From Single Enzymes to Pathways - Designs for Chiral Compounds

John Ward,

Professor of Molecular Microbiology, Structural and Molecular Biology, Darwin Building, University College London

http://www.smb.ucl.ac.uk/synbion/

Advances in Synthetic Biology, 28th-29th April 2009, London

Synthetic Biology to make small molecules

Synthetic Biology aims to define the properties of individual components so we can begin to put them together in a logical way.

To make new products, macromolecular entities or cells with defined properties.

If we want to make new small molecules and to create new routes to small molecules we need:

- -toolbox of components (enzymes)
- -knowledge of the properties of these components
- -systems to piece the components together
- -modelling to predict what the properties might be

Biocatalysis -

The use of whole cells or isolated enzymes to carry out chemical reactions.

Key reactions for organic synthesis -

Carbon-carbon bond formation

Chiral amine formation

Controlled oxidation

Halogenation

Whole cell *vs* isolated enzyme

Isolated enzyme best for:

- Reactions where substrates cannot enter cell
- Reactions where substrates would be acted on by cellular enzymes

Whole cells:

- Reactions which need co-factors (co-factor recycling)
- Reactions having several consecutive steps (2/3 or more)
- Reactions making use of a part of an endogenous pathway in the cell (providing a co-substrate or removing a product)

Biocatalysis enzyme systems studied -

Transketolase - carbon-carbon bond formation

Baeyer-Villiger mono-oxygenase - oxygen insertion

Cytochrome P450 - hydroxylation

Transaminase - chiral amine formation

Single enzymes

Two or more enzymes

de novo designed pathways

modified and chimeric natural pathways

Cytochrome P450 from Actinomycetes

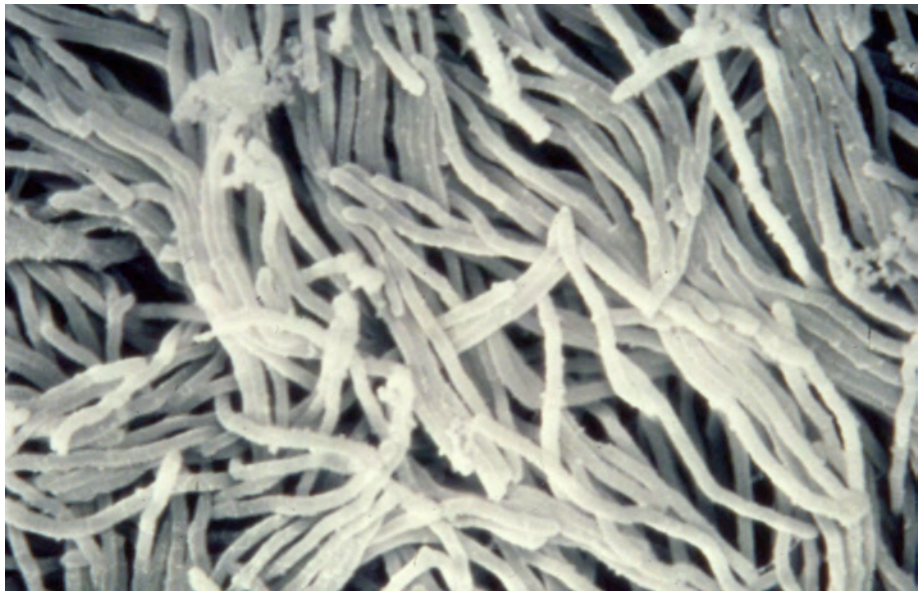
Actinomycetes - have large genomes (8-9 Mb) (for bacteria)
-have complex life cycles,
-have many degradative and detoxification pathways,
-have antibiotic biosynthesis pathways

Actinomycetes are rich in P450s, 53 full genome sequences

P450s in some some sequenced genomes:

Streptomyces coelicolor	20
Streptomyces avermitilis	33
Streptomyces peucetius	19
Mycobacterium tuberculosis	20
Mycobacterium smegmatis	40
Saccharopolyspora erythraea	33

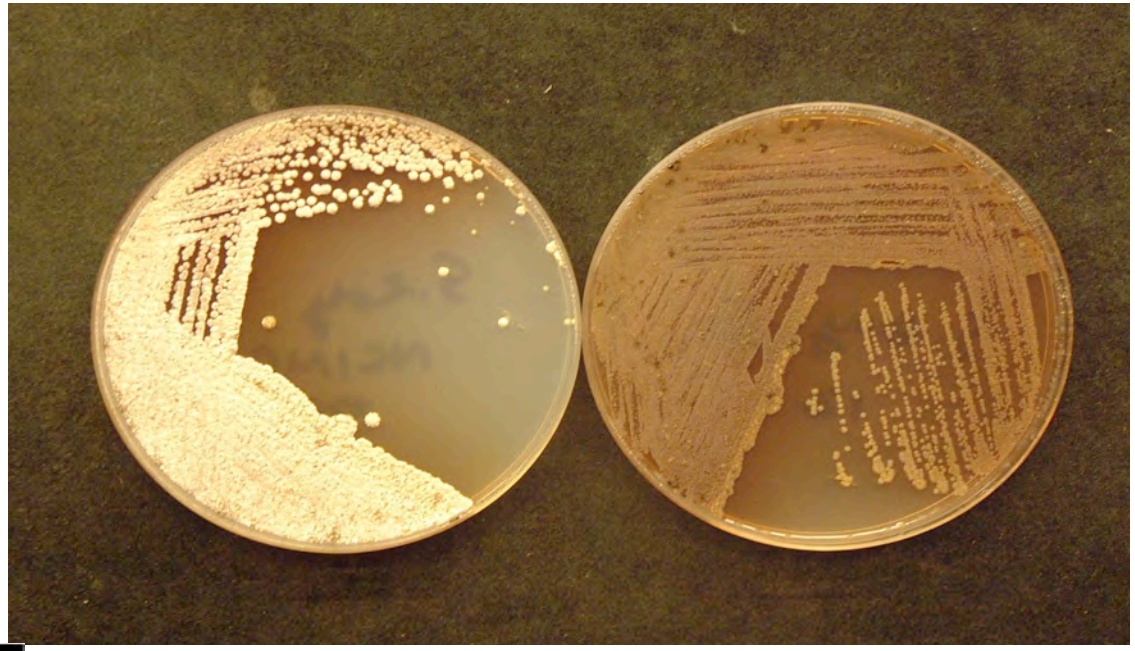
Streptomyces griseus	P450Soy	CYP105D1
S. griseolus	P450SU1	CYP105A1
S.griseolus	P450SU2	CYP105B1
Sacch. erythraea	P450EryF	
Sacch. erythraea	P450EryK	
M. tuberculosis	P450Rv1880c	



Saccharopolyspora erythraea

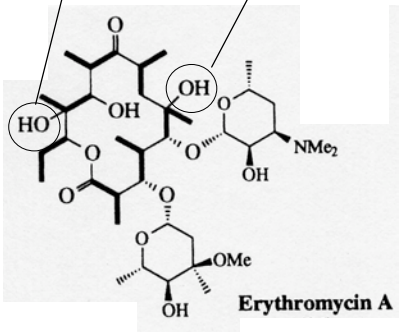
White wild type (left)

Red variant (right)



