, K. S., Behal, R. H. and Reed, L. J. (1988). Cloning and nucleotide sequence of the gene for dihydrolipoamide acetyltransferase from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7546–7550.
99. Oliver, S. G. (1996). From DNA sequence to biological function. *Nature* **379**, 597–600.
100. Orr-Weaver, T. L., Szostak, J. and Rothstein, R. J. (1981). Yeast transformation: a model system for the study of recombination. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6354–6358.
101. Oura, E. (1972). The effect of aeration on the growth energetics and biochemical composition of baker's yeast. PhD Thesis, University of Helsinki, Finland.
102. Oura, E. (1977). Reaction products of yeast fermentations. *Process Biochem.* **12**, 19–21.
103. Oura, E., Haarasilta, S. and Londesborough, J. (1980). Carbon dioxide fixation by baker's yeast in a variety of growth conditions. *J. Gen. Microbiol.* **118**, 51–58.
104. Palacián, E., de Torrontegui, G. and Losada, M. (1966). Inhibition of yeast pyruvate carboxylase by L-aspartate and oxaloacetate. *Biochem. Biophys. Res. Comm.* **24**, 644–649.
105. Pereira da Silva, L., Lindahl, M., Lundin, M. and Baltscheffsky, H. (1991). Protein phosphorylation by inorganic pyrophosphate in yeast mitochondria. *Biochem. Biophys. Res. Comm.* **178**, 1359–1364.
106. Petersen, J. G. L. and Holmberg, S. (1986). The *ILV5* gene of *Saccharomyces cerevisiae* is highly expressed. *Nucl. Acids Res.* **14**, 9631–9651.
107. Petrik, M., Kappeli, O. and Fiechter, A. (1983). An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short-term and long-term regulation. *J. Gen. Microbiol.* **129**, 43–49.
108. Porro, D., Brambilla, L., Ranzi, B. M., Martegani, E. and Alberghina, L. (1995). Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. *Biotechnol. Prog.* **11**, 294–298.
109. Postma, E., Verduyn, C., Scheffers, W. A. and van Dijken, J. P. (1989). Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **53**, 468–477.
110. Pronk, J. T., Wenzel, T. J., Luttik, M. A. H., Klaassen, C. C. M., Scheffers, W. A., Steensma, H. Y. and van Dijken, J. P. (1994). Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*. *Microbiology* **140**, 601–610.
111. Ratledge, C. and Evans, C. T. (1989). Lipids and their metabolism. In Rose, A. H. and Harrison, J. S. (Eds), *The Yeasts*, vol. 3. Academic Press, New York, pp. 367–455.
112. Reed, L. J. (1974). Multienzyme complexes. *Acc. Chem. Res.* **7**, 40–46.
113. Reed, L. J. and Yeaman, S. J. (1987). Pyruvate dehydrogenase. *The Enzymes* **28**, 77–95.
114. Rieger, M., Kappeli, O. and Fiechter, A. (1983). The role of a limited respiration in the complete oxidation of glucose by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **129**, 653–661.
115. Rohde, M., Lim, F. and Wallace, J. C. (1986). Pyruvate carboxylase from *Saccharomyces cerevisiae*. Quaternary structure, effects of allosteric ligands and binding of avidin. *Eur. J. Biochem.* **156**, 15–22.
116. Rohde, M., Lim, F. and Wallace, J. C. (1991). Electron microscopic localization of pyruvate carboxylase in rat liver and *Saccharomyces cerevisiae* by immunogold procedures. *Arch. Biochem. Biophys.* **290**, 197–201.
117. Romanos, M. A., Scorer, C. A. and Clare, J. J. (1992). Foreign gene expression in yeast: a review. *Yeast* **8**, 423–488.
118. Ross, J., Reid, G. A. and Dawes, I. W. (1988). The nucleotide sequence of the *LPDI* gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*: comparison between eukaryotic and

- prokaryotic sequences for related enzymes and identification of potential upstream control sites. *J. Gen. Microbiol.* **134**, 1131–1139.
119. Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194**, 281–301.
 120. Roy, D. J. and Dawes, I. W. (1987). Cloning and characterization of the gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **133**, 925–933.
 121. Ruiz-Amil, M., de Torrontegui, E., Palacián, E., Catalina, L. and Losada, M. (1965). Properties and function of yeast pyruvate carboxylase. *J. Biol. Chem.* **240**, 3485–3492.
 122. Ryan, E. D., Tracey, J. W. and Kohlhaw, G. B. (1973). Subcellular localization of leucine biosynthetic enzymes in yeast. *J. Bacteriol.* **116**, 222–225.
 123. Schaaff, I., Green, J. B. A., Gozalbo, D. and Hohmann, S. (1989). A deletion of the *PDC1* gene for pyruvate decarboxylase of yeast causes a different phenotype than previously isolated point mutations. *Curr. Genet.* **15**, 75–81.
 124. Schaaff, I., Heinisch, J. and Zimmermann, F. K. (1989). Overproduction of glycolytic enzymes in yeast. *Yeast* **5**, 285–290.
 125. Scheffers, W. A. (1966). Stimulation of fermentation in yeasts by acetoin and oxygen. *Nature* **210**, 533–534.
 126. Schmalix, W. and Bandlow, W. (1993). The ethanol-inducible *YAT1* gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase. *J. Biol. Chem.* **268**, 27428–27439.
 127. Schmitt, H. D. and Zimmermann, F. K. (1982). Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis. *J. Bacteriol.* **151**, 1146–1152.
 128. Schmitt, H. D., Ciriacy, M. and Zimmermann, F. K. (1983). The synthesis of yeast pyruvate decarboxylase is regulated by large variations in the messenger RNA level. *Mol. Gen. Genet.* **192**, 247–252.
 129. Scholer, A. and Schuller, H. J. (1993). Structure and function of the isocitrate lyase gene *ICL1* from the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **23**, 375–381.
 130. Scrutton, M. C. (1978). Fine control of the conversion of pyruvate (phosphoenolpyruvate) to oxaloacetate in various species. *FEBS Lett.* **89**, 1–9.
 131. Seeboth, P. G., Bohnsack, K. and Hollenberg, C. P. (1990). *pdc1⁰* mutants of *Saccharomyces cerevisiae* give evidence for an additional structural *PDC* gene: cloning of *PDC5*, a gene homologous to *PDC1*. *J. Bacteriol.* **172**, 678–685.
 132. Sierkstra, L. N., Verbakel, J. M. A. and Verrrips, C. T. (1992). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**, 2559–2566.
 133. Sims, A. P. and Barnett, J. A. (1978). The requirement of oxygen for the utilization of maltose, cellobiose and D-galactose by certain anaerobically fermenting yeasts (Kluyver effect). *J. Gen. Microbiol.* **106**, 277–288.
 134. Sinclair, D. A. and Dawes, I. W. (1995). Genetics of the synthesis of serine from glycine and the utilization of glycine sole nitrogen source by *Saccharomyces cerevisiae*. *Genetics* **140**, 1213–1222.
 135. Skoog, K. and Hahn-Hagerdal, B. (1990). Effect of oxygenation on xylose fermentation by *Pichia stipitis*. *Appl. Environ. Microbiol.* **56**, 3389–3394.
 136. Steensma, H. Y., Holterman, L., Dekker, I., van Sluis, C. A. and Wenzel, T. J. (1990). Molecular cloning of the gene for the E1 α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **191**, 769–774.
 137. Stucka, R., Dequin, S., Salmon, J. M. and Gancedo, C. (1991). DNA sequences in chromosomes II and VII code for pyruvate carboxylase isoenzymes in *Saccharomyces cerevisiae*: analysis of pyruvate carboxylase-deficient strains. *Mol. Gen. Genet.* **229**, 307–315.
 138. Sundaram, T. K., Cazzulo, J. J. and Kornberg, H. L. (1971). Pyruvate holocarboxylase formation from the apoenzyme and D-biotin in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **143**, 609–616.
 139. Teixeira de Mattos, M. J. and Tempest, D. W. (1983). Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat cultures. *Arch. Microbiol.* **134**, 80–85.
 140. Theobald, U. (1995). Untersuchungen zur Dynamik des Crabtree-Effektes. PhD Thesis, Universität Stuttgart, Stuttgart, Germany.
 141. Thevelein, J. M. (1994). Signal transduction in yeast. *Yeast* **10**, 1753–1790.
 142. Uhlinger, D. J., Yang, C. Y. and Reed, L. J. (1986). Phosphorylation-dephosphorylation of pyruvate dehydrogenase from yeast. *Biochemistry* **25**, 5673–5677.
 143. van Urk, H., Schipper, D., Breedveld, G. J., Mak, P. R., Scheffers, W. A. and van Dijken, J. P. (1989). Localization and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. *Biochim. Biophys. Acta* **992**, 78–86.
 144. van Urk, H., Voll, W. S. L., Scheffers, W. A. and van Dijken, J. P. (1990). Transient-state analysis of metabolic fluxes in Crabtree-positive and Crabtree-negative yeasts. *Appl. Environ. Microbiol.* **56**, 282–286.

145. Venturin, C., Boze, H., Moulin, G. and Galzy, P. (1995). Influence of oxygen limitation on glucose metabolism in *Hanseniaspora uvarum* K₅ grown in chemostat. *Biotechnol. Lett.* **17**, 537–542.
146. Verduyn, C., Postma, E., Scheffers, W. A. and van Dijken, J. P. (1990). Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J. Gen. Microbiol.* **136**, 405–412.
147. Verduyn, C., Postma, E., Scheffers, W. A. and van Dijken, J. P. (1990). Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J. Gen. Microbiol.* **136**, 395–403.
148. Verduyn, C. (1991). Physiology of yeasts in relation to growth yields. *Antonie van Leeuwenhoek* **60**, 325–353.
149. Verduyn, C., Stouthamer, A. H., Scheffers, W. A. and van Dijken, J. P. (1991). A theoretical evaluation of growth yields of yeasts. *Antonie van Leeuwenhoek* **59**, 49–63.
150. Visser, W., Batenburg-van der Vchte, W. M., Scheffers, W. A. and van Dijken, J. P. (1990). Oxygen requirements of yeasts. *Appl. Environ. Microbiol.* **56**, 3785–3792.
151. Visser, W., van Spronsen, E. A., Nanninga, N., Pronk, J. T., Kuenen, J. G. and van Dijken, J. P. (1995). Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*. *Antonie van Leeuwenhoek* **67**, 243–253.
152. Wach, A., Brachat, A., Pohlmann, R. and Philippse, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**, 1793–1808.
153. Walker, M. E., Vale, D. L., Rohde, M., Devenish, R. J. and Wallace, J. C. (1991). Yeast pyruvate carboxylase: identification of two genes encoding isoenzymes. *Biochem. Biophys. Res. Comm.* **176**, 1210–1217.
154. Wenzel, T. J., van den Berg, M. A., Visser, W., van den Berg, J. A. and Steensma, H. Y. (1992). Characterization of *Saccharomyces cerevisiae* mutants lacking the E1 α subunit of the pyruvate dehydrogenase complex. *Eur. J. Biochem.* **209**, 697–705.
155. Wenzel, T. J., Luttik, M. A. H., van den Berg, J. A. and Steensma, H. Y. (1993). Regulation of the *PDA1* gene encoding the E1 α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **218**, 405–411.
156. Wenzel, T. J., Zuurmond, A. M., Bergmans, A., van den Berg, J. A. and Steensma, H. Y. (1994). Promoter analysis of the *PDA1* gene encoding the E1 α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Yeast* **10**, 297–308.
157. Wenzel, T. J., Teunissen, A. W. R. H. and Steensma, H. Y. (1995). *PDA1* mRNA: a standard for quantitation of mRNA in *Saccharomyces cerevisiae* superior to *ACT1* mRNA. *Nucl. Acids Res.* **23**, 883–884.
158. Weusthuis, R. A., Visser, W., Pronk, J. T., Scheffers, W. A. and van Dijken, J. P. (1994). Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. *Microbiology* **140**, 703–715.
159. de Winde, J. H. and Grivell, L. A. (1993). Global regulation of mitochondrial biosynthesis in *Saccharomyces cerevisiae*. *Progr. Nucl. Acids Res. Mol. Biol.* **46**, 51–91.
160. Wright, A. P. H., Png, H. L. and Hartley, B. S. (1989). Identification, cloning and characterization of a new gene required for full pyruvate decarboxylase activity. *Curr. Genet.* **15**, 171–176.
161. Yeaman, S. J. (1989). The 2-oxo acid dehydrogenase complexes: recent advances. *Biochem. J.* **257**, 625–632.
162. Zelenaya-Troitskaya, O., Perlman, P. S. and Butow, R. A. (1995). An enzyme in yeast mitochondria that catalyzes a step in branched-chain amino acid biosynthesis also functions in mitochondrial DNA stability. *EMBO J.* **14**, 3268–3276.

