

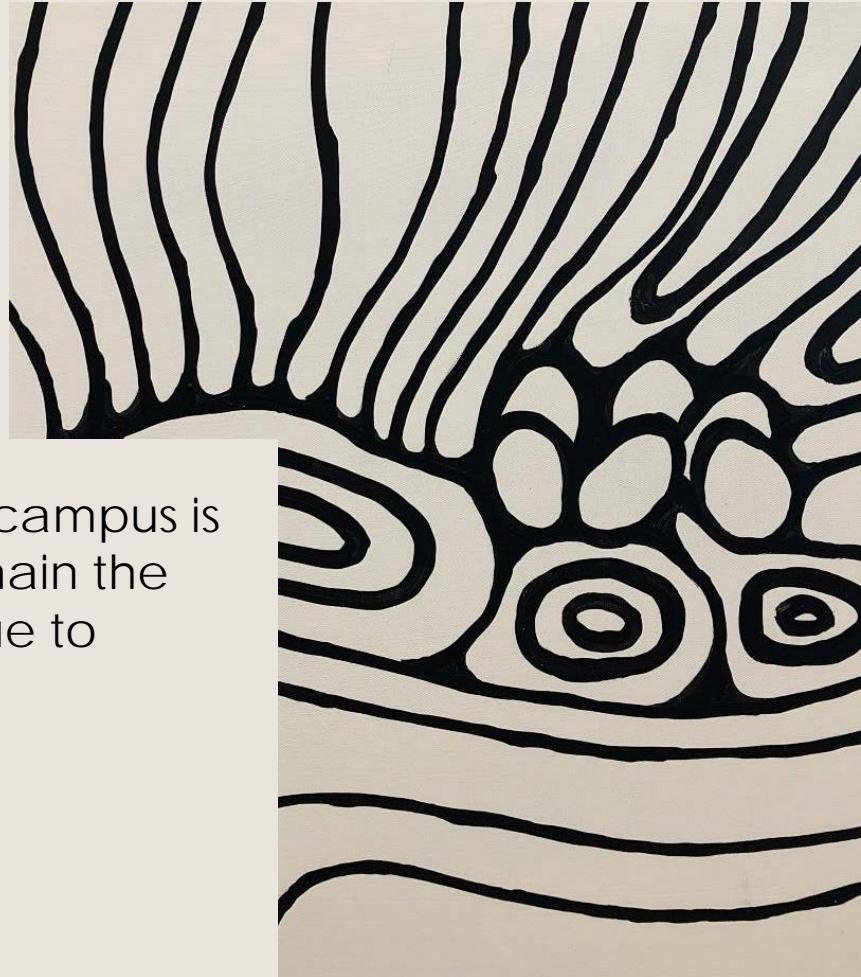
Engineering Microbial Cells as Chemical Factories I (a)

From Chassis organisms to metabolic pathway engineering strategies



Acknowledgement of country

The University of Western Australia acknowledges that its campus is situated on Noongar land, and that Noongar people remain the spiritual and cultural custodians of their land, and continue to practise their values, languages, beliefs and knowledge.



Traditional of Human use of Microorganisms



bread/sourdough



yogurt / villi / dahi



cheese



tempeh/natto



alcoholic beverages



vinegar/cider/kombucha



kimchi/sauerkraut



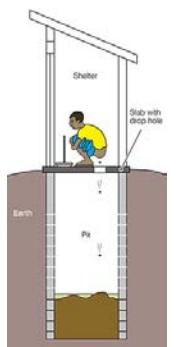
Traditional medicine



Textile (Retting e.g. flax, hemp)



Composting/sewage treatment





The Advent of Biotechnology (Synthetic Biology): *E. coli* synthesized Insulin

In 1978, Genentech scientist Dennis Kleid toured a factory in Indiana where insulin was made from pigs and cattle.

"There was a line of train cars filled with frozen pancreases," he says. At the time, it took 8,000 pounds of pancreas glands from 23,500 animals to make one pound of insulin.

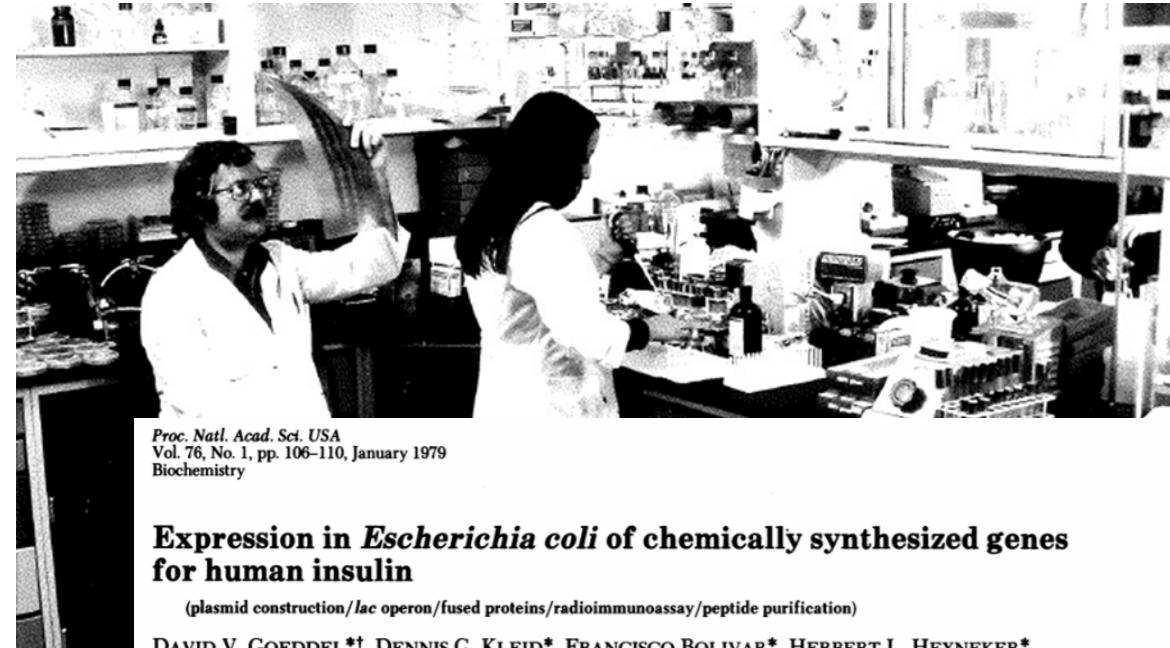
Alternative: Synthetic human insulin from *E. coli*

Swanson told Kleid "I don't want to hear that word, impossible. Tell me what you need to get it done."

"Dave was the early person, and I was the night person," Kleid says. They hardly slept, ate, or saw their families. "We kept the experiment going 24 hours a day."

Finally, in the early hours of August 21, 1978, Goeddel succeeded in reconstituting the two amino acid chains into one molecule: human insulin.

In 1982, the FDA approved human insulin and it was on the market by 1983. Since then, millions of people have used the medicine, and it has almost completely replaced insulin created from animals.



Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 106-110, January 1979
Biochemistry

Expression in *Escherichia coli* of chemically synthesized genes for human insulin

(plasmid construction/*lac* operon/fused proteins/radioimmunoassay/peptide purification)

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*Division of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080; and ‡Division of Biology, City of Hope National Medical Center, Duarte, California 91010

Communicated by Ernest Beutler, October 3, 1978

ABSTRACT Synthetic genes for human insulin A and B chains were cloned separately in plasmid pBR322. The cloned synthetic genes were then fused to an *Escherichia coli* β -galactosidase gene to provide efficient transcription and translation and a stable precursor protein. The insulin peptides were cleaved from β -galactosidase, detected by radioimmunoassay, and purified. Complete purification of the A chain and partial purification of the B chain were achieved. These products were mixed, reduced, and reoxidized. The presence of insulin was detected by radioimmunoassay.

Enzymes and DNA Preparations. T4 DNA ligase and T4 polynucleotide kinase were purified as described (6). Restriction endonuclease EcoRI was purified by the procedure of Greene *et al.* (7). HindIII was purified by a method developed by D. Goeddel (unpublished). Restriction endonuclease BamHI was purchased from Bethesda Research (Rockville, MD); *E. coli* alkaline phosphatase was purchased from Worthington.

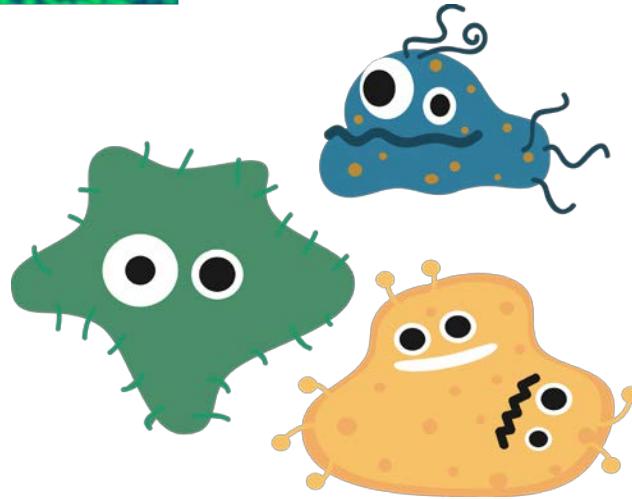
Plasmids, including pBR322 (8), were isolated by a published procedure (9) with some modifications. The chemical synthesis

<https://www.gene.com/stories/cloning-insulin>





Microbial Cell Factories



Microbial cells



DEGRADATION

- conversion of polymers to energy-rich smaller molecules
- bioremediation



SYNTHESIS and BIOTRANSFORMATION

- biomass
- biofuels
- value added biochemicals

OPERATION

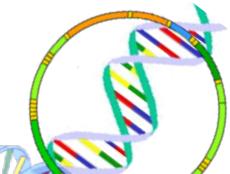
- biosensor
- killing target cells



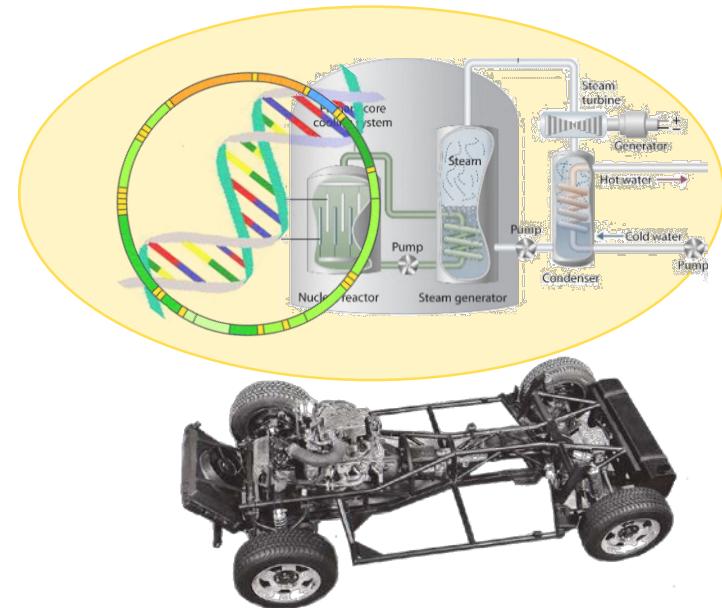
Design construct



Synthesize/assembly construct

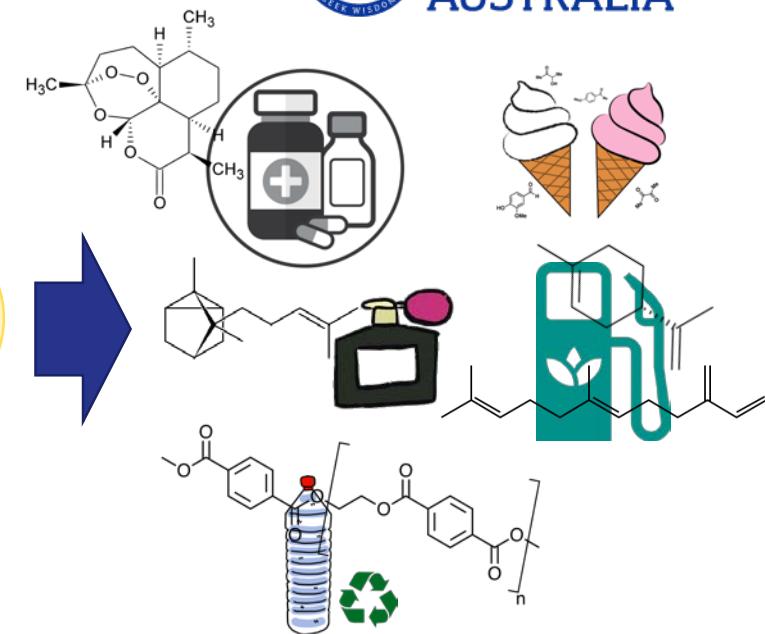


Chassis cell



Our ability to read, copy n' paste,
edit, and rewrite genomes of organism
has changed the way we can manipulate
microbial cells