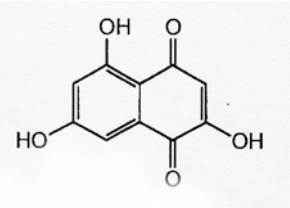
EryK

EryF



Erythromycin A

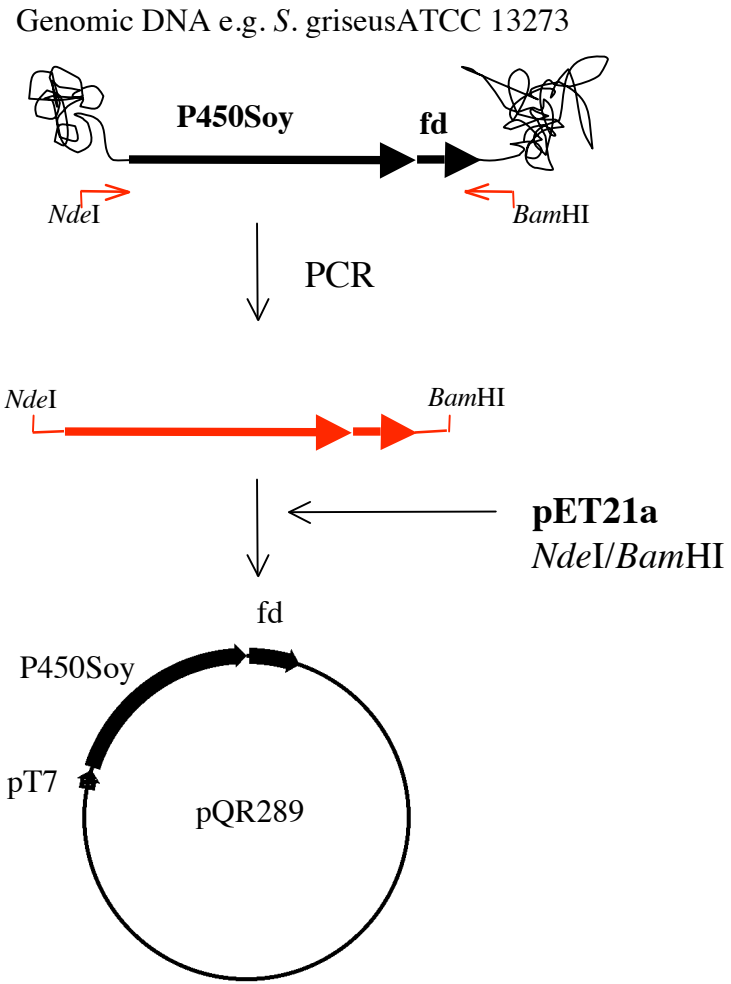
RppB

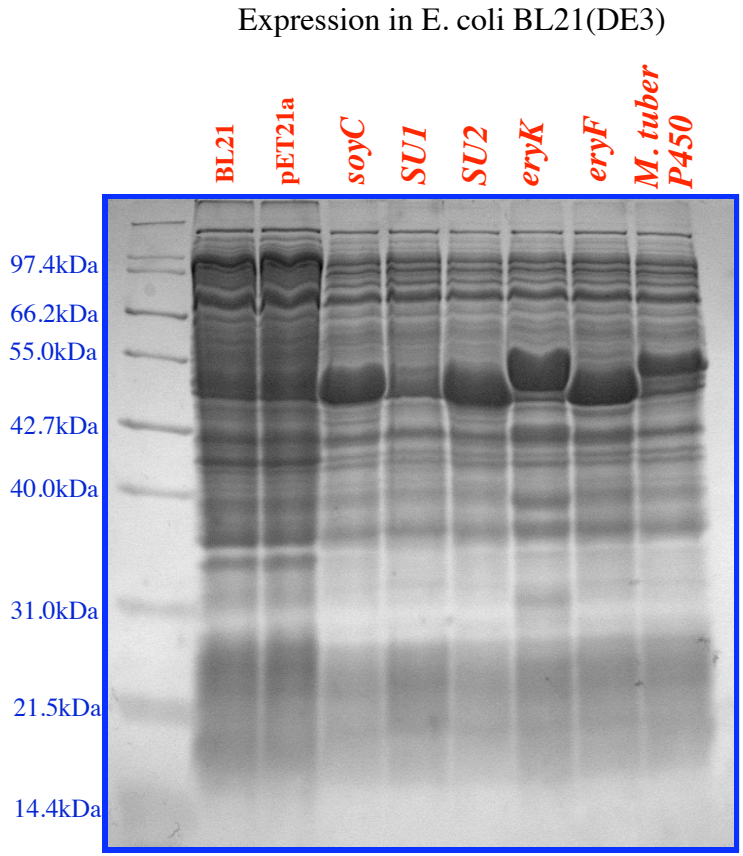


Flaviolin

EryF

Expression of 6 P450s in *E. coli*





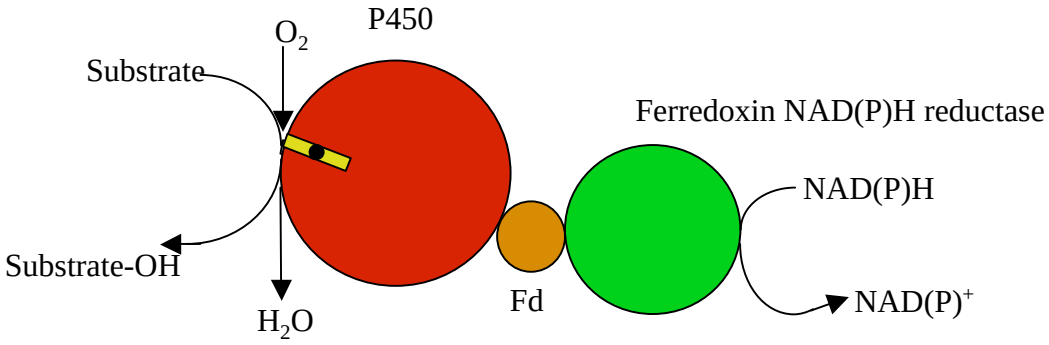
Bacterial Cytochrome P450s

They operate as a three enzyme redox system

- cannot use isolated enzymes as this would use stoichiometric amounts of NAD(P)H

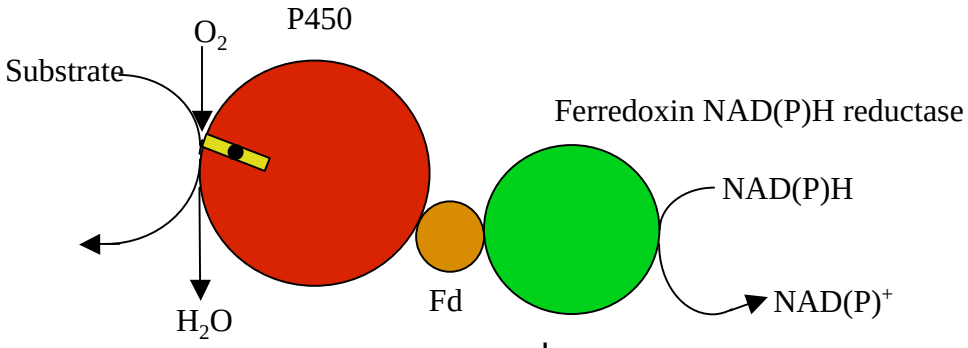
Therefore need to use whole cells as biocatalysts

Cytochrome P450 with its electron transfer proteins



Fd = ferredoxin

3 different P450s



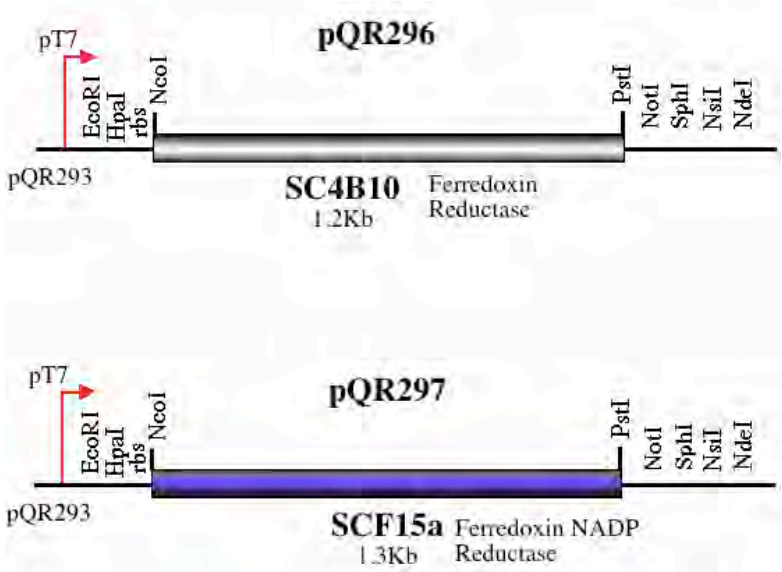
Fd = ferredoxin

3 different P450's
In pET

2 different
Fd reductases

Using a 'Mix and Match' system we were able to test 2 ferredoxin reductases and 3 P450s to get the most active combination.