MicroReview

Transcriptional control of the *GAL/MEL* regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction

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Summary

In the yeast *Saccharomyces cerevisiae*, the interplay between Gal3p, Gal80p and Gal4p determines the transcriptional status of the genes needed for galactose utilization. The interaction between Gal80p and Gal4p has been studied in great detail; however, our understanding of the mechanism of Gal3p in transducing the signal from galactose to Gal4p has only begun to emerge recently. Historically, Gal3p was believed to be an enzyme (catalytic model) that converts galactose to an inducer or co-inducer, which was thought to interact with GAL80p, the repressor of the system. However, recent genetic analyses indicate an alternative ‘protein–protein interaction model’. According to this model, Gal3p is activated by galactose, which leads to its interaction with Gal80p. Biochemical and genetic experiments that support this model provided new insights into how Gal3p interacts with the Gal80p–Gal4p complex, alleviates the repression of Gal80p and thus allows Gal4p to activate transcription. Recently, a galactose-independent signal was suggested to coordinate the induction of *GAL* genes with the energy status of the cell.

Introduction

All organisms need to adapt to constant fluctuations in their intra- and extracellular environments. They have evolved functional capabilities to face such challenges by regulating gene expression. The evolution of an efficient regulatory mechanism to control gene expression is

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therefore vital for the growth and reproduction of an organism in a given environment. Gene expression can be controlled in many different ways; however, it is becoming increasingly clear that transcriptional regulation is the predominant mechanism. Macromolecular interactions are the key to gene regulation, with small and large molecules serving as signals for these regulatory activities. These interactions provide the flexibility to coordinate gene expression in response to different combinations of signals.

The galactose/melibiose (*GAL/MEL*) regulon of *Saccharomyces cerevisiae* is one of the best characterized eukaryotic systems of transcriptional regulation. The structural genes of this regulon, the *GAL* genes, are regulated at the level of transcription in a carbon source-dependent manner (Johnston and Carlson, 1992; Lohr *et al.*, 1995). Yeast growing in a non-fermentable carbon source, such as ethanol or glycerol, do not express the *GAL* genes. The addition of galactose causes the rapid induction of *GAL* genes. However, the expression of *GAL* genes is rapidly shut off upon addition of glucose; this phenomenon is known as glucose repression (Johnston, 1999). The mechanisms of repression and induction of the *GAL/MEL* regulon have been studied in great detail; however, the nature of the initial intracellular signals that trigger subsequent events have only begun to emerge in the past decade. In this review, we focus on the recent elucidation of the galactose signalling pathway in the *GAL/MEL* regulon of *S. cerevisiae*. Some early observations will also be discussed to facilitate an understanding of recent work and to enable older data to be reinterpreted in the light of recent findings.

Elements of the *GAL/MEL* regulon: transcriptional induction by interplay of regulatory proteins

Galactose is transported across the membrane by the permease encoded by *GAL2*. Intracellular galactose is converted to glucose 1-phosphate by the sequential action of galactokinase, galactose-1-phosphate uridyl transferase and uridine diphosphoglucose epimerase (Fig. 1). These enzymes are encoded by *GAL1*, *GAL7*

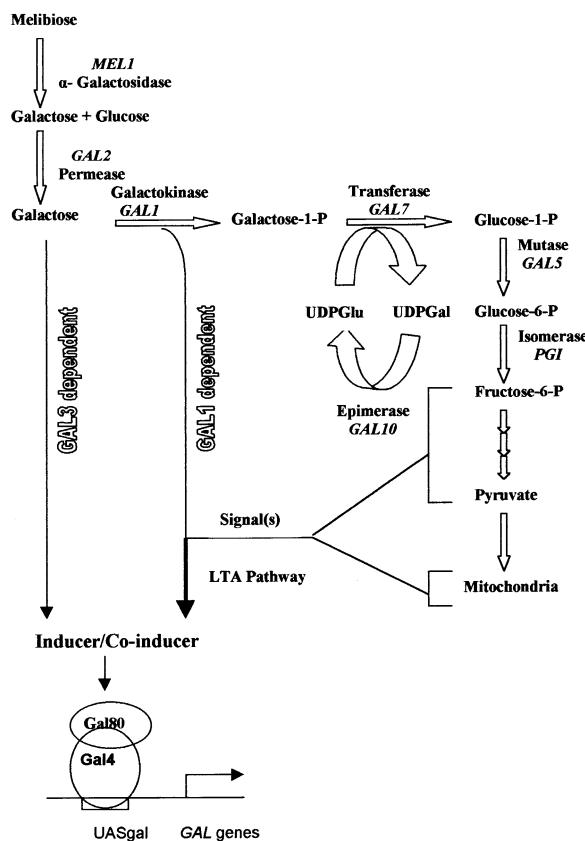


Fig. 1. Galactose metabolic pathway and schematic representation of the 'catalytic model'. Inducer/co-inducer was thought to interact with Gal80 to alleviate the repression, so that Gal4 can activate transcription. This model was later shown to be incorrect (see text for details). UASgal represents the upstream activating sequences to which Gal4 binds.

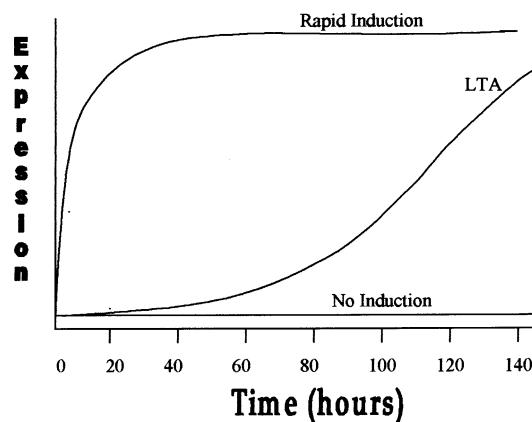


Fig. 2. Schematic representation of *GAL* gene expression in yeast strains of different genetic background in response to galactose as a function of time. Rapid induction and LTA occurs in the wild-type and *gal3* strains respectively. Induction does not occur in *gal3gal1*, *gal3gal7*, *gal3gal10*, *gal3gal5*, *gal3pgi* and *gal3rho*⁻ strains.

and *GAL10* respectively. Melibiose is hydrolysed by α-galactosidase, which is encoded by *MEL1*, and the resultant galactose enters the glycolytic pathway (Fig. 1). Phosphoglucomutase, encoded by *GAL5*, converts glucose 1-phosphate to glucose 6-phosphate, which subsequently enters the glycolytic pathway. *GAL2*, *GAL1*, *GAL7*, *GAL10* and *MEL1* are the *GAL* structural genes.

Galactose activates transcription of the *GAL* genes from undetectable or low basal levels to high levels. The complex interplay between Gal4p, Gal80p and Gal3p determines the transcriptional status of the *GAL* genes. Gal4p, the DNA-binding transcriptional activator, remains bound to upstream activating sequences in the *GAL* promoter region. However, in the absence of galactose, Gal4p is unable to activate transcription, as Gal80p interacts with Gal4p and thus inhibits the transcription of the *GAL* genes. Gal3p inhibits Gal80p repression in response to galactose, allowing Gal4p to activate the transcription of *GAL* genes. There have been many studies on *GAL4* and *GAL80* (Johnston and Carlson, 1992; Lohr *et al.*, 1995), but fewer attempts to understand the mechanism of *GAL3*, probably because of the inherent difficulties in analysing the complex phenotypes exhibited by *gal3* mutants.

Early thoughts on *GAL3*: catalytic model

In 1948, a mutant yeast strain was described that was unable to ferment galactose as rapidly as a wild-type strain, a phenotype referred to as 'long-term adaptation' (LTA) (Winge and Roberts, 1948) (Fig. 2). Genetic analysis indicated that LTA resulted from a recessive mutation in *GAL3*. Despite considerable progress in our understanding of the role of *GAL3* in the rapid induction phenotype, the mechanism of LTA remains unknown. Initial attempts to decipher the molecular mechanism of *GAL3* focused on interpreting the unique phenotypes resulting from mutations in other genes in a *gal3* context.

gal3 strains that lack mitochondria cannot use galactose efficiently (Douglas and Pelroy, 1963). However, a *gal3* strain that has been adapted to galactose continues to grow, even after treatment with ethidium bromide, which destroys mitochondrial function (Tsuyumu and Adams, 1973). Further analysis of this phenotype led to the proposal that the wild-type but not the *gal3* strain synthesizes a co-inducer that activates *GAL* genes rapidly in the presence of galactose (Tsuyumu and Adams, 1974). Thus, the *GAL3* product was proposed to have a role in the synthesis of this co-inducer. Accordingly, LTA was explained by assuming that either the mutation in *GAL3* was leaky or the co-inducer was also synthesized through other pathways, albeit inefficiently.

Strains carrying mutations in *GAL3* and in any of the *GAL1*, *GAL7* or *GAL10* genes cannot be induced (Fig. 2),

whereas strains that harbour mutations in the galactose catabolic pathway, but not in *GAL3*, can still be induced (Broach, 1979). For example, a *gal3gal7* strain growing in glycerol does not express Gal1p or Gal10p upon the addition of galactose. This observation led to the suggestion that an intermediate of galactose metabolism, synthesized by Gal1p, Gal7p and Gal10p, is an intracellular inducer. This implies that the *GAL3* product converts galactose to an inducer that is also an intermediate of galactose metabolism. Accordingly, the absence of *GAL3* and *GAL1*, *GAL7* or *GAL10* leads to non-inducibility. The slow induction in a *gal3* strain was thought to result from the low activity of *GAL1*, *7* and *10* products, and it was believed that these products eventually built and initiated the LTA. This is known as the 'catalytic model'. However, a *gal3gal5* and *gal3 pgi* strain (in which *GAL3* is disrupted and the catabolism of glucose 1-phosphate is blocked as a result of disruption of phosphoglucomutase or phosphoglucoisomerase) is also non-inducible (Bhat *et al.*, 1990), like *gal3gal1*, *gal3gal7*, *gal3gal10* or *gal3rho⁻* (Douglas and Pelroy, 1963; Broach, 1979), suggesting that the catalytic model might not be correct.