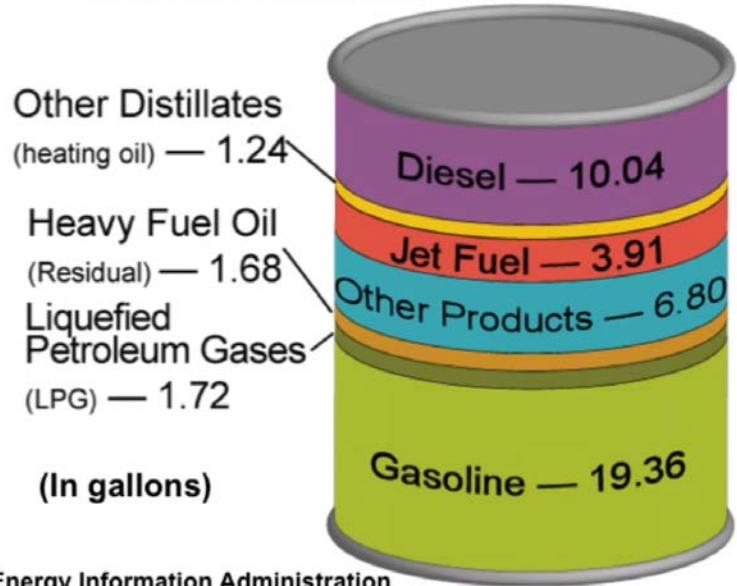
Chemicals/Products



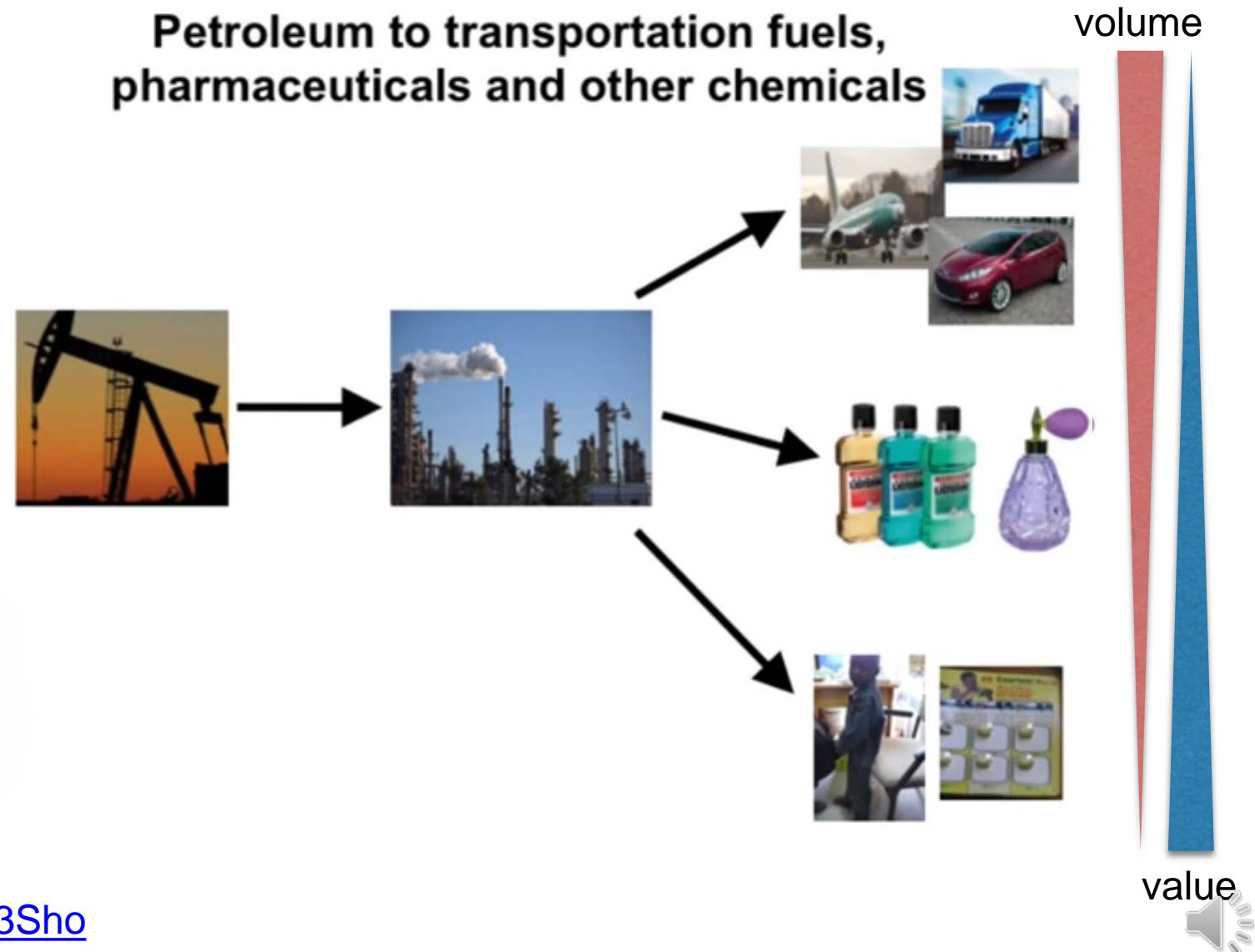


Engineering Microbes to Solve Global Challenges

15% of a barrel of oil produces the many non-fuel chemicals we use

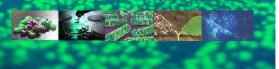


Petroleum to transportation fuels, pharmaceuticals and other chemicals



Watch: iBiology lecture by Jay Keasling

<https://www.youtube.com/watch?v=N0iOB0a3Sho>



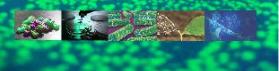
Some products derived from petroleum



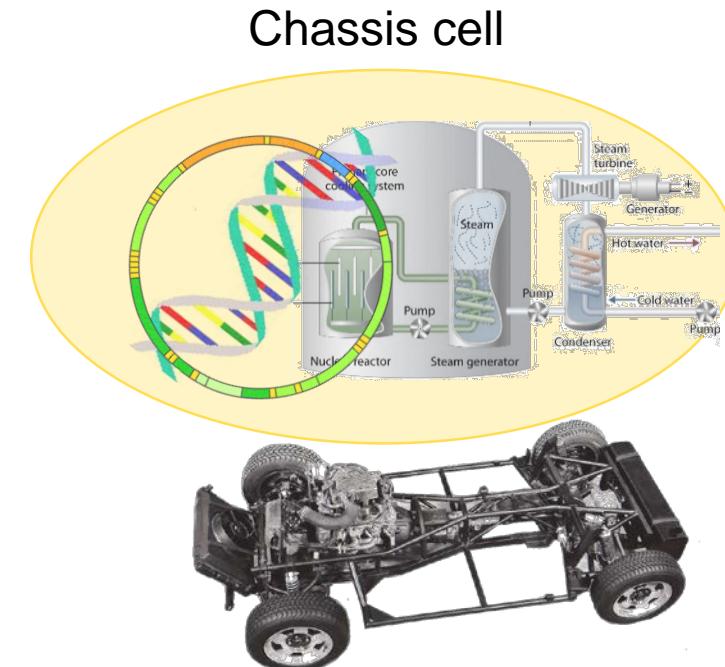
Tents	Heart Valves	Perfumes	Cassettes	Dresses
Crayons	Candles	TV Cabinets	Dishwasher parts	Tires
Parachutes	Trash Bags	Shag Rugs	Tool Boxes	Golf Bags
Telephones	House Paint	Electrician's Tape	Shoe Polish	Percolators
Enamel	Water Pipes	Tool Racks	Motorcycle Helmet	Life Jackets
Pillows	Hand Lotion	Car Battery Cases	Caulking	Rubbing Alcohol
Dishes	Roller Skates	Epoxy	Petroleum Jelly	Tennis Rackets
Cameras	Surf Boards	Paint	Transparent Tape	Rubber Cement
Anesthetics	Shampoo	Mops	CD Player	Fishing Boots
Artificial Turf	Wheels		Faucet Washers	Vaporizers
Artificial limbs	Paint Rollers		Antiseptics	Balloons
Bandages	Shower Curtain		Clothesline	Sun Glasses
Dentures	Guitar Strings		Curtains	Solvents
Model Cars	Luggage		Food Preservatives	Diesel fuel
Folding Doors	Aspirin		Basketballs	Motor Oil
Hair Curlers	Safety Glasses		Soap	Bearing Grease
Cold cream	Antifreeze		Vitamin Capsules	Ink
Movie film	Football Helmets		Antihistamines	Floor Wax
Soft Contact lenses	Awnings		Purses	Ballpoint Pens
Drinking Cups	Eyeglasses		Shoes	Football Cleats
Fan Belts	Clothes		Dashboards	Upholstery
Car Enamel	Toothbrushes		Cortisone	Sweaters
Shaving Cream	Ice Chests		Deodorant	Boats
Ammonia	Footballs		Footballs	Insecticides
Refrigerators	Combs		Putty	Bicycle Tires
Golf Balls	CD's & DVD's		Dyes	Sports Car Bodies
Toothpaste	Paint Brushes		Panty Hose	Nail Polish
Gasoline	Detergents		Glycerin	Fishing lures