2 Ferredoxins



SC4B10 and SCF15a are two Ferredoxin reductases from *Streptomyces coelicolor.*

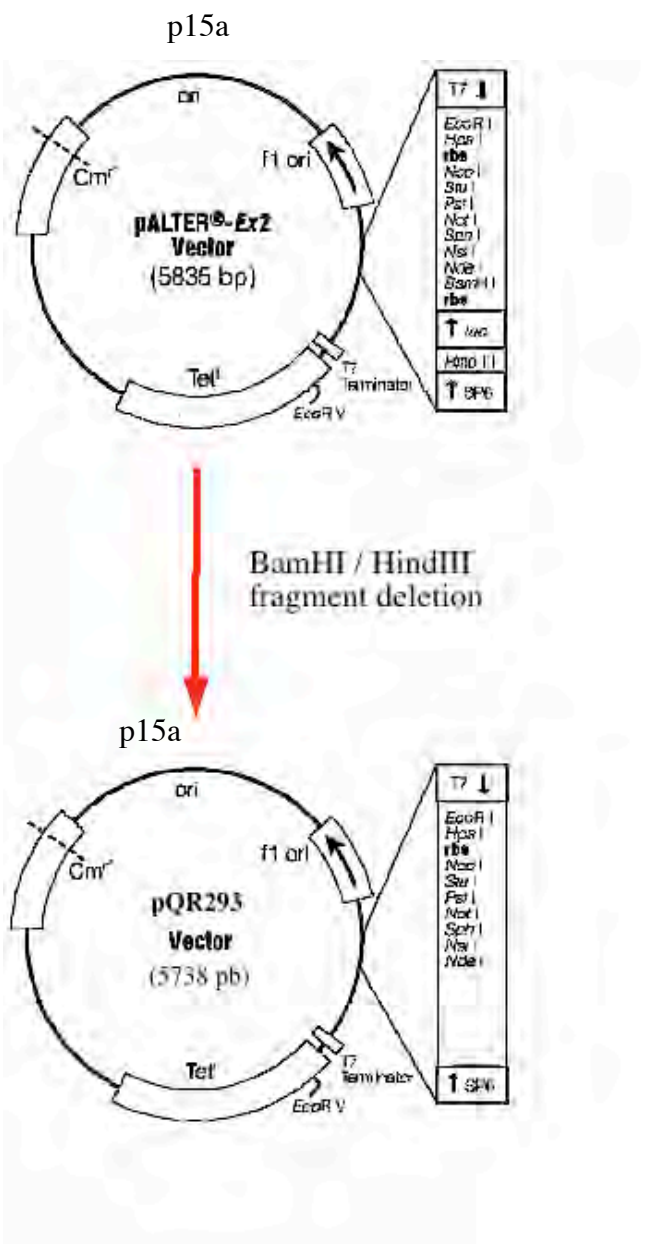
Cloned into the p15a replicon pQR293.

‘Mix and Match’ expression

Problem - how to co-express two or more proteins in the same cell?

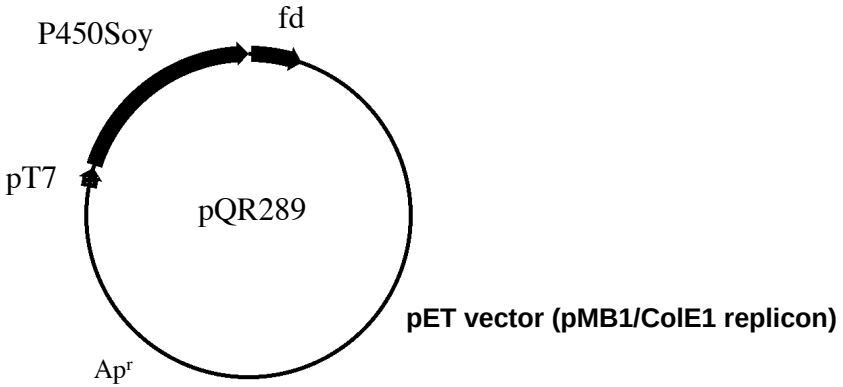
Most expression vectors are all based on the same plasmid replication origin.

Solution - construct expression vectors based on different replicons so they will Stably co-exist in the same cell.



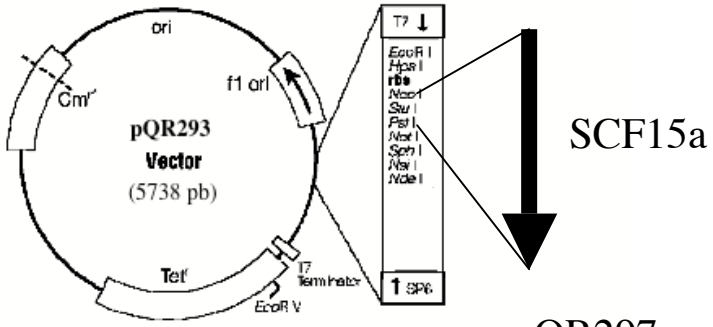
Hussain and Ward. (2003). Appl. Env. Microbiol. 69. 373-382

Mix and Match expression



pET vector (pMB1/ColE1 replicon)