A functional galactose catabolic pathway is required for the initiation of LTA and for the maintenance of the induced state in *gal3* cells (Nogi, 1986; Torchia and Hopper, 1986). Thus, a *gal7gal3^{ts}* strain growing in ethanol expresses Gal10p upon addition of galactose at permissive temperatures, but is not expressed at non-permissive temperatures. In contrast, a *gal3^{ts}* strain continues to express Gal10p when shifted to a non-permissive temperature after induction (Nogi, 1986).

Conditions such as defective *GAL80* (leading to the activation of *GAL* genes in the absence of galactose as a result of the inability of mutant Gal80p to repress Gal4p) or *GAL4^c* mutation (which is not repressed by Gal80p) that confer constitutive *GAL* gene expression suppress the non-inducible phenotype of *gal3 rho⁻* strains (Douglas and Pelroy, 1963; Douglas and Hawthorne, 1966). The *gal3 gal1gal7* induction block is also suppressed by conditions that result in constitutive induction (Torchia and Hopper, 1986). These observations suggest that the inducer acts upstream of *GAL80* (Fig. 1).

Molecular genetics of *GAL3*: cloning and sequencing of *GAL3*

A strain in which *GAL3* is disrupted behaves like the original *gal3* isolate. This indicates that LTA is not caused by the leaky nature of the *gal3* mutation (Torchia and Hopper, 1986). *GAL3* is expressed at a basal level when cells are grown in glycerol. It is induced three- to fivefold in response to galactose, but is severely repressed in the presence of glucose (Bajwa *et al.*, 1988). This explains why *GAL* genes are expressed within minutes of

galactose addition in a strain growing in glycerol. The *GAL3* promoter has consensus Gal4p binding sites, consistent with the induction profile in response to galactose (Bajwa *et al.*, 1988).

The mechanism of Gal3p began to unfold after its amino acid sequence was shown to be strikingly similar to galactokinase of *Escherichia coli* (Bajwa *et al.*, 1988). *GAL3* encodes a 520-amino-acid protein, the amino acid sequence of which is strikingly similar to many galactokinases in prokaryotes and eukaryotes (Vollenbroich *et al.*, 1999; Platt *et al.*, 2000). However, Gal3p has no detectable galactokinase activity (Bhat *et al.*, 1990). Expression of yeast galactokinase from a multicopy plasmid partially suppressed the LTA phenotype of a *gal3* strain (Bhat *et al.*, 1990; Meyer *et al.*, 1991). Expression of yeast galactokinase from the *ADHII* promoter in *gal7gal3*, *gal3gal10* or *gal3 rho⁻* strains leads to the activation of *GAL* genes in response to galactose. This suggests that galactokinase is the limiting factor in strains that are compromised for *GAL3* function, galactose metabolic pathway and mitochondrial function (Bhat and Hopper, 1991). This also suggests that the *GAL1*-dependent LTA phenotype requires additional signal(s) from the galactose catabolic pathway as well as mitochondrial function, whereas the *GAL3*-dependent rapid induction phenotype does not (Fig. 1).

Unlike Gal3p, Gal1p has both kinase- and Gal3p-like activity (Bhat *et al.*, 1990; Meyer *et al.*, 1990). Gal3p function is unlikely to be mediated through galactose 1-phosphate, because *E. coli* galactokinase cannot suppress LTA (Bhat *et al.*, 1990). Attempts to identify the presumed Gal3p-associated enzyme activity were not successful (Bhat *et al.*, 1990). Moreover, attempts to detect a change in the interaction between Gal80p and Gal4p *in vitro* in the presence of various galactose derivatives were also unsuccessful (Lue *et al.*, 1987; Chasman and Kornberg, 1990; Yun *et al.*, 1990). These studies found that the *GAL3* product might not convert galactose to an intracellular inducer and questioned the basic tenet of the 'catalytic model'.

GAL/LAC regulon of *Kluyveromyces lactis*: lessons learnt

The regulation of transcription induction of the *GAL/MEL* and *GAL/LAC* regulons is very similar in *S. cerevisiae* and *K. lactis* (Michael and Robert, 1984; Salmeron and Johnston, 1986; Riley *et al.*, 1987; Pan *et al.*, 1990; Zenke *et al.*, 1993). Signal transduction pathways in these two organisms show some subtle but interesting differences. For example, a *GAL3* homologue is not present in *K. lactis*, and its function is performed by galactokinase, encoded by *KIGAL1* (Meyer *et al.*, 1990; 1991). As expected, *GAL3*, *GAL1* and *KIGAL1* show significant

amino acid sequence similarity (Vollenbroich *et al.*, 1999). Although a *GAL3* homologue is absent in *K. lactis*, it does not show LTA, probably because the basal expression of *KIGAL1* is higher than the undetectable basal expression of this gene in *S. cerevisiae*. Another difference is that a *gal7* *K. lactis* mutant constitutively expresses the *GAL* genes in stationary phase (Michael and Robert, 1984). A detailed study of this phenotype provided independent evidence that galactose is the intracellular inducer and not a galactose derivative (Cardinali *et al.*, 1997). Although *KIGAL1* can function as a signal transducer in both *S. cerevisiae* and *K. lactis*, both *GAL3* and *GAL1* are partially active in *K. lactis* (Zenke *et al.*, 1996). However, if *GAL80* is substituted for *KIGAL80* in *K. lactis*, both *GAL3* and *GAL1* can transduce the signal efficiently (Zenke *et al.*, 1996).

Interaction between Gal3p and Gal80p: the key for signal transduction

The lack of experimental evidence for the catalytic model suggested that galactose probably activates Gal3p or Gal1p so that they are capable of inactivating Gal80p, and Gal4p can activate transcription (Bhat and Hopper, 1992). According to this 'protein–protein interaction model', Gal3p and Gal1p can exist in inactive and active conformations. In the absence of galactose, the concentration of the inactive form is below the threshold required to alter the interaction between Gal80p and Gal4p. The addition of galactose shifts this equilibrium in favour of the active form, relieving Gal80p inhibition. This model was initially supported by the following observations. (i) Overexpression of Gal3p or Gal1p in the absence of galactose activates *GAL* gene expression to 40% of its maximum level and, as expected, galactose increases this level further (Bhat and Hopper, 1992). (ii) High-level expression of a missense *GAL1* allele (C175Y) lacking galactokinase activity leads to the constitutive expression of *GAL* genes, but the addition of galactose does not increase the induction level further as it does with a wild-type *GAL1* allele (Bhat and Hopper, 1992). (iii) Constitutive *GAL3* (*GAL3^c*) and *KIGAL1* alleles activate the *GAL* genes in the absence of galactose (Blank *et al.*, 1997; Vollenbroich *et al.*, 1999). These mutant alleles differ from the wild-type alleles in that they need not be expressed at high levels for the constitutive induction of *GAL* genes. (iv) Galactose-independent activation of *GAL* genes upon overexpression of Gal3p is reduced by the concomitant overproduction of Gal80p (Suzuki-Fujimoto *et al.*, 1996). These observations are consistent with the protein–protein interaction model, but not with the catalytic model.

A direct interaction between Gal3p and Gal80p under *in vitro* and *in vivo* conditions would support the protein–protein interaction model. Indeed, co-precipitation

showed that epitope-tagged Gal3p expressed in a wild-type strain forms a complex with Gal80p (Suzuki-Fujimoto *et al.*, 1996). The amount of Gal80p associated with Gal3p is higher when cells are grown in the presence of galactose (Suzuki-Fujimoto *et al.*, 1996). However, when both proteins are overexpressed, the presence of galactose in the growth medium does not alter the amount of Gal80p associated with Gal3p. This is consistent with the previous observation that the overexpression of Gal3p causes the constitutive expression of *GAL* genes (Bhat and Hopper, 1992). Epitope-tagged *KIGal80p* also interacts with *KIGal1p* in whole-cell extracts (Zenke *et al.*, 1996). This interaction is dependent on the presence of both galactose and ATP. Weak interactions between *KIGal1p* and *KIGal80p* could be demonstrated in extracts from cells grown in galactose, but not from cells grown in the absence of galactose (Zenke *et al.*, 1996). Gal3p functions less efficiently than *KIGal1p* in *K. lactis*, which is consistent with the weak interaction detected between Gal3p and *KIGal80p* (Zenke *et al.*, 1996). The interactions between purified proteins depend on galactose, but mannose, maltose, glucose and fructose have no effect (Zenke *et al.*, 1996; Yano and Fukasawa, 1997). However, ATP can be substituted by ADP, UTP, GTP and ATP-γ-S, indicating that the hydrolysis of a high-energy phosphate bond is not required for the interaction (Zenke *et al.*, 1996). In the absence of galactose and ATP, the interaction is sensitive to the presence of NaCl, suggesting that galactose and ATP might stabilize the interaction (Yano and Fukasawa, 1997).

The physiological relevance of the interaction between Gal3p and Gal80p was also analysed rigorously using variants of Gal80p and Gal3p, or *KIGal1p*. Variants of Gal3p or *KIGal1p* lacking induction activity cannot interact with wild-type Gal80p or *KIGal80p* respectively (Suzuki-Fujimoto *et al.*, 1996; Zenke *et al.*, 1996). Likewise, a defective form of Gal80p that confers a non-inducible phenotype cannot interact with wild-type Gal3p, and *GAL3^c* mutant proteins interact with Gal80p *in vitro* even in the absence of galactose and ATP (Blank *et al.*, 1997; Yano and Fukasawa, 1997). Galactose-dependent interactions between full-length *KIGal1p* and *KIGal80p* were also demonstrated by two-hybrid analysis (Vollenbroich *et al.*, 1999).