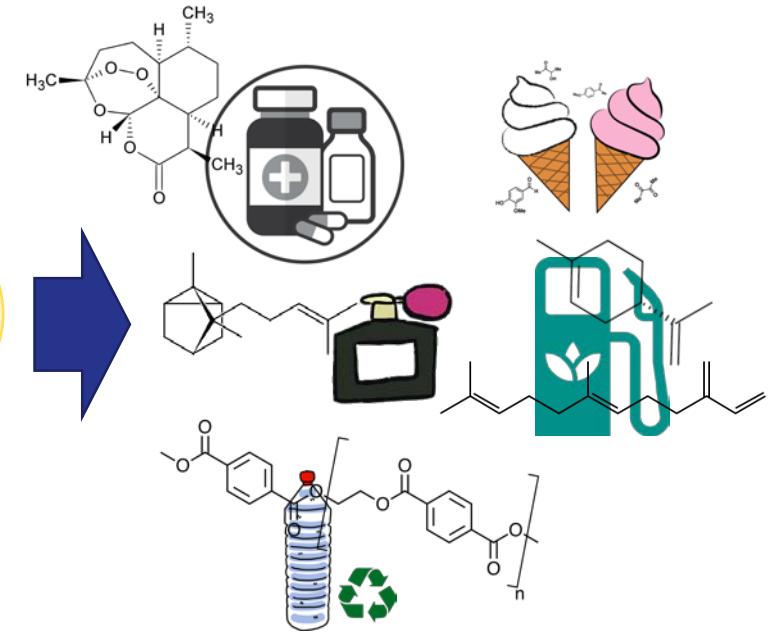
Can we replace these with plant-derived microbial-processed products?



Design construct

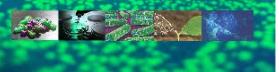
Synthesize/assembly
construct

Chassis cell



Chemicals/Products

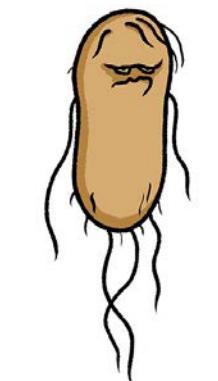




Microbial Chassis cell

Chassis cell - a platform cell for the production of a variety of chemicals or enzymes by integrating corresponding synthetic biology modules into the cell.

Common chassis cell often started from model organisms or organisms known to be GRAS (Generally regarded as safe).



gram -ve

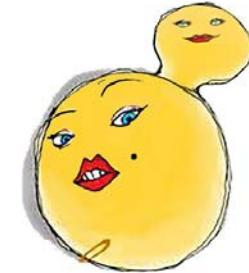
E. coli
P. aeruginosa
V. natriegens



gram +ve

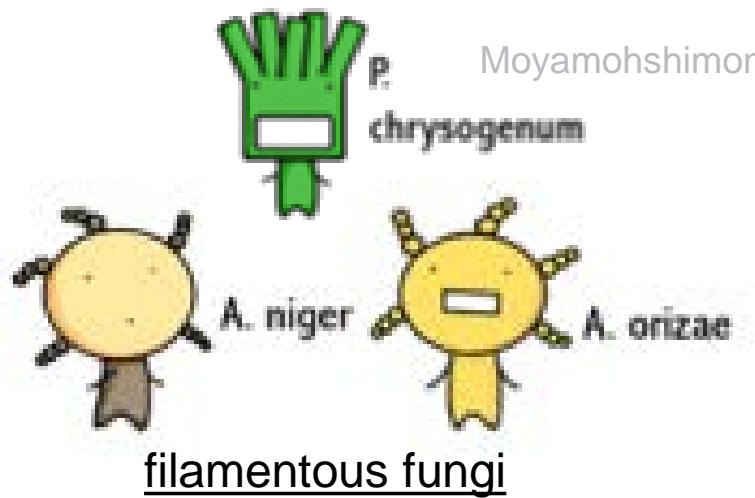
B. subtilis
C. glutamicum
C. acetobutylicum
Streptomyces sp.

2016.igem.org



yeast

S. cerevisiae
S. pombe
Y. lipolytica
P. pastoris



filamentous fungi

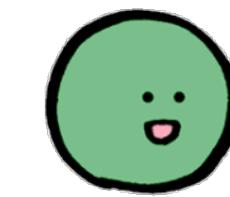
Aspergillus sp.
Penicillium sp.

Moyamohshimon

P. chrysogenum

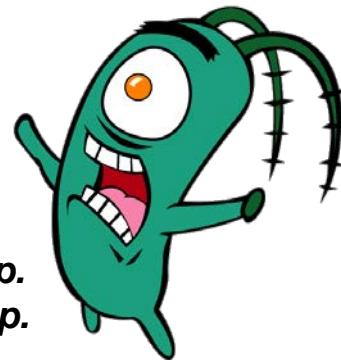
A. niger

A. orizae



cyanobacteria
Synechocystis sp.
Synechococcus sp.

Spongebob



algae
Chlamydomonas sp.