+



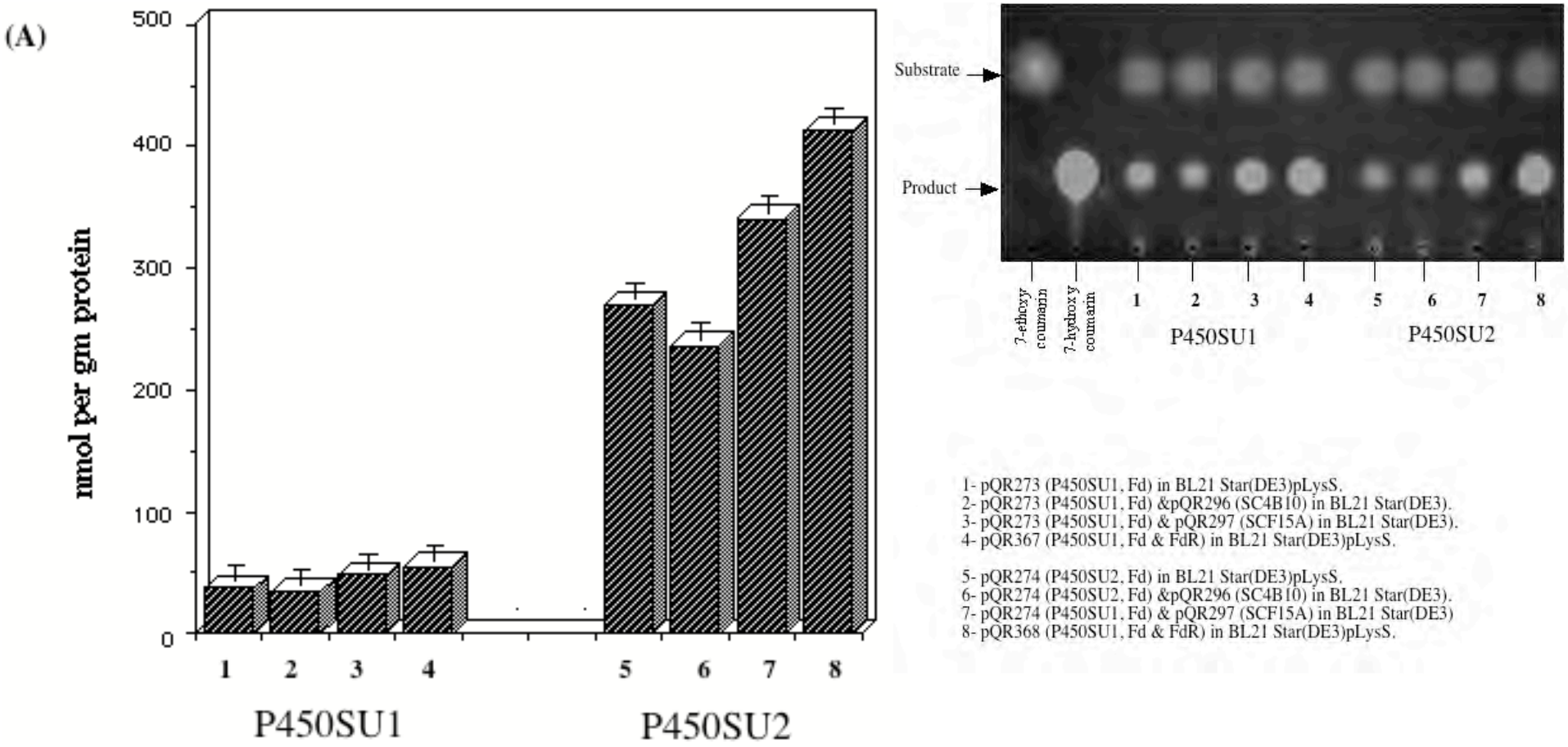
SCF15a

pQR297

Transform into same *E. coli*BL21Star (DE3)pLysS

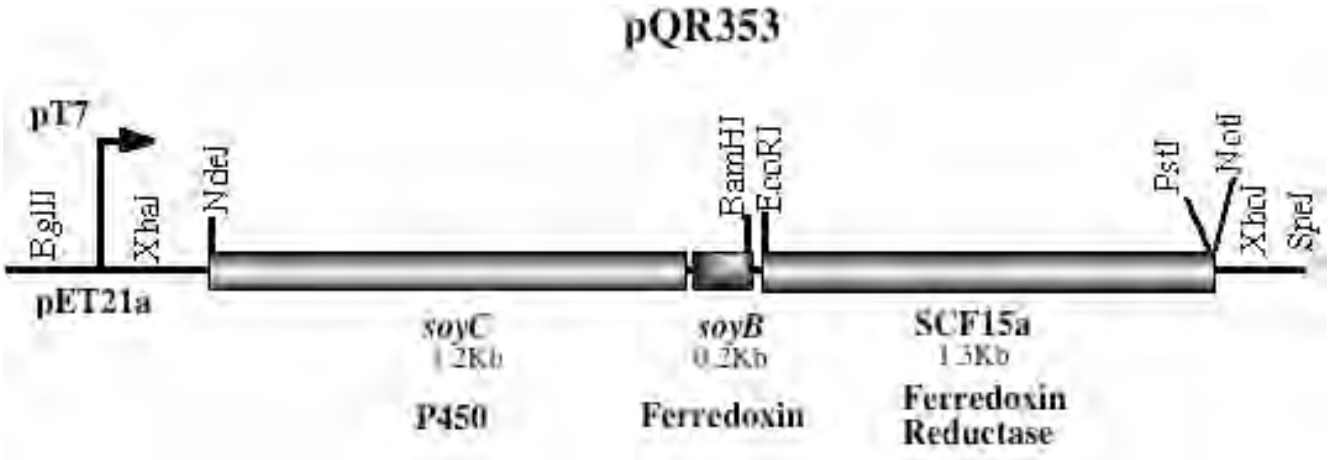
Using this Mix and Match system we were able to test 2 ferredoxin reductases and 3 P450s

Activity of SU1 and SU2 with different Ferredoxin Reductases



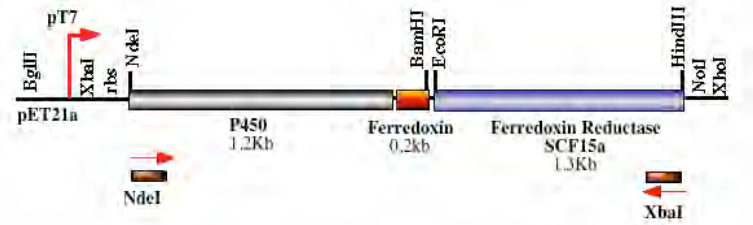
1 and 5 just the P450; 2 and 6 with SC4B10; 3 and 7 with SCF15A; 4 and 8 operon of P450, Ferredoxin and SCF15A

Operon of P450Soy, Fd and Ferredoxin reductase



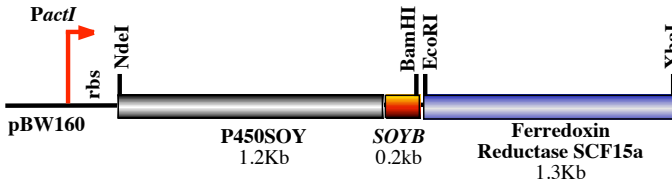
Hussain and Ward. (2003) Enzyme and Microbial Technology 32. 790-800