Mutational analysis of *GAL3*, *GAL1* and *KIGAL1*: domain structure

Amino acid substitutions in five different *GAL3^c* alleles are not confined to any specific region of the primary sequence, making it difficult to identify functional domains even tentatively (Blank *et al.*, 1997). Two missense *GAL1* mutations, G166D and C175T, disrupt kinase function but not *GAL3* function (Bhat and Hopper, 1992). Missense

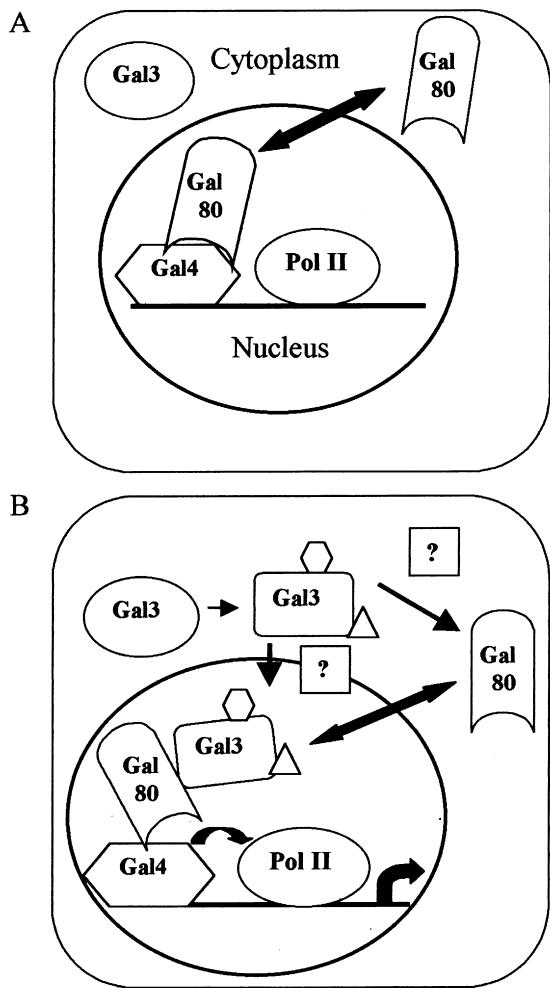


Fig. 3. Schematic representation of Gal3-dependent signal transduction. In the uninduced state (A), Gal3 is located in the cytoplasm, and Gal80 is distributed in the cytoplasm and nucleus. In the induced state (B), Gal3 is activated in the presence of galactose (hexagon) and ATP (triangle) and interacts with Gal80 in the nucleus or in the cytoplasm (see text for details). Gal3 interaction with Gal80 allows Gal4 to activate transcription by interacting with the basal transcription machinery.

mutations have also been isolated that specifically inactivate kinase or *GAL3* functions of KIGal1p (Meyer *et al.*, 1990; Vollenbroich *et al.*, 1999). Mutations in *GAL1* or *KIGAL1* that abolish either kinase or *GAL3* activity indicated that the loss of one of the two activities is not the result of major structural alterations. These mutants should prove useful in the structural elucidation of these proteins. Gal3p lacks the consecutive serine and alanine residues within the highly conserved GLSSSAA motif that is present in all known galactokinases. Insertion of serine and alanine at this position of Gal3p converted it to a weak galactokinase (Platt *et al.*, 2000). Insertion of alanine alone did not confer galactokinase activity, but resulted in the loss of *GAL3* activity. Insertion of a serine or two alanine residues abolished both the kinase and the Gal3p activities.

A two-hybrid assay, involving *VP16-GAL80* and the *lexA* DNA-binding domain fused to different deletion segments of *KIGAL1*, indicated that the entire *GAL1* sequence is required for function (Vollenbroich *et al.*, 1999). *KIGAL1* and *GAL3* chimeras can interact with ScGAL80 but not with KIGAL80, suggesting that the species-specific interaction of KIGal1p and Gal80p involves both the N- and C-terminal regions of KIGal1p (Vollenbroich *et al.*, 1999). Attempts to study the properties of the different deletion derivatives of Gal3p indicated that it is sensitive to the removal of amino acid residues from both the C- and N-terminals (Murthy and Bhat, 2000; Platt *et al.*, 2000).

Mechanism of signal transduction by Gal3p3: fate of Gal80p upon induction

Elucidating the fate of Gal80p upon interaction with Gal3p is central to the understanding of the molecular mechanism of the galactose 'genetic switch'. Two-hybrid analysis showed that Gal80p remains associated with Gal4p even under inducing conditions, implying that the interaction of Gal3p and Gal80p alters the Gal80p–Gal4p interaction (Leuther and Johnston, 1992). *In vitro* transcription studies showed that galactose-mediated induction results from the formation of a tripartite complex consisting of Gal3p/Gal1p, Gal80p and Gal4p (Platt and Reece, 1998). These results are consistent with the following experimental observations. (i) Gal1p can substitute for Gal3p for activation but only when present at a 40-fold molar excess of the amount of Gal3p required for activation. (ii) Gal3^{c-322}p can activate transcription even in the absence of galactose, whereas the addition of galactose increases transcription even further. Thirty-fold more galactose is required for the system to be fully activated by wild-type Gal3p than by Gal3^{c-322}p. (iii) Although Gal80^{s-2}p can interact with Gal4p and repress its activity, it does not respond to either wild-type Gal3p or *GAL3*^{c-322}p. (iv) Gel mobility shift assays showed that *GAL3*^{c-322} forms a complex with Gal80p–Gal4p DNA in a galactose- and ATP-dependent manner. Gal3^{c-322}p was used instead of wild-type Gal3p to circumvent technical difficulties. These results suggest that, under *in vivo* conditions, the interaction of Gal3p and Gal80p alters the interaction between Gal80p and Gal4p, without necessarily causing them to dissociate (Fig. 3).

More recent biochemical and genetic experiments suggest that the interaction of Gal3p and Gal80p relocates Gal80p from its original binding site on Gal4p to a second site (Sil *et al.*, 1999). Two-hybrid analysis indicated that the second Gal80p-binding region spans amino acid residues 225–797 of Gal4p. This two-site model does not differentiate between the possibility that Gal80p might simultaneously recognize two Gal4p binding sites or that

the interaction of Gal3p and Gal80p might destabilize the interaction that inhibits transcription (Sil *et al.*, 1999).

The activation of Gal4p involves the interaction of active Gal3p and the Gal80p–Gal4p complex bound to DNA, but it is not clear how the signal is transmitted to the nucleus because Gal3p is a cytoplasmic protein and appears to be excluded from the nucleus (Peng and Hopper, 2000). Although *GAL80* has an NLS sequence (Nogi and Fukasawa, 1989), subcellular fractionation, immunofluorescence and studies conducted to determine whether Gal80p shuttles between nuclei in yeast heterokaryons have shown that Gal80p is found in both the cytoplasm and the nucleus. Targeting Gal80p to the nucleus by adding exogenous NLS resulted in a twofold decrease in transcription. The decrease in transcription observed when NLS-Gal80p is expressed can be partially restored by expressing NLS-Gal3p. (Peng and Hopper, 2000) These studies suggest that the dynamics of Gal80p distribution between the cytoplasm and the nucleus are critical for the cytoplasm–nuclear signal transduction process (Peng and Hopper, 2000). It has been suggested that either active Gal3p traps Gal80p in the cytoplasm (cytoplasmic trapping) or active Gal3p modifies Gal80p, thus altering its affinity for Gal4p (Peng and Hopper, 2000).

Phosphorylation of Gal4p at S699, mediated by RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase encoded by *SRB10*, occurs concomitantly with transcriptional induction and may modulate the interaction between Gal80p and Gal4p (Sadowski *et al.*, 1996; Hirst *et al.*, 1999). This is supported by the recent observation that phosphorylation of S699 is not required when the inducing signal is not limited, implying that a strong signal is sufficient to inactivate Gal80p (Rohde *et al.*, 2000). However, when the induction signal is weak, phosphorylation of S699 becomes dominant and maintains Gal80p in an inactive state. Phosphorylation of S699 may be involved in the initiation of induction in a *gal3* mutant (weak induction signal) by stabilizing a spontaneous change in the interaction between Gal80p and Gal4p and leading to slow transcription induction as seen in LTA. Thus, the need for an intact galactose metabolic pathway and for mitochondrial function for the manifestation of LTA in a *gal3* strain might reflect a positive signal of cellular energy status to the galactose genetic switch (Bhat *et al.*, 1990). *SRB10* is probably a component of this relay system (Rohde *et al.*, 2000), which maintains the ‘ON’ and ‘OFF’ status of the galactose genetic switch according to the overall energy status of the cell.

Evolutionary aspects: design of novel regulatory strategies

The galactose genetic switch provides an excellent

opportunity to understand how different regulatory circuits have evolved to suit a given environmental and physiological context. For example, unlike the *lac* operon in *E. coli*, the *GAL/MEL* regulon of yeast has incorporated both positive and negative regulation by recruiting Gal3p and Gal80p in the induction cascade. Expression of *GAL3* and *GAL80* is in turn regulated by the galactose genetic switch (Shimada and Fukasawa, 1985; Bajwa *et al.*, 1988). This is known as autogenous regulation (Goldberg, 1975). Based on a modelling approach, it was suggested that, in addition to co-operativity, autogenous regulation of Gal3p and Gal80p might provide an additional mechanism that allows the switch to respond within narrow limits of the inducer concentration (Venkatesh *et al.*, 1999). Such a protein–protein interaction cascade (Fig. 3) would allow the cell to attain the ‘ON’ or ‘OFF’ states without too many intermediates. Clearly, tight regulation of biological processes provides distinct evolutionary advantages to any organism over its competitors.

Conclusions: future prospects

The *GAL/MEL* regulon is a powerful model for studying transcriptional regulation in eukaryotes and has been used in a variety of pioneering studies. For example, knowledge of Gal4p and Gal80p interaction was used to identify genes encoding interacting proteins and to identify protein–protein interaction domains. The recurring biological theme in this system is how a specific constellation of proteins orchestrates the optimal expression of genes in response to physiological and environmental changes. We have made great progress in understanding this ‘genetic switch’, but have only recently begun to elucidate the molecular mechanism of Gal3p-mediated signal transduction. However, we still need to resolve a number of important questions. How does a predominantly cytoplasmic protein, such as Gal3p, transmit the signal to the Gal80p–Gal4p complex that is located in the nucleus? How does galactose transform Gal3p from an inactive state to an active state? It is believed that the activation of Gal3p only involves a conformational change, but there is no experimental evidence for this. How does the interaction between Gal3p and Gal80p alter Gal80p–Gal4p interactions and activate transcription? How does a gene-specific activator, such as Gal4p, sense and respond to intracellular signals, such as the energy status of the cell? Now that the genes and proteins involved have been identified and their interactions have been characterized at the genetic and biochemical level, the next step is to study these processes using more refined techniques. Biochemical, genetic and biophysical experiments coupled with quantitative studies are required to understand the functioning of the galactose ‘genetic switch’.