Different advantages and disadvantages





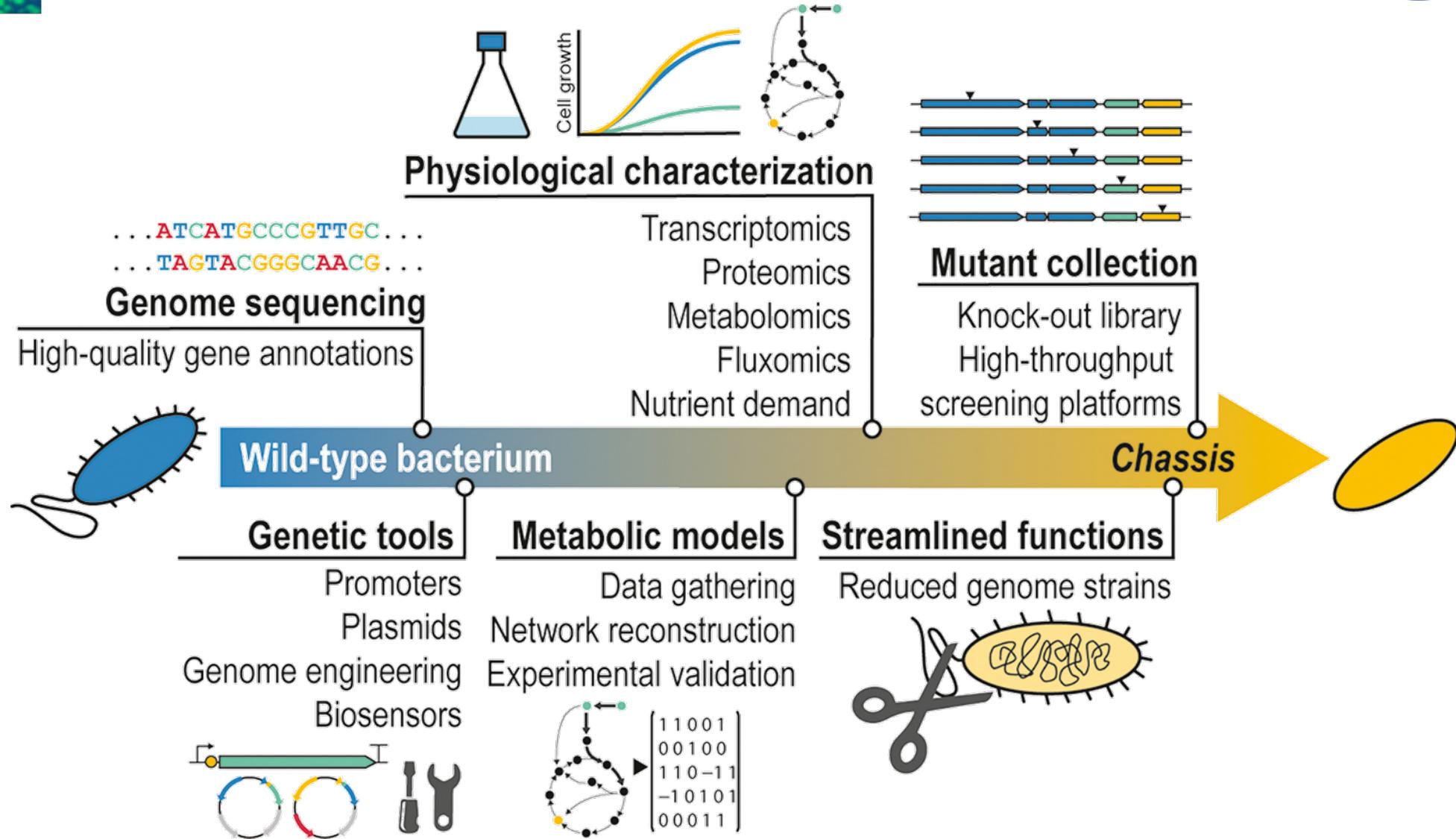
Criteria for Choosing a Chassis Organism

Similar to criteria for choosing a model organism

- Reproduction is rapid
- Many progeny are produced
- Asexual reproduction simplifies the isolation of genetically pure strains
- Growth in the laboratory is easy and requires minimal infrastructures or space
- Genome are relatively small (easy to model)
- Techniques are available for isolating and manipulating their genes
- They can be genetically engineered to produce substance of commercial value
 - Further selected based on the type of pathways or products
 - Matching with natural talent (e.g. high flux of certain pathway) and environment it thrives in (e.g. solvent or heat tolerant)
 - Origin of the genes to build the pathway (e.g. some enzymes need compartmentalisation or post-translational modifications)

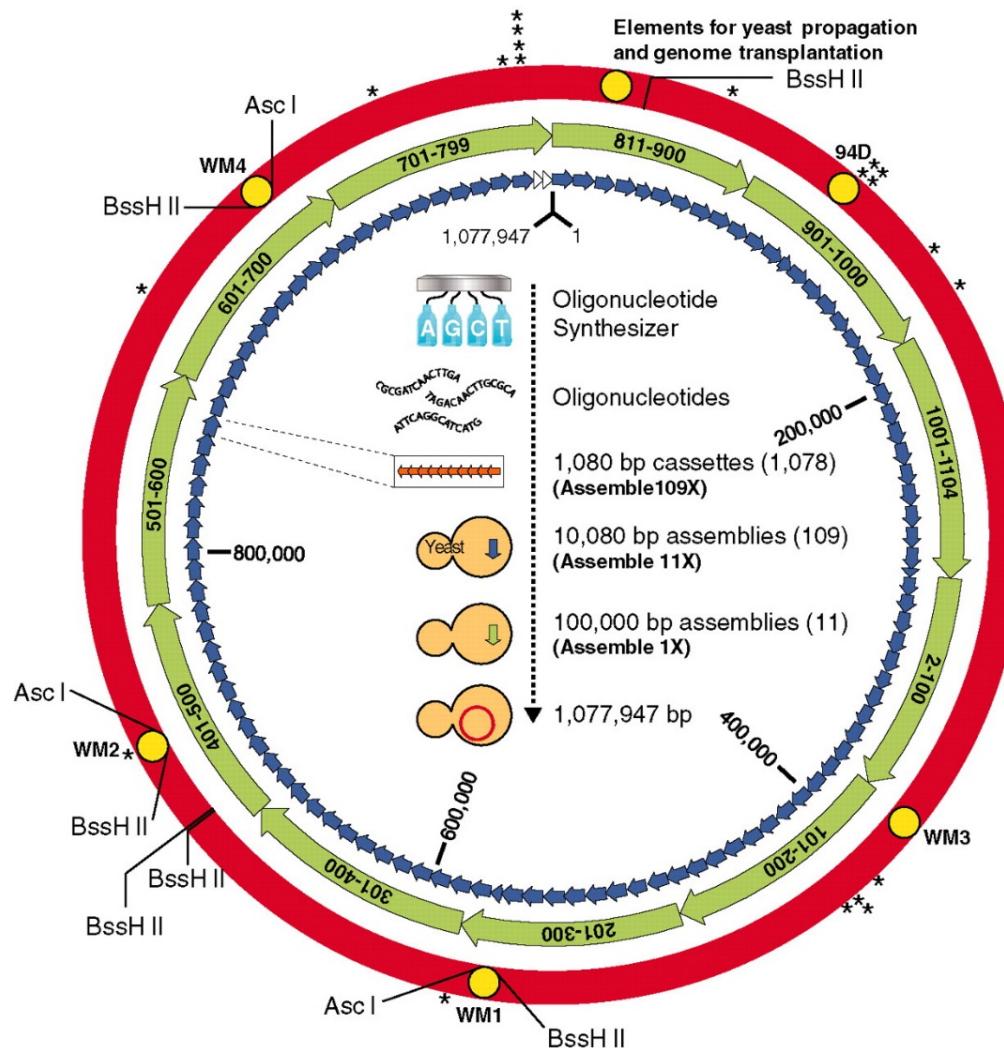


From Wild Organism to Chassis



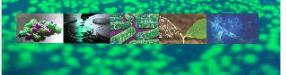


Synthetic Chassis cells



- A fully synthetic microbial cell
- 1080bp cassettes synthesised based on *Mycoplasma mycoides* genomic data (orange), 80 bp overlap between cassettes. Each cassette flanked by *NotI* restriction sites.
- Combined by Gibson cloning or yeast TAR into 10kb cassettes (blue). Screening done in *E. coli*.
- Clones from picked from *E. coli* screen combined in yeast into 100kb cassettes (green). DNA product size screening had to be done.
- These cassettes combined for final DNA, which was transplanted into *M. capricolum* shell (cell with chromosome removed).