P450Soy → For *E. coli*



pQR353

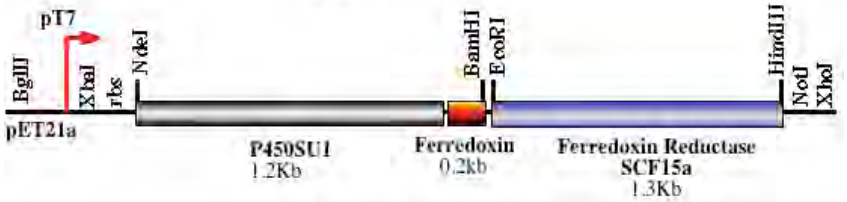
P450Soy → For *S. lividans*



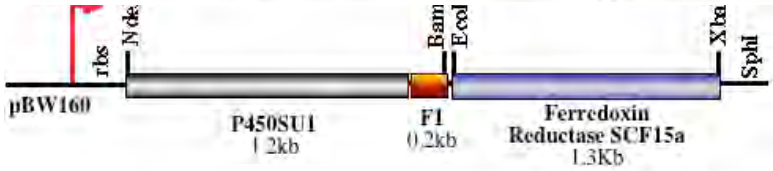
pQR357

pQR368

P450SU1 → For *E. coli*

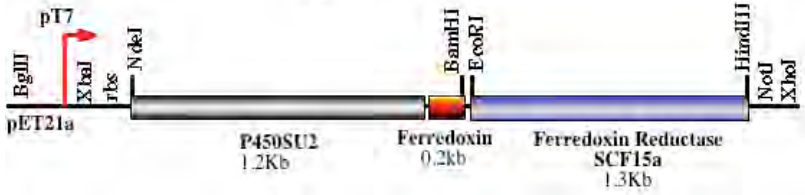


P450SU1 → For *S. lividans*



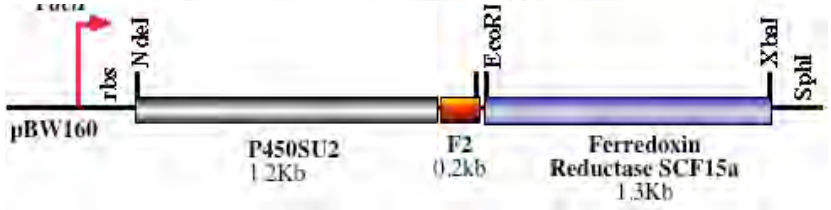
pQR373

P450SU2 → For *E. coli*



pQR368

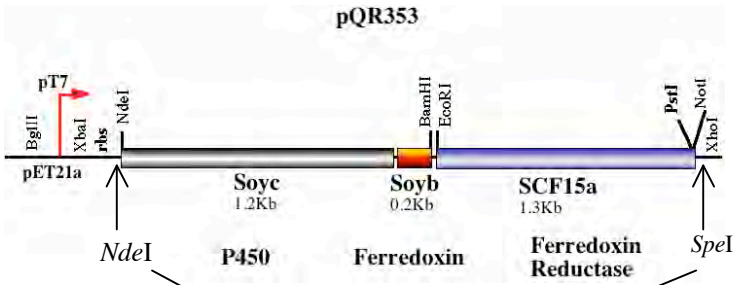
P450SU2 → For *S. lividans*



pQR374

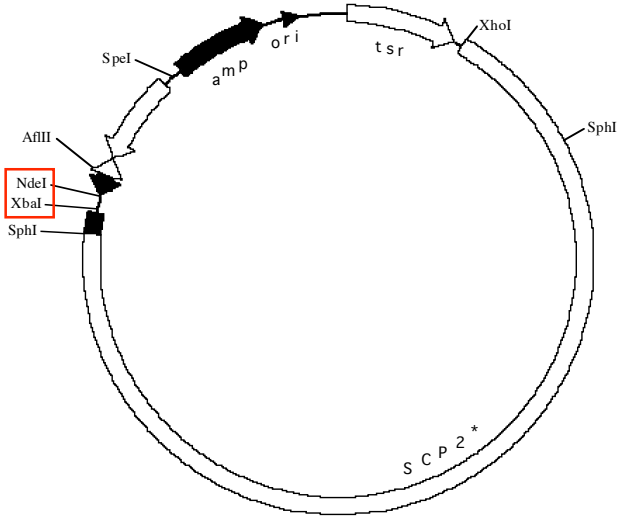
Expression in a novel chassis

Cloning P450Soy operon into *Streptomyces lividans*



\+

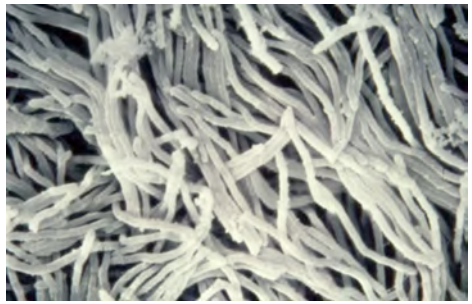
*Nde*I-*Xba*I



pBW160

→ **pQR357**

Conjugate from *E. coli* to *S. lividans*



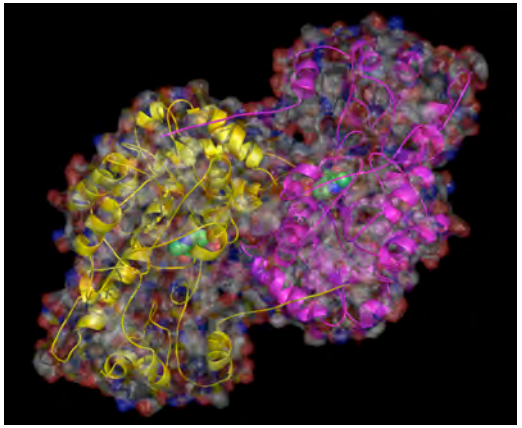
Test Biotransformation of 7-ethoxycoumarin

7-ethoxycoumarin (substrate)

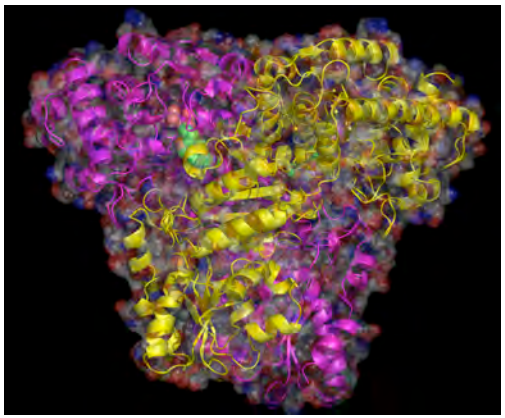
7-hydroxycoumarin (product)

Biotransformation Active for 70 hr.

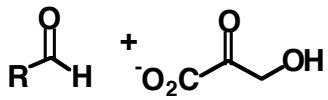
Making aminodiols



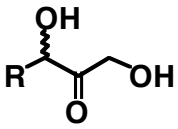
Transaminase



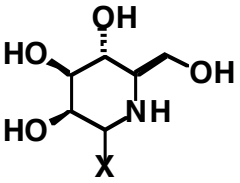
Transketolase



TK (Transketolase)

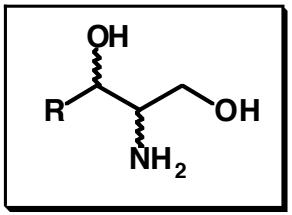


TAm (Transaminase)

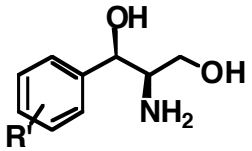


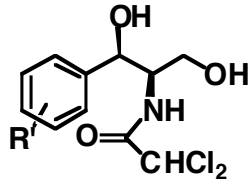
Azasugars,
e.g. mannojirimycin
X = OH

R = CHOCH(OH)CH(OH)



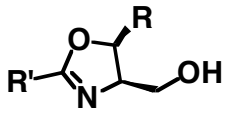
R= aryl



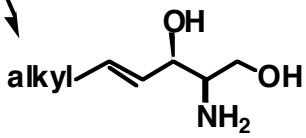


antibiotics

R = unsaturated alkyl chain,

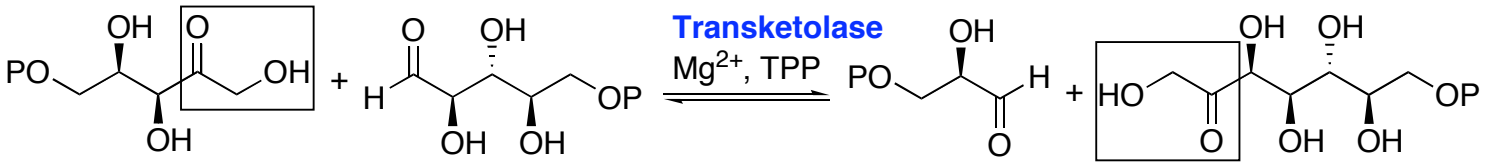


Oxazolines

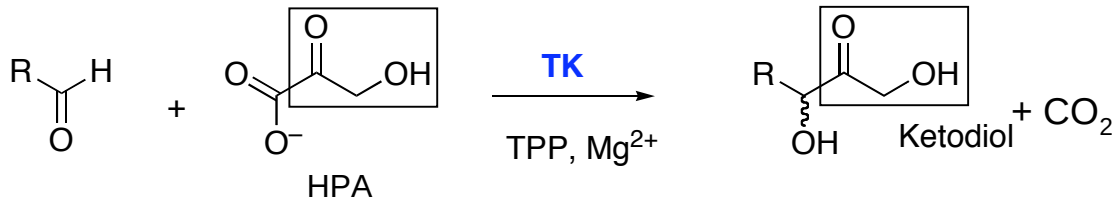


Sphingosines

Transketolase



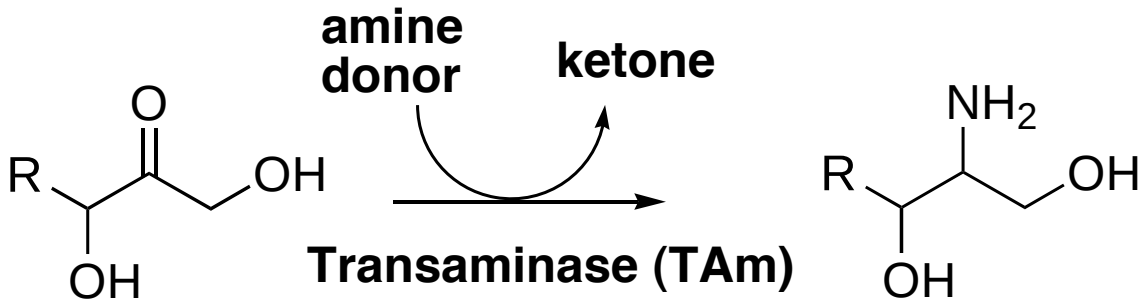
D-xylulose 5-P **D-ribose 5-P**



In vivo transketolase catalyses the transfer of the 2 carbon ketol unit from D-xylulose-5-phosphate to D-ribose-5-phosphate.