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References

- Bajwa, W., Torchia, T.E., and Hopper, J.E. (1988) Yeast regulatory gene *GAL3*: carbon regulation; UAS gal elements in common with *GAL1*, *GAL2*, *GAL7*, *GAL10*, *GAL80*, and *MEL1*; encoded protein strikingly similar to yeast and *Escherichia coli* galactokinase. *Mol Cell Biol* **8**: 3439–3447.
- Bhat, P.J., and Hopper, J.E. (1991) The mechanism of inducer formation in *gal3* mutants of the yeast galactose system is independent of normal galactose metabolism and mitochondrial respiratory function. *Genetics* **128**: 233–239.
- Bhat, P.J., and Hopper, J.E. (1992) Overproduction of the *GAL1* or *GAL3* protein causes galactose-independent activation of the *GAL4* protein: evidence for a new model of induction for the yeast *GAL/MEL* regulon. *Mol Cell Biol* **12**: 2701–2707.
- Bhat, P.J., Oh, D., and Hopper, J.E. (1990) Analysis of the *GAL3* signal transduction pathway activating *GAL4* protein-dependent transcription in *Saccharomyces cerevisiae*. *Genetics* **125**: 281–291.
- Blank, T.E., Woods, M.P., Lebo, C.M., Xin, P., and Hopper, J.E. (1997) Novel Gal3 proteins showing altered Gal80p binding cause constitutive transcription of Gal4p-activated genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**: 2566–2575.
- Broach, J.R. (1979) Galactose utilization in *Saccharomyces cerevisiae*. The enzymes encoded by the *GAL7,1,10* cluster are co-ordinately controlled and separately translated. *J Mol Biol* **131**: 41–53.
- Cardinali, G., Vollenbroich, V., Jeon, M.D.E., Graff, A.A., and Hollenberg, C.P. (1997) Constitutive expression in *gal7* mutants of *Kluyveromyces lactis* is due to internal production of galactose as an inducer of the *GAL/LAC* regulon. *Mol Cell Biol* **17**: 1722–1730.
- Chasman, D.I., and Kornberg, R.D. (1990) *GAL4* protein: purification, association with *GAL80* protein and conserved domain structure. *Mol Cell Biol* **10**: 2196–2923.
- Douglas, H.C., and Hawthorne, D.C. (1966) Regulation of genes controlling synthesis of the galactose pathway. Enzymes in yeast. *Genetics* **54**: 911–916.
- Douglas, H.C., and Pelroy, G. (1963) A gene controlling the inducibility of galactose pathway enzymes in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **68**: 155–156.
- Goldberg, R.F. (1975) Autogenous regulation of gene expression. *Science* **183**: 810–816.
- Hirst, M., Kobor, M., Kuriakose, N., Greenblat, J., and Sadowski, I. (1999) *GAL4* is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase *SRB10/CDJ8*. *Mol Cell Biol* **3**: 673–678.
- Johnston, M. (1999) Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. *Trends Genet* **15**: 29–33.
- Johnston, M., and Carlson, M. (1992) Regulation of carbon and phosphate utilisation. In *The Molecular and Cellular Biology of the Yeast Saccharomyces*, Vol. 2. Jones, E.W., Pringle, J.R., and Broach, J.R. (eds). New York: Cold Spring Harbor Laboratory Press, pp. 193–281.
- Leuther, K.K., and Johnston, S.A. (1992) Nondissociation of *GAL4* and *GAL80* *in vivo* after galactose induction. *Science* **256**: 1333–1335.
- Lohr, D., Venkov, P., and Zlatanova, J. (1995) Transcriptional regulation in the yeast *GAL* gene family: a complex genetic network. *FASEB J* **9**: 777–787.
- Lue, N.F., Chasman, D.L., Buchman, A.R., and Kornberg, R.D. (1987) Interaction of *GAL4* and *GAL80* gene regulatory proteins *in vitro*. *Mol Cell Biol* **7**: 3446–3451.
- Meyer, J., Walker, J.A., and Hollenberg, C.P. (1990) *GAL1* of *Kluyveromyces lactis* is required for the induction of the Lelior enzymes and can complement the *gal3* long term adaptation phenotype in *Saccharomyces cerevisiae*. *Yeast* **6**: 591–596.
- Meyer, J., Walker, J.A., and Hollenberg, C.P. (1991) Galactokinase encoded by *GAL1* is a bifunctional protein required for the induction of the *GAL* genes in *Kluyveromyces lactis* and able to suppress the *gal3* phenotype in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**: 5454–5461.
- Michael, I.R., and Robert, C.D. (1984) Genetic and biochemical characterization of the galactose gene cluster in *Kluyveromyces lactis*. *J Bacteriol* **158**: 705–712.
- Murthy, T.V.S., and Bhat, P.J. (2000) Disruption of galactokinase signature sequence in *GAL3* of *Saccharomyces cerevisiae* does not lead to loss of signal transduction function. *Biochem Biophys Res Commun* **273**: 824–828.
- Nogi, Y. (1986) *GAL3* gene product is required for maintenance of the induced state of the *GAL* cluster genes in *Saccharomyces cerevisiae*. *J Bacteriol* **165**: 101–106.
- Nogi, Y., and Fukasawa, T. (1989) Functional domains of a negative regulatory protein, *GAL80*, of *Saccharomyces cerevisiae*. *Mol Cell Biol* **9**: 3009–3017.
- Pan, T., Halvorsen, Y.D., Dickson, R.C., and Coleman, J.E. (1990) The transcription factor *LAC9* from *Kluyveromyces lactis*-like *GAL4* from *Saccharomyces cerevisiae* forms a Zn(II)2Cys6 binuclear cluster. *J Biol Chem* **265**: 21427–21429.
- Peng, G., and Hopper, J.E. (2000) Evidence for Gal3p's cytoplasmic location and Gal80p's dual cytoplasmic-nuclear location implicates new mechanisms for controlling Gal4p activity in *Saccharomyces cerevisiae*. *Mol Cell Biol* **20**: 5140–5148.
- Platt, A., and Reece, R.J. (1998) The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex. *EMBO J* **17**: 4086–4091.
- Platt, A., Ross, H.C., Hankin, S., and Reece, R.J. (2000) Insertion of two amino acids into a transcriptional inducer converts it into a galactokinase. *Proc Natl Acad Sci USA* **97**: 3154–3159.
- Riley, M.I., Hopper, J.E., Johnston, S.A., and Dickson, R.C. (1987) *GAL4* of *Saccharomyces cerevisiae* activates the lactose-galactose regulon of *Kluyveromyces lactis* and creates a new phenotype: glucose repression of the regulon. *Mol Cell Biol* **7**: 780–786.
- Rohde, J.R., Trinh, J., and Sadowski, I. (2000) Multiple

- signals regulate *GAL* transcription in yeast. *Mol Cell Biol* **20**: 3880–3886.
- Sadowski, I., Costa, C., and Dhanawansa, R. (1996) Phosphorylation of Gal4p at a single C-terminal residue is necessary for galactose inducible transcription. *Mol Cell Biol* **16**: 4879–4887.
- Salmeron, J.M., Jr, and Johnston, S.A. (1986) Analysis of the *Kluyveromyces lactis* positive regulatory gene *LAC9* reveals functional homology to, but sequence divergence from the *Saccharomyces cerevisiae* *GAL4* gene. *Nucleic Acids Res* **14**: 7767–7781.
- Shimada, H., and Fukasawa, T. (1985) Controlled transcription of the yeast regulatory gene *GAL80*. *Gene* **39**: 1–9.
- Sil, A.K., Alam, S., Xin, P., Ma, L., Morgan, M., Lebo, C.M., et al. (1999) The Gal3p–Gal80p–Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p–Gal4p complex in response to galactose and ATP. *Mol Cell Biol* **19**: 7828–7840.
- Suzuki-Fujimoto, T., Fukuma, M., Yano, K.-I., Sakurai, H., Vonika, A., Johnston, S.A., and Fukasawa, T. (1996) Analysis of the galactose signal transduction pathway in *Saccharomyces cerevisiae* interaction between Gal3p and Gal80p. *Mol Cell Biol* **16**: 2504–2508.
- Torchia, T.E., and Hopper, J.E. (1986) Genetic and molecular analysis of the *GAL3* gene in the expression of the galactose/melibiose regulon of *Saccharomyces cerevisiae*. *Genetics* **113**: 229–246.
- Tsuyumu, S., and Adams, B.G. (1973) Population analysis of the deinduction kinetics of galactose long term adaptation mutants of yeast. *Proc Natl Acad Sci USA* **70**: 919–923.
- Tsuyumu, S., and Adams, B.G. (1974) Dilution kinetic studies of yeast populations: *in vivo* aggregation of galactose utilizing enzymes and positive regulator molecules. *Genetics* **77**: 494–505.
- Venkatesh, K.V., Bhat, P.J., Kumar, R.A., and Doshi, P. (1999) Quantitative model for Gal4p-mediated expression of the galactose/melibiose regulon in *Saccharomyces cerevisiae*. *Biotechnol Prog* **15**: 51–57.
- Vollenbroich, V., Meyer, J., Engels, R., Cardinali, G., Menezes, R.A., and Hollenberg, C.P. (1999) Galactose induction in yeast involves association of Gal80p with Gal1p or Gal3p. *Mol Gen Genet* **261**: 495–507.
- Winge, O., and Roberts, C. (1948) Inheritance of enzymatic characters in yeast and the phenomenon of long term adaptation. *CR Trav Lab Carlsberg Ser Physiol* **24**: 263–315.
- Yano, K.I., and Fukasawa, T. (1997) Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **94**: 1721–1726.
- Yun, S.J., Hiraoka, Y., Nishizawa, M., Takio, K., Titani, K., Nigi, Y., and Fukasawa, T. (1990) Purification and characterization of the yeast negative regulatory protein *GAL80*. *J Biol Chem* **266**: 693–697.
- Zenke, F.T., Zachariae, W., Lunkes, A., and Breunig, K. (1993) Gal80 proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are highly conserved but contribute differently to glucose repression of the galactose regulon. *Mol Cell Biol* **13**: 7566–7576.
- Zenke, F.T., Engles, R., Vollenbroich, V., Meyer, J., Hollenberg, C.P., and Breunig, K.D. (1996) Activation of Gal4p by galactose-dependent interaction of galactokinase and Gal80p. *Science* **272**: 1662–1665.

Glucose and sucrose: hazardous fast-food for industrial yeast?

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Yeast cells often encounter a mixture of different carbohydrates in industrial processes. However, glucose and sucrose are always consumed first. The presence of these sugars causes repression of gluconeogenesis, the glyoxylate cycle, respiration and the uptake of less-preferred carbohydrates. Glucose and sucrose also trigger unexpected, hormone-like effects, including the activation of cellular growth, the mobilization of storage compounds and the diminution of cellular stress resistance. In an industrial context, these effects lead to several yeast-related problems, such as slow or incomplete fermentation, 'off flavors' and poor maintenance of yeast vitality. Recent studies indicate that the use of mutants with altered responses to carbohydrates can significantly increase productivity. Alternatively, avoiding unnecessary exposure to glucose and sucrose could also improve the performance of industrial yeasts.

Unlike most mammalian cells, which function in an environment with virtually constant concentrations of only one sugar, namely glucose, yeast cells encounter variable concentrations of many different carbon sources. To overcome this lack of homeostasis and to survive in a highly competitive ecosystem, the common brewer's and baker's yeast *Saccharomyces cerevisiae* has evolved the capacity to take up and to metabolize different carbohydrates. *S. cerevisiae* cells also have several mechanisms for sensing the nutritional status of the environment, enabling them to adapt their uptake and metabolism of nutrients to specific conditions. Although these signaling pathways have been studied mostly as a model for nutrient-induced signaling cascades in higher eukaryotes, their unraveling has relevance for several applications of yeast

biotechnology, including alcoholic fermentation, bread production and the fabrication of biopharmaceuticals.

In this review, we begin by summarizing the role of the two best-known glucose-triggered signaling cascades in *S. cerevisiae*: the main glucose repression pathway (also known as the catabolite repression pathway), and the Ras/cAMP/protein kinase A (PKA) pathway. We then consider the role and implications of sugar signaling in industrial yeast-based processes.

Glucose regulation of carbohydrate uptake and metabolism

In response to glucose and sucrose, the main glucose repression pathway downregulates several genes involved in the uptake and metabolism of alternative carbohydrates, as well as genes involved in gluconeogenesis and respiration. The repression of respiration by glucose and sucrose, which is known as the 'Crabtree effect', might seem counterproductive but, although respiration is a more efficient method of energy production, fermentation offers the advantage of ethanol production, which hampers the growth of competing microorganisms. In addition, glucose concentrations, and possibly levels of glycolytic intermediates, regulate the expression of several glucose transporters and some glycolytic genes (Figure 1 and reviewed in Refs [1–4]). Thus, the main glucose repression pathway ensures that the preferred sugars are metabolized before the consumption of alternative carbohydrates, such as maltose and galactose.