Lessons from creating a synthetic minimal cell

Shrink that genome

Syn 3.0 has the fewest genes of any known free-living organism.

Human	~20,000–25,000
<i>Escherichia coli</i> (K12 strain)	~4500
Syn 1.0	901
<i>Mycoplasma genitalium</i> *	525
Syn 3.0	473

*previous fewest

<https://www.sciencemag.org/news/2016/03/synthetic-microbe-lives-fewer-500-genes>

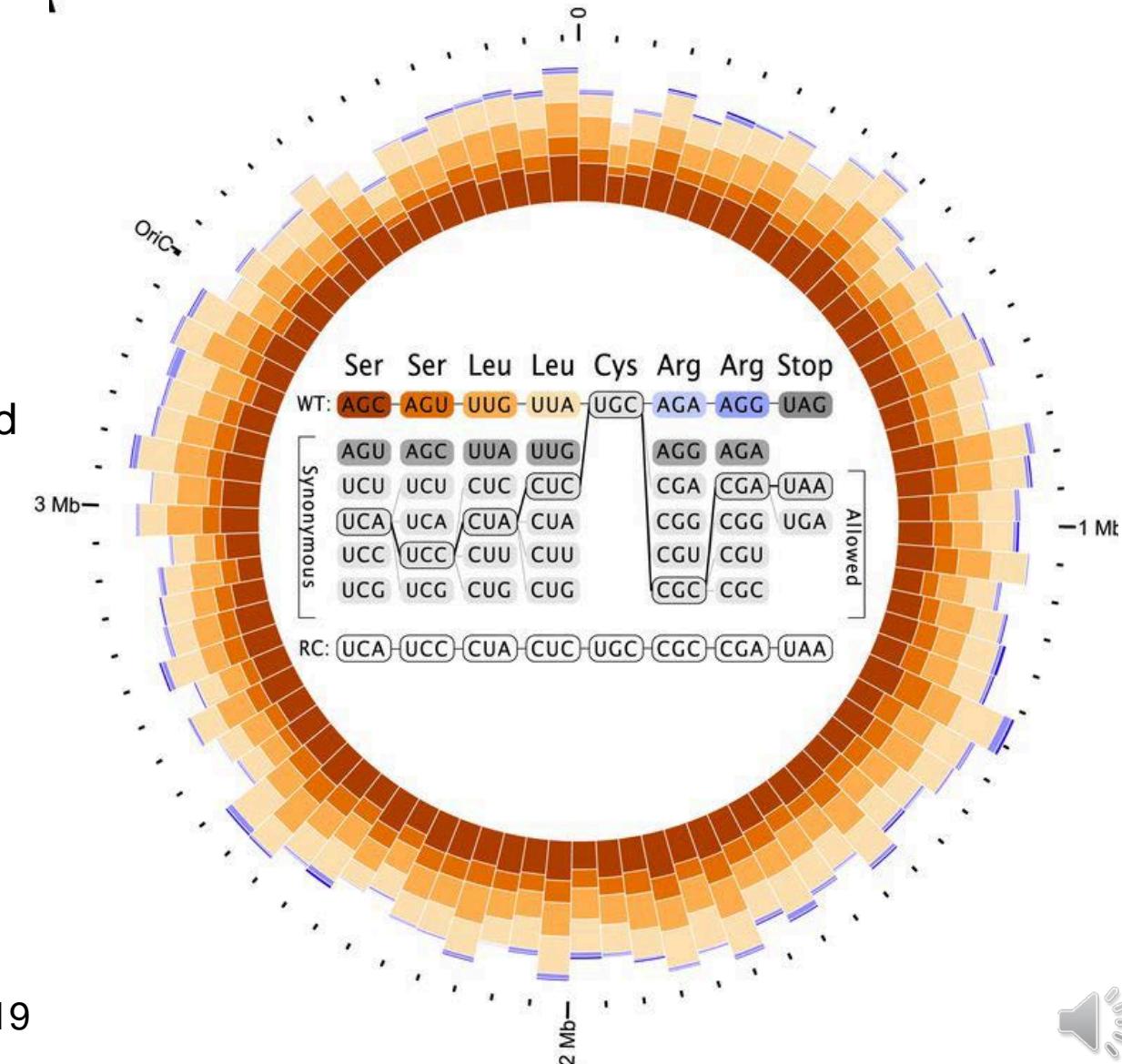
*Richard Feynman,
'what I cannot create, I do
not understand'*

- JCVI-syn3.0 in Hutchinson et al. *Science* (2016) attempt to create a synthetic *Mycoplasma* with minimal genome
 - removed genes responsible for the production of nutrients that could be provided externally, and other “unnecessary” DNA sequences
 - essential (375) genes determined by single gene knockouts → failed!
 - developed a ‘design-build-and-test’ cycle; broke genome into eight DNA segments and mixed and matched; see which combinations produced viable cells; lessons learned from each cycle informed next
 - This process highlighted DNA sequences that do not encode proteins but that are still needed because they direct the expression of essential genes, as well as pairs of genes that perform the same essential task
 - Eventually → a 531,000-base, 473-gene synthetic genome
 - *M. genitalium* takes weeks to double Syn 3.0 takes 3 hrs (*E. coli* 20 min)