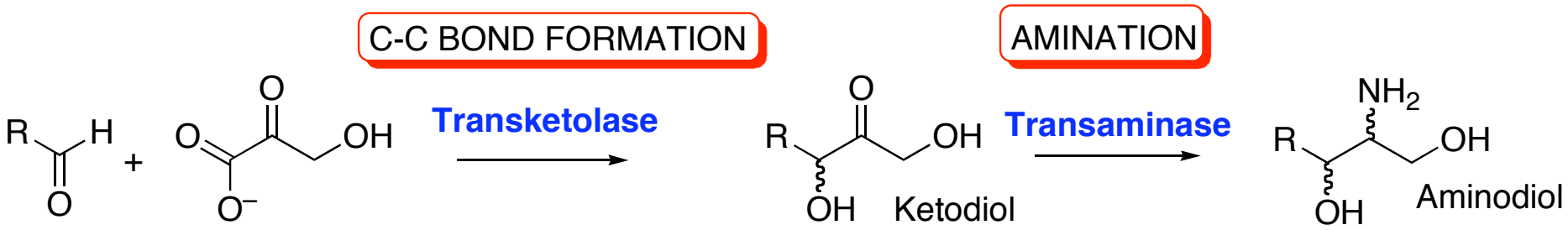
When β-hydroxypyruvic acid (HPA) is used as a donor the loss of CO_2, renders the reaction irreversible.

Transaminase



Amine donor required e.g. alanine which generates pyruvate; β-alanine which generates 3-oxopropanoate; *S*-α-methylbenzylamine (MBA) which generates acetophenone

Building a new pathway from individual components



Need a toolkit of components -Transketolases and transaminases

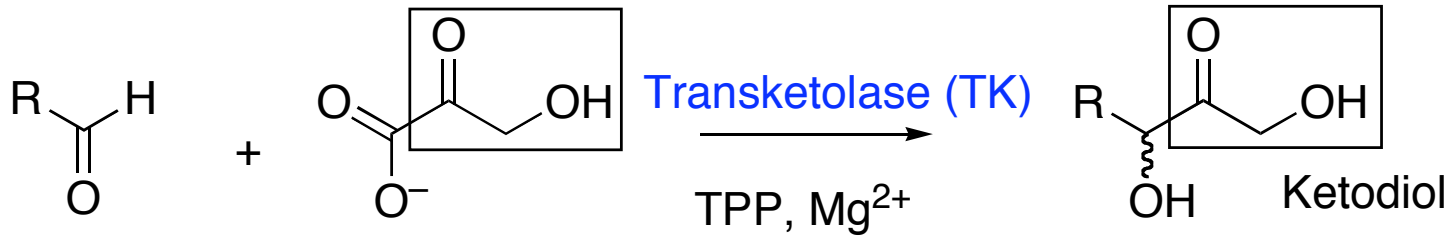
The Transketolase toolkit:

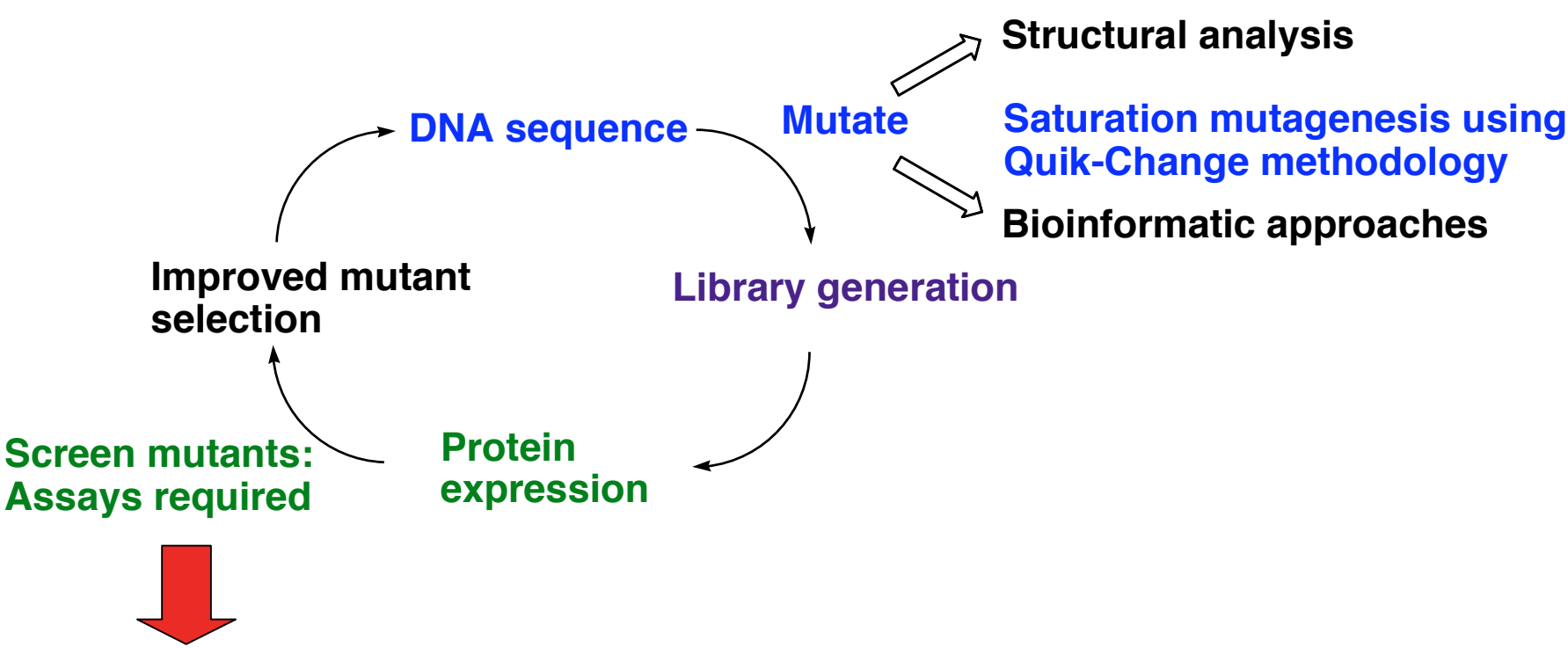
E. coli over-expressing the wild type *E. coli* TK engineered at UCL. This is an efficient catalyst and has been scaled up to 400 L and substrate concentrations of 300-500 mM.
From this a large range of mutant transketolase derivatives have been derived.