Glucose also slows down the uptake of fructose because both sugars are imported by the same carriers, which have a greater affinity for glucose than for fructose. Besides this competitive inhibition of fructose uptake, recent research shows that glucose can repress the expression of specific fructose transporters such as

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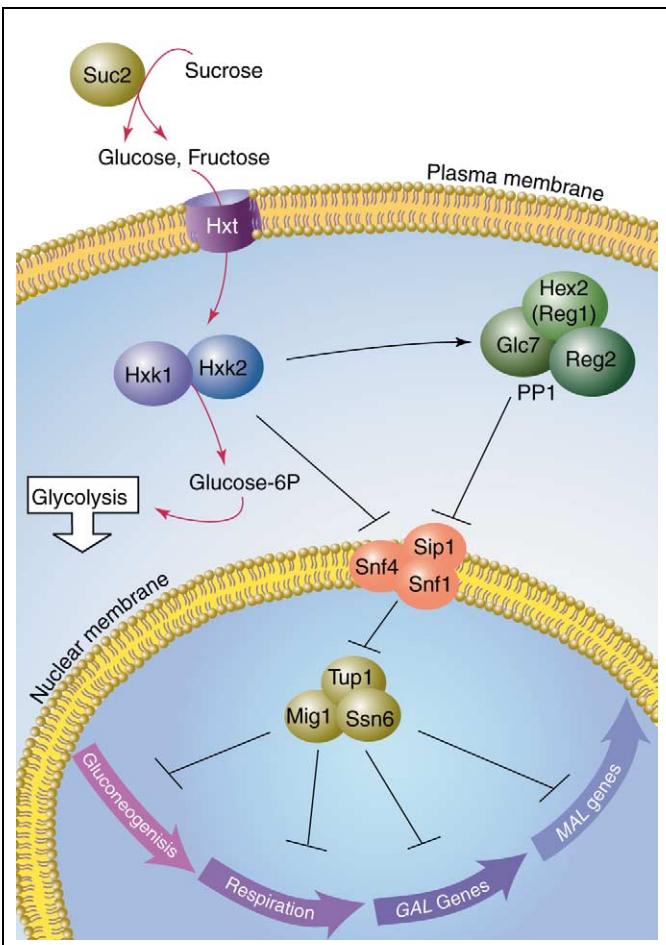


Figure 1. The main glucose repression pathway in *Saccharomyces cerevisiae*. Extracellular glucose is taken up through one of the hexose transporters (Hxt) and subsequently phosphorylated to glucose 6-phosphate (glucose-6P) by one of the hexokinases (Hxk). This phosphorylation process and/or the depletion of AMP owing to the increased production of ATP inactivates the central protein kinase Snf1 [59]. Inactivation of Snf1 occurs via either inhibition of its phosphorylation or stimulation of the Snf1-dephosphorylation activity of the phosphatase complex PP1 [60,61]. When Snf1 is inactive, the DNA-binding protein Mig1 is translocated from the cytoplasm to the nucleus [62]. In the nucleus, Mig1 recruits the general repressors Tup1 and Ssn6 and binds to the promoters of several glucose-repressed genes, including genes involved in gluconeogenesis, respiration and the uptake and breakdown of alternative carbon sources, such as maltose (*MAL* genes) and galactose (*GAL* genes). When extracellular glucose is depleted, the Snf1 complex is activated, causing the translocation of Mig1 back to the cytoplasm, where it can no longer repress its targets [62,63]. Thus, glucose repression is relieved and alternative carbon sources can be taken up. Fructose and sucrose, which is extracellularly hydrolyzed into glucose and fructose, exert catabolite repression effects similar to those of glucose, although glucose is taken up preferentially [1]. For details, see reviews by Gancedo [1], Johnston [2], Carlson [3] and Winderickx *et al.* [4].

Fsy1 [5–7]. In addition to regulating the uptake of alternative sugars, the main glucose repression pathway prevents futile cycling in carbohydrate metabolism by shutting down *de novo* synthesis of glucose by the gluconeogenic pathway.

Regulation of the hormone-like effects of glucose and sucrose

Another principal carbon signaling pathway, the Ras/cAMP/PKA pathway, controls the expression of various genes involved in metabolism, proliferation and stress resistance in response to glucose (Figure 2 and reviewed in Refs [4,8,9]). Because full activation of this

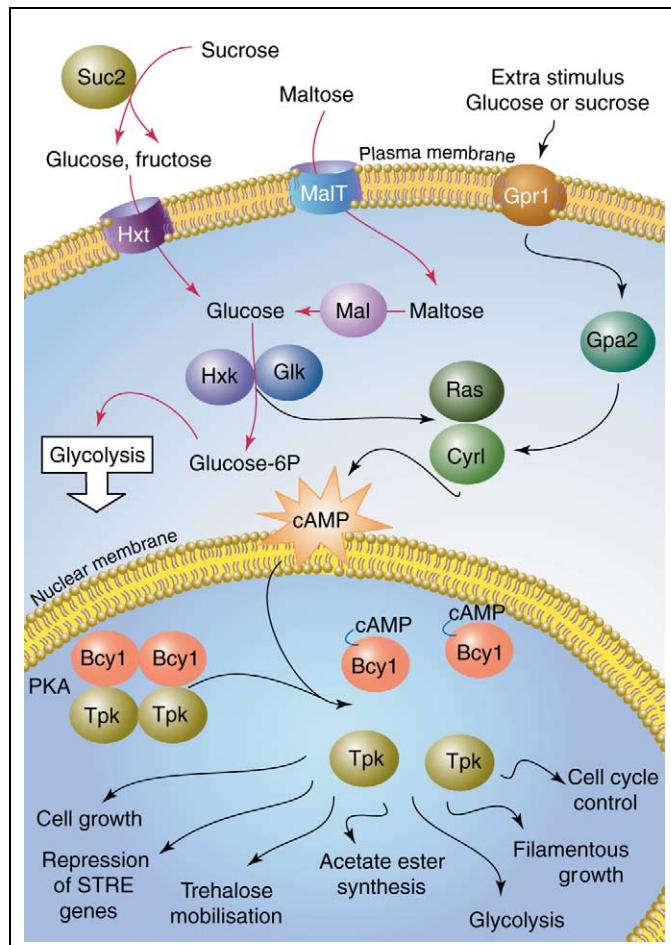


Figure 2. The Ras/cAMP/protein kinase A (PKA) nutrient signaling pathway in *Saccharomyces cerevisiae*. Full activation of this pathway requires a dual signal. First, the intracellular phosphorylation of glucose to glucose 6-phosphate (glucose-6P) enhances the activity of adenylate cyclase Cyr1. Second, extracellular glucose or sucrose is sensed by a G-protein-coupled receptor system, consisting of the receptor Gpr1 and the $\text{G}\alpha$ protein Gpa2 [10]. This second signal strongly enhances Cyr1 activity, resulting in a transient peak of cAMP immediately after the addition of glucose or sucrose [9,10,57,64]. The rise in cAMP causes the regulatory subunits (Bcy1) of the PKA complex to bind cAMP and to dissociate from the Tpk catalytic subunits of PKA [65,66]. The free Tpk kinases then phosphorylate various target proteins, which eventually leads to acclimatization to high glucose levels (Figure 3). Although the role of the small G proteins Ras1 and Ras2 in this process is not completely understood, these proteins are essential for basal Cyr1 activity [67,68]. For details, see reviews by Johnston [2] and Winderickx *et al.* [4]. For clarity, note that Gpa2 and Cyr1 are not depicted as attached to the plasma membrane and the shuttling of the free PKA catalytic subunits out of the nucleus [69] is not shown.

pathway requires extracellular glucose or sucrose [10], other sugars such as fructose, maltose, maltotriose and galactose cannot trigger a strong cAMP/PKA response [10,11] (Figure 2).

Among the targets regulated by the Ras/cAMP/PKA pathway are genes encoding heat-shock proteins, such as *HSP12* and *HSP104*, which are rapidly repressed on activation of this pathway [12,13] (Figure 3). These proteins have important roles in various processes that help yeast cells to cope with a broad array of stresses, including heat and ethanol stress [14–16]. Furthermore, high PKA activity also causes repression of the *TPS1* and *TPS2* genes encoding trehalose synthase [17]. Trehalose has a prominent role in cellular stress resistance because it protects membranes from desiccation and prevents protein denaturation [18].

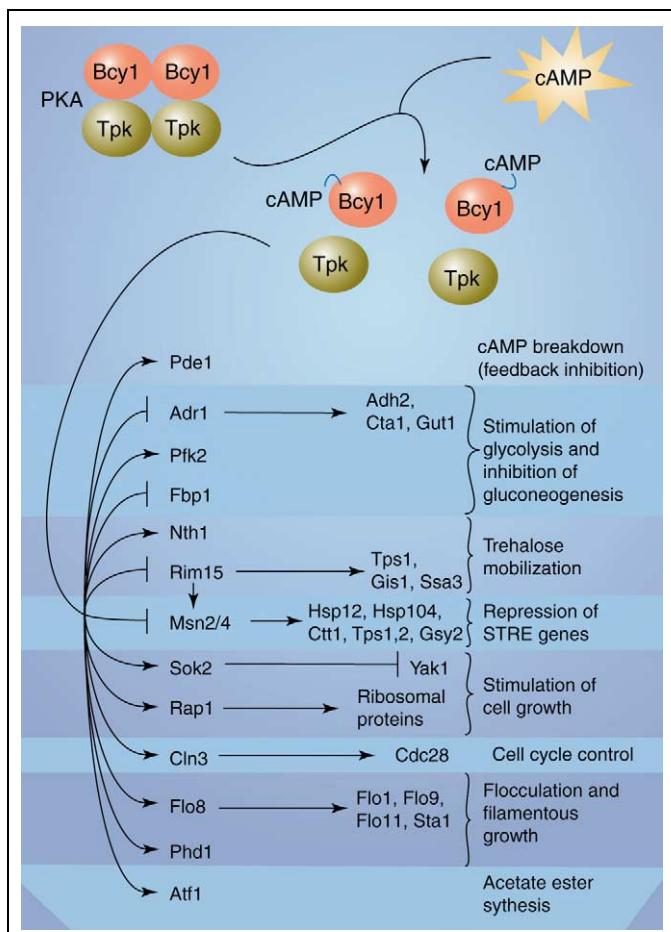


Figure 3. Targets of the Ras/cAMP/PKA nutrient signaling pathway. Targets include key proteins involved in the control of cell growth, glucose metabolism, stress resistance (including trehalose metabolism), flocculation and filamentous growth, and volatile ester synthesis, as well as feedback mechanisms. For example, one target of this pathway is the phosphodiesterase Pde1, which is responsible for the rapid breakdown of cAMP [4].

Glucose and sucrose signaling in industrial fermentations

In most commercial processes, the fermentation medium is a complex mixture of different fermentable sugars. The main sugars in grape must are glucose and fructose; by contrast, beer wort contains glucose, fructose, sucrose, maltose and maltotriose, and the fermentation medium for bioethanol production is usually a mixture of any of these sugars in variable concentrations, depending on the origin of the molasses [19,20].

In most applications, the initial concentration of glucose and/or sucrose in the growth medium is above the threshold concentrations (20–40 mM) for inducing the sugar signaling cascades [21,22]. Thus, both the main glucose repression pathway and the Ras/cAMP/PKA pathway are triggered at the start of the process. Activation of these pathways has three main consequences: repression of respiration, arrest of the consumption of other carbohydrates, and loss of cellular stress resistance. Although these effects presumably help *S. cerevisiae* to survive in its natural habitat, sugar signaling causes several problems in various yeast-based industrial processes.