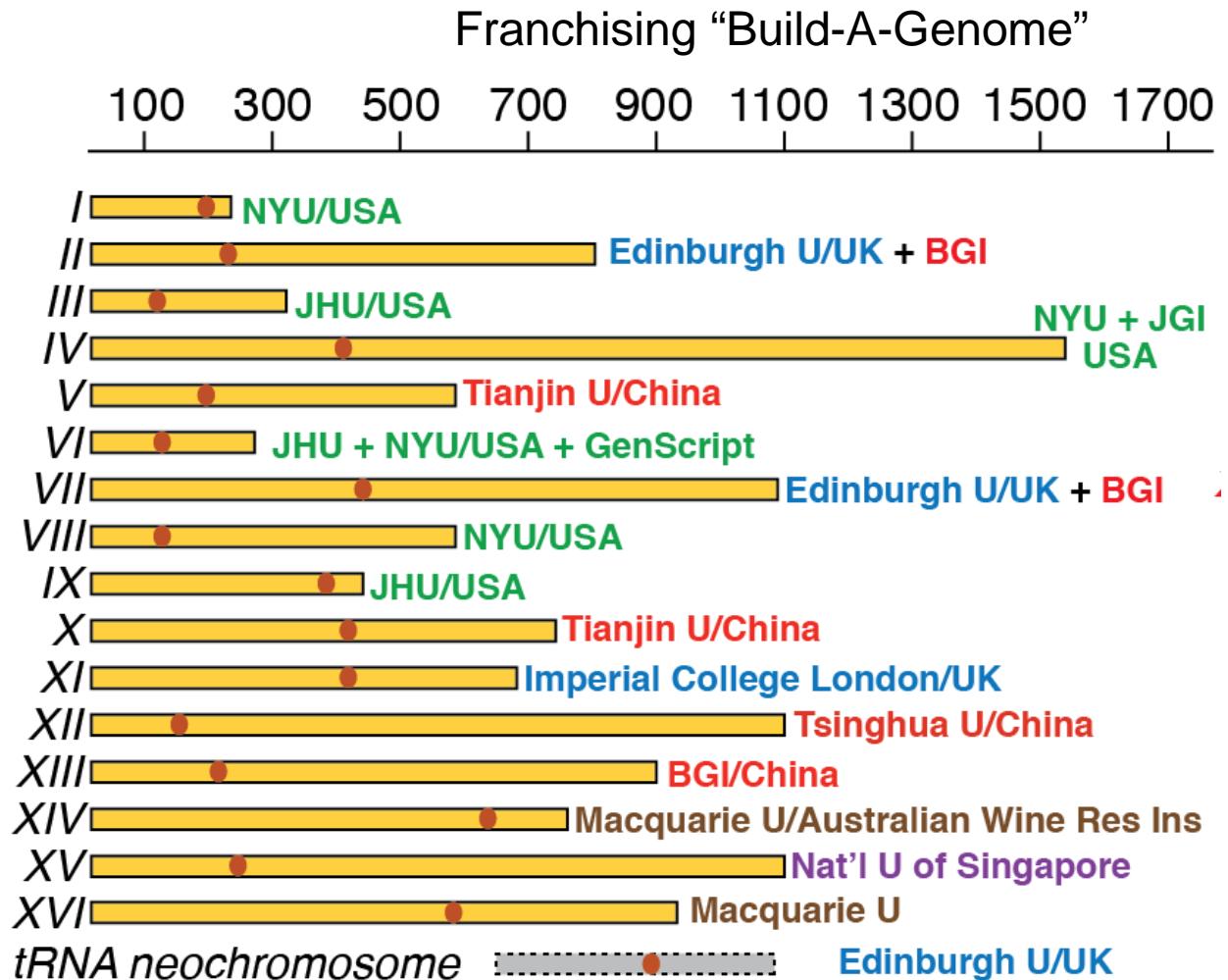
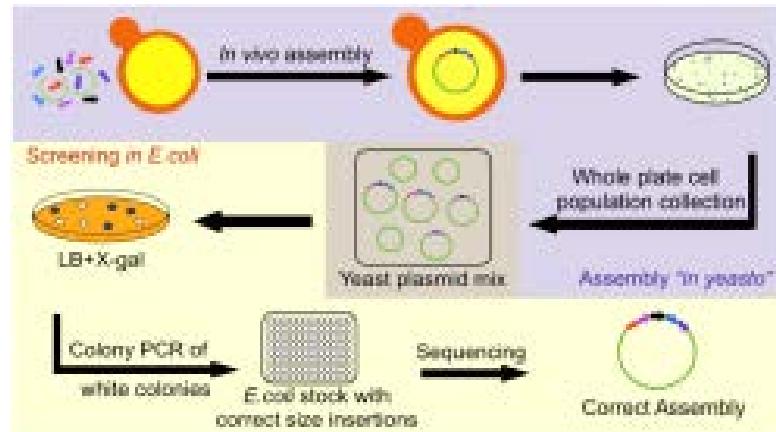


Other Minimal Chassis cells

- *rE. coli* - recoding *E. coli* (~4.4 Mb)
- Multiple-deletion series (MDS) strains – reduced genome (Pósfai et al. 2006, Science)
- New genetic code: genetic code reduced from 64 to 57 by removing instances of the UAG stop codon and excising two arginine codons, two leucine codons, and two serine codons.
- Use of unnatural amino acids.
- Improved biocontainment

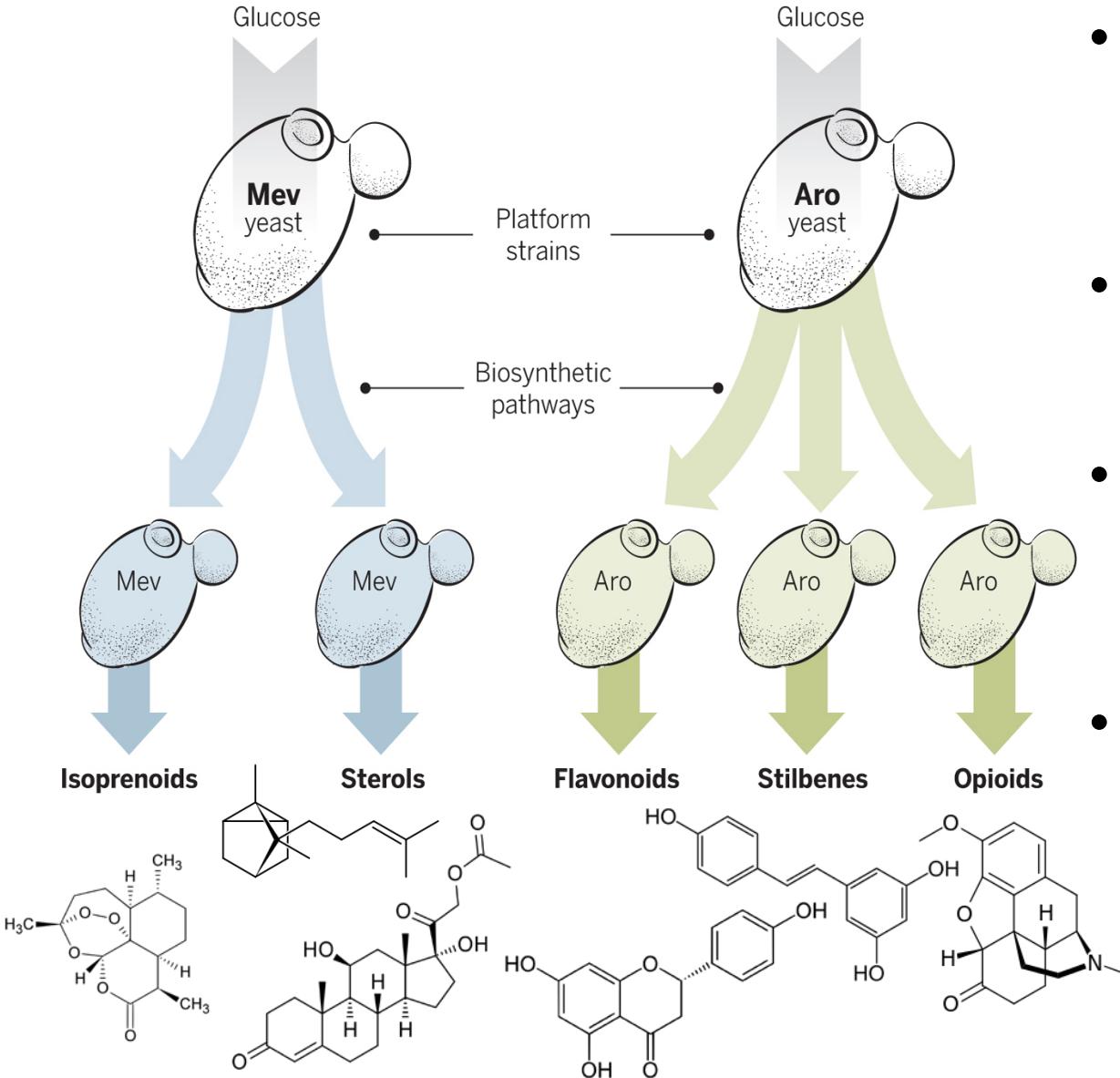


- Synthetic Yeast 2.0
- Synthetic chromosomes
- Homologous recombination in yeast
- “bottom up” approach
 - Replacing 30-60 kb at a time
 - Removed “unnecessary” non-coding DNA (though many discovered to be essential)