The Transaminase tookit:

Screen the sequenced microbial genomes using key proteins in a Blast search

Expanding the toolkit for Transketolase

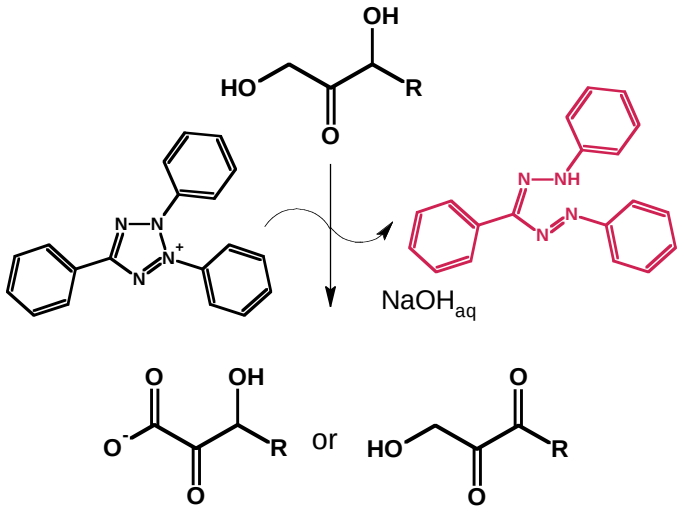


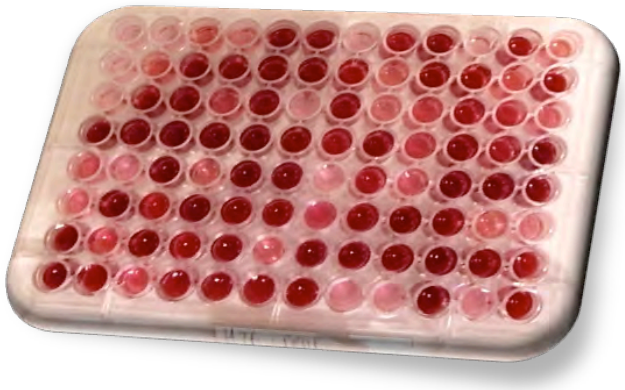


- Development of assays to identify active mutants

A colorimetric screening method for Transketolase activity[1]

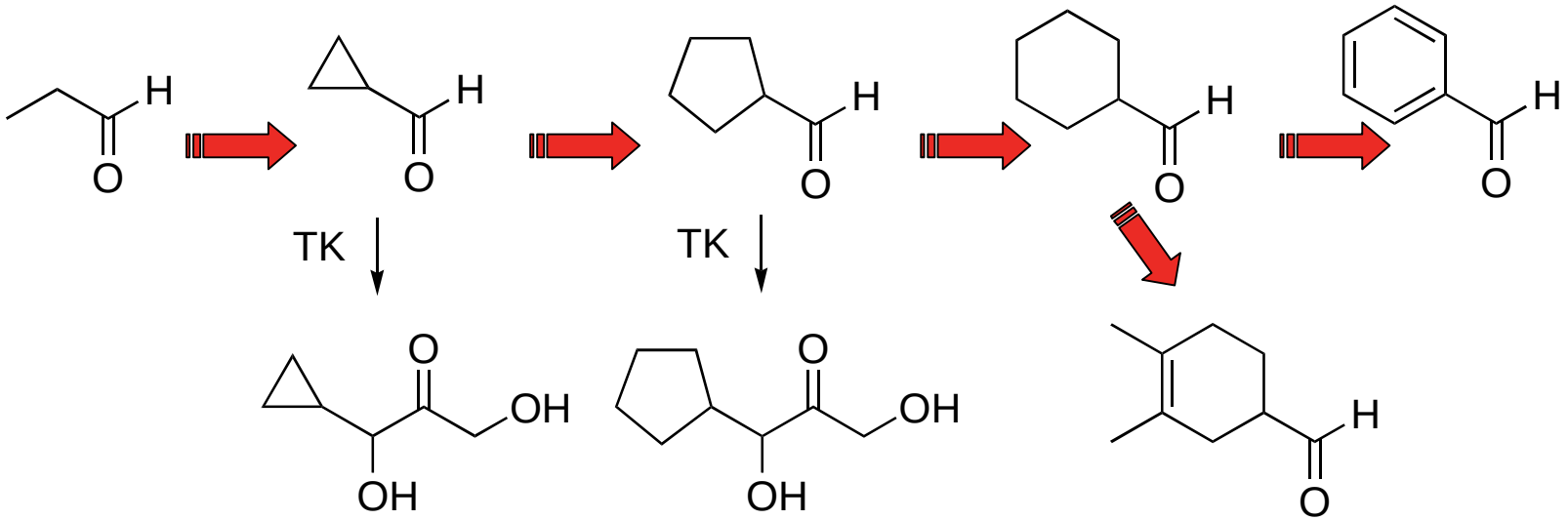
The transketolase product - a ketodiol - can reduce a tetrazolum dye producing a colored (red) product.

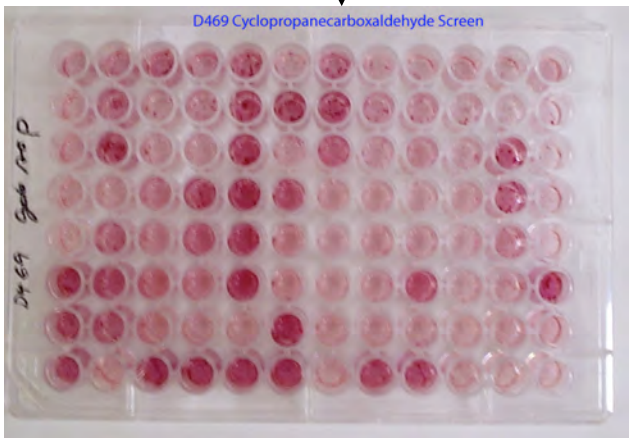


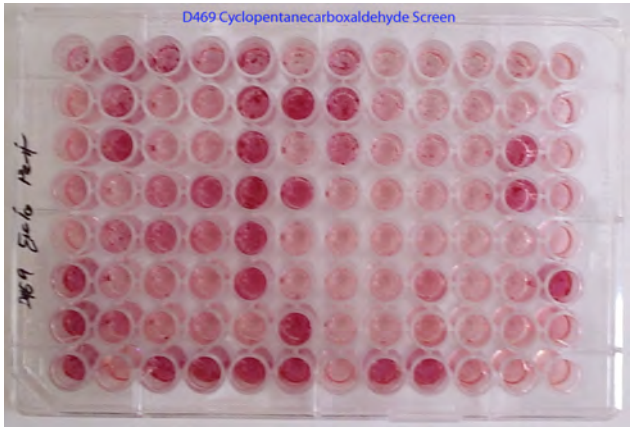


[1]M. E. B. Smith, U. Kaulmann, J. M. Ward, H. C. Hailes, *Bioorg. Med. Chem.* **2006**, *14*, 7062.