The negative effects of catabolite repression on yeast performance

In an industrial context, one of the best-known adverse effects of the main glucose repression pathway on yeast performance is seen in the production of baker's yeast, during which the switch from respiration to fermentation induced by glucose or sucrose causes a marked drop in biomass yield. Similarly, in the production of biopharmaceuticals, repression of respiration decreases the yield of product [23,24]. Furthermore, the ethanol generated during fermentation is an additional stress factor for the yeast cells and can also negatively affect the stability of the pharmaceutical product. To avoid these effects, yeasts are grown mostly in aerated fed-batch reactors, in which the glucose and sucrose concentrations are maintained constantly below the threshold concentration for inducing the Crabtree effect [23,25].

By contrast, the production of alcoholic beverages and bioethanol requires fermentation. Apart from the first few hours, these fermentations are carried out anaerobically so that, even in the absence of the Crabtree effect, the yeast cells cannot respire. In this set-up, the negative effect of the main glucose repression pathway lies in the repressed uptake and metabolism of other sugars, such as galactose, maltose and maltotriose. The resulting reduced consumption of alternative sugars negatively affects the fermentation rate [26]. Remarkably, catabolite repression is not always relieved immediately once glucose and sucrose are depleted; instead, an initial preincubation in glucose leads to the slower metabolism of other sugars over several hours [21].

Empirical data obtained in various breweries show that extended propagation and cultivation in sucrose often leads to a permanent loss of maltose-fermenting capacity. Similarly, it has been recently shown that several genes encoding maltose transporters and maltase remain repressed when yeast cells are inoculated into maltose-containing medium after a long-term cultivation in glucose-rich medium [27]. This continued repression suggests that sustained favorable growth conditions, such as the availability of glucose, might cause a long-term decrease in the capacity of yeast to metabolize alternative carbon sources.

The negative effects of Ras/cAMP/PKA activation on yeast performance

As mentioned above, in addition to catabolite repression, glucose and sucrose also trigger activation of the Ras/cAMP/PKA pathway, which in turn leads to a decrease in the stress resistance of yeast cells. During industrial processes, yeast cells encounter various severe stress conditions, including shear stress; marked shifts in oxygen levels, temperature, osmolarity and pH; high ethanol and carbon dioxide concentrations; and a lack of nutrients [28,29]. Notably, Brosnan *et al.* [30] and Rossignol *et al.* [31] have reported that industrial yeast strains do not fully activate several stress responses during the first phases of industrial fermentations, which could be due to the presence of glucose and sucrose.

The inability of yeast cells to respond to unfavorable, stressful conditions leads to sluggish fermentations and

cell autolysis [32]. The latter process causes severe off flavors in alcoholic beverages, including a highly unpleasant goat-like aroma caused by the release of fatty acids, and a decrease in fruity aromas owing to the hydrolysis of volatile esters by esterases leaking out of damaged cells [33,34]. Furthermore, sustained high concentrations of glucose cause a decrease in the replicative lifespan of yeast, whereas the use of maltose-rich media generally leads to an increase in cell longevity [35,36]. Other reported effects of high glucose include an increase in the instability of short chromosomes [37] and a strong increase in the production of aroma-active esters through activation of the Ras/cAMP/PKA pathway [38,39]. Although moderate levels of these volatile esters are essential for the desired fruity flavors of beer and wine, high glucose concentrations lead to an undesirable over-production of these compounds [39].

In addition to the Ras/cAMP/PKA, other closely related nutrient signaling pathways, such as the 'target of rapamycin' pathway and the 'fermentable growth medium-induced' pathway can influence the fermentation performance of yeast. In contrast to the short-term response of the Ras/cAMP/PKA pathway, which essentially responds to changes in glucose and sucrose levels, these pathways are thought to integrate the overall nutritional and/or energy status of cells [4,40,41]. This implies that, even in the presence of glucose and sucrose, some cellular stress responses can be activated when other essential nutrients (e.g. nitrogen sources) are depleted, as can occur in the last phase of wine fermentations [42]. Given the limited data, however, it is premature to estimate the importance of these signaling cascades in industrial processes.

Yeast carbon signaling mutants with improved industrial performance

Considering the various adverse effects of carbohydrate signaling on the performance of industrial yeasts, the use of strains with altered responses to glucose and sucrose should lead to a significant improvement in productivity. In addition, mutations in the regulatory systems might provoke 'balanced' changes in the expression of several genes involved in fermentation performance [43,44].

Indirect phenotypic evidence suggests that many of the currently used industrial strains have already acquired altered sugar signaling properties. Some brewer's strains, for example, do not break down trehalose in the presence of glucose [45]. Furthermore, many brewer's strains show constitutive uptake of maltose, independent of the presence of glucose [21]. Interestingly, in most cases glucose-mediated repression of the uptake of other less-preferred sugars, such as galactose, is still present. This indicates that if the alternative sugar-usage patterns are caused by alterations in the glucose-sensing pathways, these alterations are most probably present in a downstream part of the pathway [21]. Taken together, many industrial strains have acquired alternative glucose signaling phenotypes, enabling them to complete their tasks better in industrial processes.

Most of these yeasts are not the result of a specific, intentional selection procedure, but have presumably out-

competed the ancestral parent strain over long periods of time under stressful industrial conditions [46]. Although this natural selection can lead to vast improvements in yeast performance, especially when yeasts are re-used for multiple fermentations, as they are in beer production, further improvements can be achieved by a more direct approach; for example, the use of mutagenesis by ultraviolet irradiation or chemical mutagens such as ethyl methane sulfonate, followed by a screening or selection procedure. Alternatively, superior yeast strains can be developed by genetic engineering. Both methods have their pros and cons, which have been extensively discussed elsewhere [47,48]. Mutagenesis followed by selection does not require any knowledge of target genes or metabolic pathways and is relatively easy to perform. Furthermore, strains acquired through this process are not considered as 'genetically modified', which makes them virtually ready to use; however, a good selection procedure is required to facilitate rapid isolation of the few useful mutants from billions of 'uninteresting' cells. This selection requirement severely limits the use of this method for the isolation of yeasts with specific fermentation characteristics.

Many of the stress responses are not specific for particular stress conditions [13,16]; thus, a potential strategy is the selection of mutants that are more resistant to lethal stresses, such as heat or ethanol stress, in the presence of glucose or sucrose. After this first selection, the isolated mutants can be screened for improved fermentation properties under industrial stress conditions. In our experience, approaches in which the actual stressful fermentation conditions are mimicked as closely as possible greatly increase the chance of success [49]. Ideally, all screens should be therefore done in pilot-scale fermenters under growth conditions and medium similar to those used in the industrial-scale process. Even so, the mutant that performs best in pilot-scale trials is often not the best strain for full-scale production. Thus, it is vital to select several mutants for production-scale trials.

Recent progress in our understanding of the yeast carbohydrate signaling pathways makes it possible to improve strains by genetic engineering, leading to the introduction of such traits as increased trehalose levels and constitutive expression of targeted carbohydrate transporters [48,50,51]. Brewers and wine producers are reluctant to use genetically modified yeasts, however, mainly because of the complex legislation and the negative public perception. Furthermore, drastic changes in the vital carbon signaling pathways frequently affect other commercially important properties.

The mutations found in superior strains selected after random mutagenesis are often more subtle and can lead to more balanced performances [49,52]. Moreover, techniques similar to genome shuffling in bacteria [53] provide an efficient way with which to combine different subtle improvements. Thus, although genetic engineering and self-cloning are certainly the most promising methods for the future, random mutagenesis and selection remain the most realistic method with which to obtain improved yeast strains for short-term introduction into industrial food production.

These arguments do not apply to the production of heterologous proteins and biopharmaceuticals, in which the use of genetically modified organisms is less controversial. Moreover, the growth medium often contains only a single carbon source in these processes, and thus the metabolic and regulatory complications associated with complex sugar mixtures are avoided. A good example, developed and patented by Boles *et al.* [54], is a strain that has been genetically modified to express a chimeric hexose transporter. This strain is relieved of the Crabtree effect, and thus there is less need for sophisticated cultivation techniques to reach optimal production rates. Another interesting strain has been developed by Ostergaard *et al.* [43], who have used genetic engineering of the *GAL* regulatory system to increase galactose metabolism.

Selecting interesting carbon signaling mutants: a practical example

Relatively few carbon signaling mutants of industrial yeast strains have been described or investigated, in part, owing to the polyploid or aneuploid nature of these strains, which makes their genetic analysis much more difficult as compared with haploid laboratory strains. Perhaps the best examples of carbon signaling mutants are the so-called 'fermentation-induced loss of stress resistance' (*fil*) mutants of industrial baker's yeast, which show improved resistance to freezing and thawing in the presence of glucose and sucrose.

Freeze-thaw resistance is required for the increasingly popular use of frozen dough, which permits the separation of dough production and baking. Before freezing, yeast is mixed with the dough. Because of the high glucose and/or sucrose concentrations in the dough, however, yeast cells rapidly activate the Ras/cAMP/PKA pathway and lose their stress resistance, resulting in considerable loss of vitality during freezing and thawing [49,55]. By irradiating baker's yeast with ultraviolet, preparing dough with the mutants and subsequently subjecting the dough to numerous freeze-thaw cycles, it has been possible to isolate several mutants with improved freeze tolerance, although only a few strains retain all of the other desirable characteristics of industrial baker's yeast and perform superiorly in full-scale processes of dough freezing and thawing [49,56]. The exact mutations in these industrial strains are not known.

fil mutants have been also isolated from laboratory yeast strains, and two of these mutations have been identified. One strain contains a mutation in the *GPR1* gene, which encodes a G-protein-coupled receptor involved in glucose and sucrose sensing [57] (Figure 2). The other strain contains a partially inactivating mutation in the *CYR1* gene encoding adenylate cyclase, which is also an essential component of the Ras/cAMP/PKA pathway [44,52]. These mutations in Ras/cAMP/PKA signaling prevent the strains from losing their stress resistance in glucose and sucrose media. Remarkably, some of the laboratory *fil* strains were selected for heat-resistance in glucose medium, but the superior mutants also show improved resistance to various other stresses [44,52].

A healthy diet for optimal performance?

The selection or creation of superior yeast strains is not the only way to improve the productivity of industrial yeasts. Relatively simple changes in yeast handling can also lead to significant improvements. Considering the many adverse effects of glucose and sucrose on yeast performance, it is advisable to avoid using high concentrations of these sugars. In many applications, the composition of the fermentation medium cannot be changed without affecting product quality and cost, but sometimes it is possible to use alternative carbon sources. For example, when high-carbon syrups are added to the medium, as is often done in high-gravity beer fermentation or the production of bioethanol or biopharmaceuticals, it might be better to use maltose-rich syrups instead of sucrose-containing syrups.

Furthermore, when yeasts are propagated for industrial use, it is beneficial to combine glucose or sucrose with alternative sugars, such as maltose syrups, or with non-fermentable carbon sources, such as mannitol or sorbitol [58]. The preferred sugars will be used first, facilitating maximal biomass production rates, whereas the maltose or mannitol will be metabolized just before the production phase or storage of the yeast cells. Avoiding contact with glucose and sucrose just before the fermentation process might lead to increased levels of stored carbohydrates, better stress resistance and a faster fermentation rate. In the specific case of bioethanol production, it has proved advantageous to remove sugar monomers continuously from the saccharification vessel to maximize the breakdown and use of more complex sugars [46].