Chromosome 1-6, 8,9, 11 and 12 are in various stages
Syntheticyeast.org

Pathway-specialised Platform Yeast strains



- To ensure sufficient production of the natural products, it is necessary to boost the endogenous yeast pathway for efficient provision of precursors
- Tailored platform strains for production of different class of compounds can be generated using metabolic engineering tools
- Example: high mevalonate-producing strain for production of isoprenoid-derived biofuels, sesquiterpenoid fragrance, antimalarial drug artemisinin, steroids
- Example : high aromatic amino acid-producing strain for production of plant flavonoids, antioxidant/anti-aging resveratrol and opioid analgesics

Other “reduced genome” microbial chassis

Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism

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^cDepartment of Chemistry, Box H, Brown University, Providence, RI 02912-9108

Contributed by Satoshi Omura, January 4, 2010 (sent for review November 27, 2009)

To construct a versatile model host for heterologous expression of genes encoding secondary metabolite biosynthesis, the genome of the industrial microorganism *Streptomyces avermitilis* was systematically deleted to remove nonessential genes. A region of more than 1.4 Mb was deleted stepwise from the 9.02-Mb *S. avermitilis* linear chromosome to generate a series of defined deletion mutants, corresponding to 83.12–81.46% of the wild-type chromosome, that did not produce any of the major endogenous secondary metabolites found in the parent strain. The suitability of the mutants as hosts for efficient production of foreign metabolites was shown by heterologous expression of three different exogenous biosynthetic gene clusters encoding the biosynthesis of streptomycin (from *S. griseus* Institute for Fermentation, Osaka [IFO] 13350), cephamycin C (from *S. clavuligerus* American type culture collection (ATCC) 27064), and pladienolide (from *S. platensis* Mer-11107). Both streptomycin and cephamycin C were efficiently produced by individual transformants at levels higher than those of the native-producing species. Although pladienolide was not produced by a deletion mutant transformed with the corresponding intact biosynthetic gene cluster, production of the macrolide was enabled by introduction of an extra copy of the regulatory gene *pldR* expressed under control of an alternative promoter. Another mutant optimized for terpenoid production efficiently produced the plant terpenoid intermediate, amorph-4,11-diene, by introduction of a synthetic gene optimized for *Streptomyces* codon usage. These findings highlight the strength and flexibility of engineered *S. avermitilis* as a model host for heterologous gene expression, resulting in the production of exogenous natural and unnatural metabolites.

Results

these biosynthetic gene clusters will allow the production of analogs by combinatorial biosynthesis. A critical requirement for such applications is the availability of the relevant biosynthetic gene clusters controlling the production of a secondary metabolite of interest as well as appropriate genetic systems for the *in vivo* manipulation of the corresponding genes in heterologous hosts. Furthermore, successful production of the desired products requires an optimum relationship of timing and flux between primary and secondary cellular metabolism, because all secondary metabolites are ultimately derived from primary metabolic building blocks and require an adequate source of energy as well as reducing equivalents derived from primary metabolism such as ATP and NAD(P)H.

There has been considerable recent interest in the development of engineered bacterial strains for the efficient heterologous production of secondary metabolites (6–8). *S. avermitilis*, which is used for the industrial production of the important anthelmintic agent avermectin, has already proven to be a highly efficient producer of secondary metabolites. Because this strain is already optimized for the efficient supply of primary metabolic precursors and biochemical energy to support multistep biosynthesis, it is, therefore, an attractive host for the heterologous production of secondary metabolites. We now report on the construction of a versatile host for the efficient production of natural products by the controlled minimization of the genome of the *S. avermitilis*.



Communications

Angewandte
Chemie
International Edition

Natural Products

International Edition: DOI: 10.1002/anie.201507097

German Edition: DOI: 10.1002/ange.201507097

Development of Genetic Dereplication Strains in *Aspergillus nidulans* Results in the Discovery of Aspercryptin

Yi-Ming Chiang, Manmeet Ahuja, C. Elizabeth Oakley, Ruth Entwistle, Anabanadam Asokan, Christoph Zutz, Clay C. C. Wang, and Berl R. Oakley*

Abstract: To reduce the secondary metabolite background in *Aspergillus nidulans* and minimize the rediscovery of compounds and pathway intermediates, we created a “genetic dereplication” strain in which we deleted eight of the most highly expressed secondary metabolite gene clusters (more than 244,000 base pairs deleted in total). This strain allowed us to discover a novel compound that we designate aspercryptin and to propose a biosynthetic pathway for the compound. Interestingly, aspercryptin is formed from compounds produced by two separate gene clusters, one of which makes the well-known product cichorine. This raises the exciting possibility that fungi use differential regulation of expression of secondary metabolite gene clusters to increase the diversity of metabolites they produce.

Genetic and molecular genetic approaches that up-regulate secondary metabolite (SM) production have dramatically facilitated the discovery of new fungal natural products.^[1] However, these approaches often result in complex metabolite profiles owing to the production of a large number of compounds, including pathway intermediates. We thus devised a strategy we call “genetic dereplication” whereby the discovery of novel compounds is simplified by eliminating

the major known SM biosynthetic pathways in *A. nidulans*, thereby reducing the complexity of SM profiles such that novel compounds are more easily detected. Elimination of highly expressed biosynthetic pathways might also reserve pools of SM precursors such as acetyl-CoA and malonyl-CoA for pathways expressed at low levels.

We have previously shown that we can delete entire SM biosynthetic gene clusters in *A. nidulans* while recycling a selectable marker.^[2] We therefore set out to sequentially delete as many of the major SM clusters as possible. In this study, we engineered a strain (LO8030, genotype in Table S1) in which the clusters responsible for the biosynthesis of the following major SM producing clusters are deleted: sterigmatocystin,^[3] the emericellamides,^[4] asperfuranone,^[5] monodictyphenone,^[6] terrequinone,^[7] F9775A and B,^[8] aspertheicin,^[9] and both portions of the split cluster that makes austinol and dehydroaustinol.^[10] Deletion of these clusters reduced the size of the *A. nidulans* genome by 244,061 base pairs (bp). LO8030 is surprisingly healthy (Figure 1). It forms aerial hyphae upon prolonged incubation, but it is not difficult to work with or to transform. As anticipated, production of the products of the deleted SM clusters was eliminated under all conditions, thus resulting in a low SM background.

Genome-reduced *Streptomyces* and *Aspergillus* chassis for heterologous expression of actinobacterial and fungal biosynthetic pathways and genome mining for drug discovery.