Conclusion

In the constant strive for better and more efficient yeast-based industrial processes, much attention has been drawn to various high-tech solutions ranging from optimization of bioreactors to the refinement of genetic technologies. The recently unraveled negative effects of glucose and sucrose on the industrial performance of yeast are often overlooked, and these two sugars remain among the most commonly used carbon sources in yeast media.

In general, the presence of these sugars at moderate concentrations is inevitable and will not cause significant problems. In some applications, however, high concentrations of glucose or sucrose at a particular stage of the industrial process cause a significant and long-term decrease in fermentation performance. Thus, the use of sugar signaling mutants or the reduction of glucose and sucrose might be advantageous in various bioindustries, ranging from the production of alcoholic beverages and bread to the fabrication of biopharmaceuticals. Indeed, although yeast cells seem to prefer glucose and sucrose over other carbohydrates, offering them too much of this 'fast-food' negatively affects their general fitness, again demonstrating the striking analogy between yeasts and higher eukaryotes.

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References

- 1 Gancedo, J.M. (1998) Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* 62, 334–361
- 2 Johnston, M. (1999) Feasting, fasting and fermenting – glucose sensing in yeast and other cells. *Trends Genet.* 15, 29–33
- 3 Carlson, M. (1999) Glucose repression in yeast. *Curr. Opin. Microbiol.* 2, 202–207
- 4 Winderickx, J. et al. (2003) From feast to famine: adaptation to nutrient availability in yeast. In *Topics in Current Genetics, Vol. 1: Yeast Stress Responses* (Hohmann, S. and Mager, P.W.H., eds), pp. 305–386, Springer-Verlag
- 5 Berthels, N.J. et al. (2004) Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. *FEMS Yeast Res.* 4, 683–689
- 6 de Sousa, H.R. et al. (2004) Differential regulation by glucose and fructose of a gene encoding a specific fructose/H⁺ symporter in *Saccharomyces cerevisiae*. *Yeast* 21, 519–530
- 7 Goncalves, P. et al. (2000) FSY1, a novel gene encoding a specific fructose/H⁺ symporter in the type strain of *Saccharomyces carlsbergensis*. *J. Bacteriol.* 182, 5628–5630
- 8 Thevelein, J.M. and De Winde, J.H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* 33, 904–918
- 9 Verselé, M. et al. (2001) Sex and sugar in yeast: two distinct GPCR systems. *EMBO Rep.* 2, 574–579
- 10 Rolland, F. et al. (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. *Mol. Microbiol.* 38, 348–358
- 11 Rolland, F. et al. (2001) The role of hexose transport and phosphorylation in cAMP signalling in the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 1, 34–45
- 12 Varela, J.C.S. et al. (1995) The *Saccharomyces cerevisiae* HSP12 gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A. *Mol. Cell. Biol.* 15, 6232–6245
- 13 Marchler, G. et al. (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* 12, 1997–2003
- 14 Piper, P.W. et al. (1997) Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H⁺-ATPase. *Cell Stress Chaperones* 2, 12–24
- 15 Sanchez, Y. et al. (1992) HSP104 is required for tolerance to many forms of stress. *EMBO J.* 11, 2357–2364
- 16 Piper, P.W. (1995) The heat-shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol. Lett.* 134, 121–127
- 17 Winderickx, J. et al. (1996) Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? *Mol. Gen. Genet.* 252, 470–482
- 18 Wiemken, A. (1990) Trehalose in yeast: stress protectant rather than reserve carbohydrate. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* 58, 209–217
- 19 Yoon, S-H. et al. (2003) Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydr. Res.* 338, 1127–1132
- 20 Bamforth, C.W. (2003) Wort composition and beer quality. In *Brewing Yeast Fermentation Performance* (Vol. 2) (Smart, K. ed.), pp. 77–85, Blackwell Science
- 21 Meneses, J.F. et al. (2002) A survey of industrial strains of *Saccharomyces cerevisiae* reveals numerous altered patterns of maltose and sucrose utilisation. *J. Inst. Brew.* 108, 310–321
- 22 Meijer-Michelle, M.C. et al. (1998) Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. *J. Biol. Chem.* 273, 24102–24107
- 23 Calado, C.R.C. et al. (2003) Development of a fed-batch cultivation strategy for the enhanced production and secretion of cutinase by recombinant *Saccharomyces cerevisiae* SU50 strain. *J. Biosci. Bioeng.* 96, 141–148
- 24 Ejiofor, A.O. et al. (1994) A robust fed-batch feeding strategy for optimal parameter estimation for baker's yeast production. *Bioprocess Eng.* 11, 135–144
- 25 Valentiniotti, S. et al. (2003) Optimal operation of fed-batch fermentations via adaptive control of overflow metabolite. *Control Eng. Pract.* 11, 665–674
- 26 Shimizu, H. et al. (2002) Effect of carbon and nitrogen additions on consumption activity of apparent extract of yeast cells in a brewing process. *J. Am. Soc. Brew. Chem.* 60, 163–169
- 27 Kuthan, M. et al. (2003) Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology. *Mol. Microbiol.* 47, 745–754
- 28 Attfield, P.V. (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. *Nat. Biotechnol.* 15, 1351–1357
- 29 Bauer, F.F. and Pretorius, I.S. (2000) Yeast stress response and fermentation efficiency: how to survive the making of wine – a review. *S. Afr. J. Enol. Vitic.* 21, 27–51
- 30 Brosnan, M.P. et al. (2000) The stress response is repressed during fermentation of brewery strains of yeast. *J. Appl. Microbiol.* 88, 746–755
- 31 Rossignol, T. et al. (2003) Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* 20, 1369–1385
- 32 Ivorra, C. et al. (1999) An inverse correlation between stress resistance and stuck fermentations in yeast. A molecular study. *Biotechnol. Bioeng.* 64, 698–708
- 33 Neven, H. et al. (1997) Flavor evolution of top fermented beers. *MBAA Tech. Quart.* 34, 115–118
- 34 Taylor, G.T. and Kirsop, B.H. (1977) The origin of medium chain length fatty acids present in beer. *J. Inst. Brew.* 83, 241–243
- 35 Maskell, D.L. et al. (2001) Impact of carbohydrate composition of media on lager yeast replicative lifespan. *J. Am. Soc. Brew. Chem.* 59, 111–116
- 36 Lin, S.J. et al. (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* 418, 344–348
- 37 Sato, M. et al. (2002) Effect of growth media and strains on structural stability in small chromosomes (chromosomes I, VI and III) of bottom fermenting yeast. *J. Inst. Brew.* 108, 283–285
- 38 Verstrepen, K.J. et al. (2003) Flavour-active esters: adding fruitiness to beer. *J. Biosci. Bioeng.* 96, 110–118
- 39 Verstrepen, K.J. et al. (2003) The *Saccharomyces cerevisiae* alcohol acetyl transferase gene *ATF1* is a target of the cAMP/PKA and FGM nutrient signalling pathways. *FEMS Yeast Res.* 4, 285–296
- 40 Crauwels, M. et al. (1997) The Sch9 protein kinase in the yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for activation of the fermentable-growth-medium-induced (FGM) pathway. *Microbiol.* 143, 2627–2637
- 41 Pedruzzoli, I. et al. (2003) TOR and PKA signalling pathways converge on the protein kinase Rim15 to control entry into G0. *Mol. Cell* 12, 1–20
- 42 Puig, S. and Pérez-Ortin, J.E. (2000) Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. *Yeast* 16, 139–148
- 43 Ostergaard, S. et al. (2000) Increasing galactose consumption by *Saccharomyces cerevisiae* through metabolic engineering of the *GAL* gene regulatory network. *Nat. Biotechnol.* 18, 1283–1286
- 44 Verstele, M. et al. (2004) The high general stress resistance of the *Saccharomyces cerevisiae* *fil1* adenylate cyclase mutant (Cyr1^{Lys1682}) is only partially dependent on trehalose, Hsp104 and overexpression of Msn2/4-regulated genes. *Yeast* 21, 75–86
- 45 Reinman, M. and Londesborough, J. (2000) Rapid mobilization of intracellular trehalose by fermentable sugars: a comparison of different strains. In *Brewing Yeast Fermentation Performance* (Vol. 1, 1st edn) (Smart, K. ed.), Blackwell Science
- 46 Wheals, A.E. et al. (1999) Fuel ethanol after 25 years. *Trends Biotechnol.* 17, 482–487

- 47 Pretorius, I.S. (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729
- 48 Dequin, S. (2001) The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Appl. Microbiol. Biotechnol.* 56, 577–588
- 49 Teunissen, A. et al. (2002) Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. *Appl. Environ. Microbiol.* 68, 4780–4787
- 50 Pretorius, I.S. and Bauer, F.F. (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends Biotechnol.* 20, 426–432
- 51 Verstrepen, K.J. et al. (2001) Genetic modification of *Saccharomyces cerevisiae*: fitting the modern brewer's needs. *Cerevisia* 26, 89–97
- 52 Van Dijck, P. et al. (2000) A baker's yeast mutant (*fil1*) with a specific, partially inactivating mutation in adenylate cyclase maintains a high stress resistance during active fermentation and growth. *J. Mol. Microbiol. Biotechnol.* 2, 521–530
- 53 Patnaik, R. et al. (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nat. Biotechnol.* 20, 707–712
- 54 Boles, E. et al. Recombinant *Saccharomyces cerevisiae* expressing chimeric glucose transporters. Patent WO200880. Gothia Yeast Solutions AB (2002)
- 55 Park, J.I. et al. (1997) The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal transduction pathway. *Appl. Environ. Microbiol.* 63, 3818–3824
- 56 Dumortier, F. et al. (1999) New strains 'fil', stress-resistant under fermentation and/or growth conditions. Patent EP0967280. Lesaffre & Cie, France
- 57 Kraakman, L. et al. (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* 32, 1002–1012
- 58 Quain, D.E. and Boulton, B. (1987) Growth and metabolism of mannitol by strains of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* 133, 1675–1684
- 59 Wilson, W.A. et al. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* 6, 1426–1434
- 60 Ludin, K. et al. (1998) Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* 95, 6245–6250
- 61 Sanz, P. et al. (2000) Sip5 interacts with both the Reg1/Glc7 protein phosphatase and the Snf1 protein kinase of *Saccharomyces cerevisiae*. *Genetics* 154, 99–107
- 62 De Vit, M.J. et al. (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Mol. Biol. Cell* 8, 1603–1618
- 63 Östling, J. and Ronne, H. (1998) Negative control of the Mig1 repressor by Snf1-dependent phosphorylation in the absence of glucose. *Eur. J. Biochem.* 252, 162–168
- 64 Colombo, S. et al. (1998) Involvement of distinct G-proteins Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J.* 17, 3326–3341
- 65 Toda, T. et al. (1987) Cloning and characterization of Bcy1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein-kinase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7, 1871–1877
- 66 Toda, T. et al. (1987) 3 different genes in *Saccharomyces cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* 50, 277–287
- 67 Broach, J. et al. (1985) Ras proteins function exclusively to modulate adenylate cyclase activity in the yeast *Saccharomyces* DNA. *J. Mol. Cell. Biol.* 4, 64
- 68 Toda, T. et al. (1985) In yeast, Ras proteins are controlling elements of adenylate cyclase. *Cell* 40, 27–36
- 69 Griffioen, G. and Thevelein, J.M. (2002) Molecular mechanisms controlling the localisation of protein kinase A. *Curr. Genet.* 41, 199–207

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