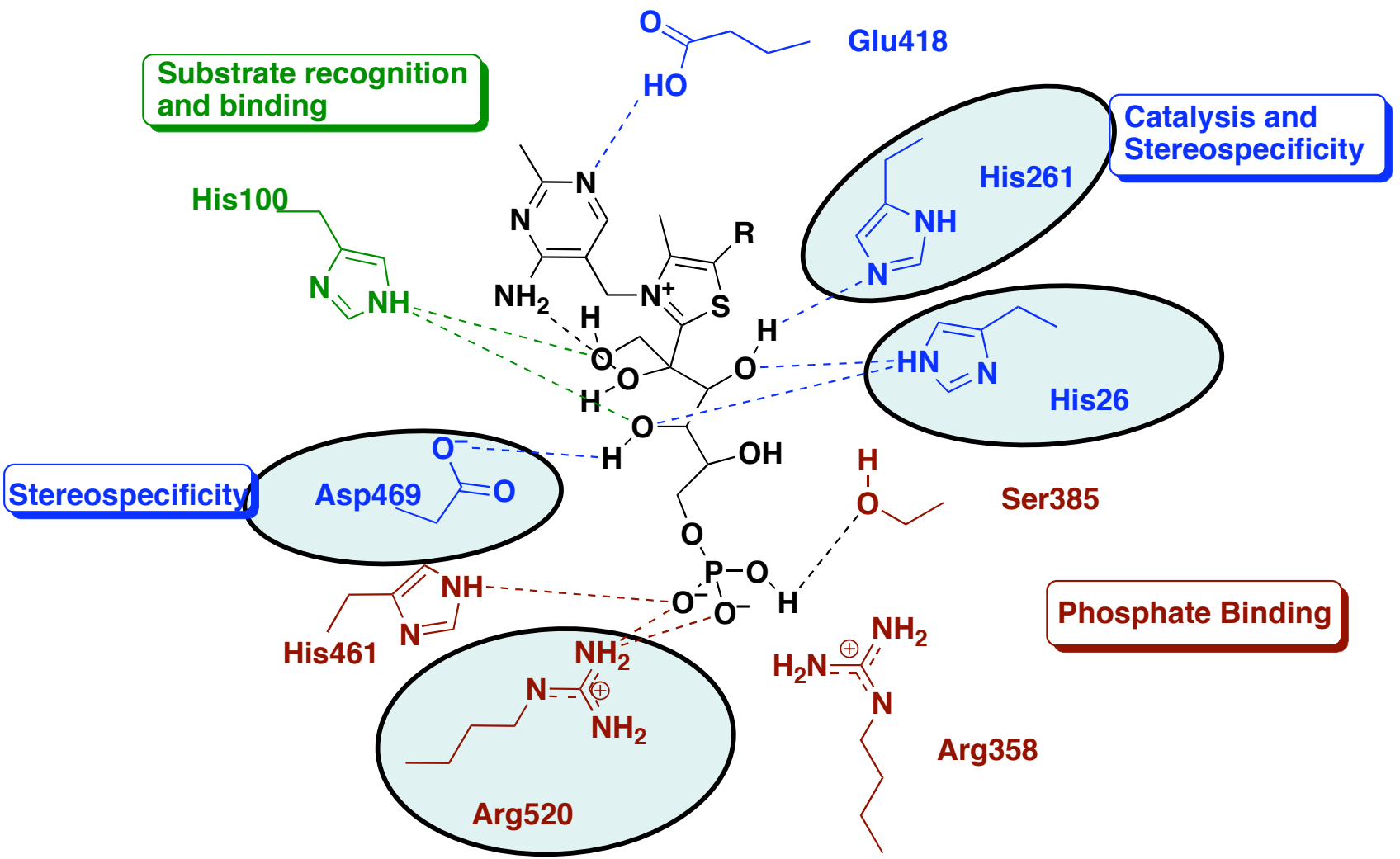
TK Libraries and cyclic aldehydes







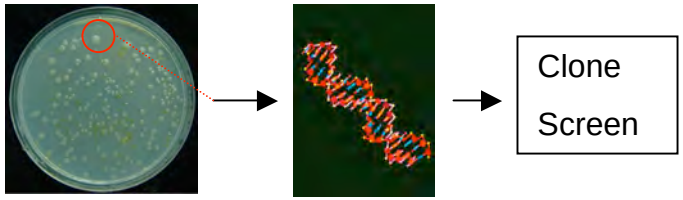
Structural Library Target Residues



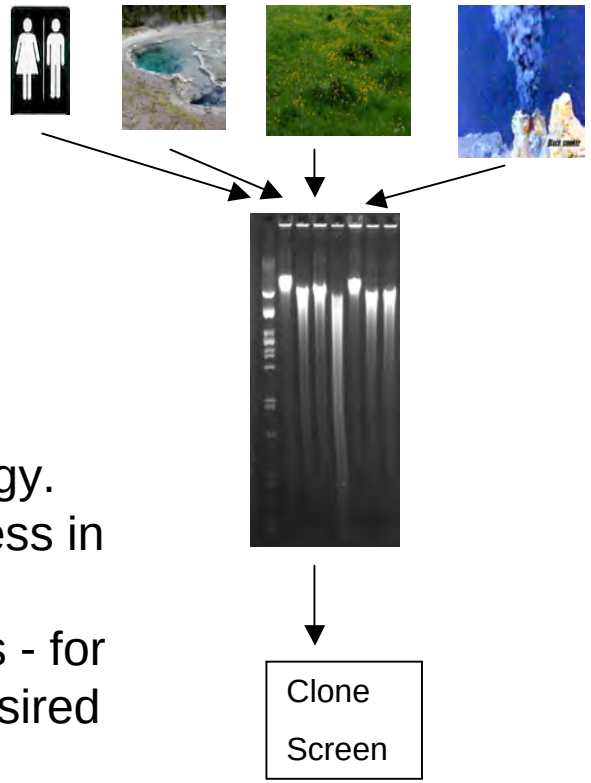
- Model of donor-substrate-TPP adjunct in active site of TK

Recruiting new enzymes for the synthetic biology toolkit

- Enrich or select wild type bacteria



- Metagenomics (culture independent)



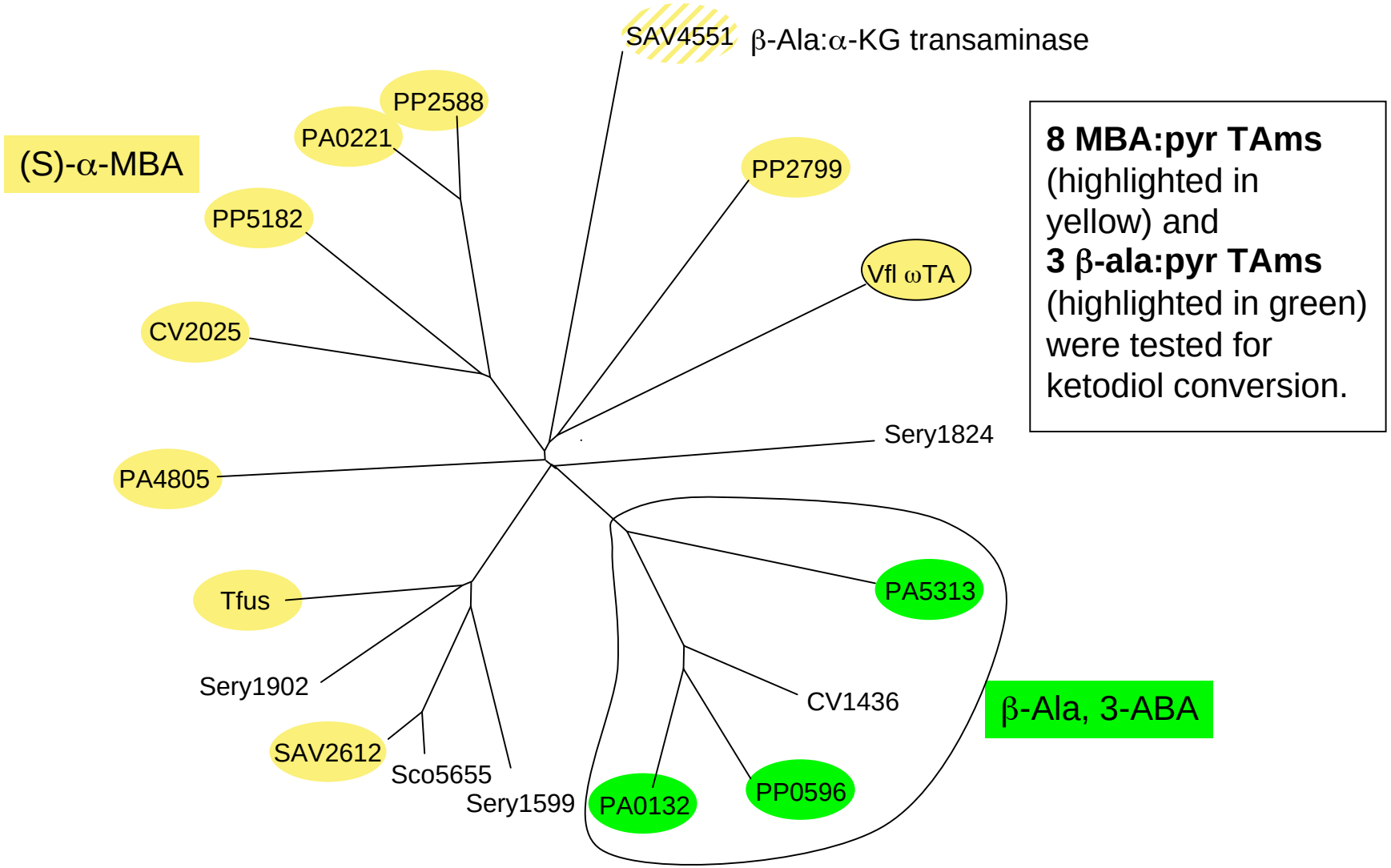
- Bioinformatics - 1607 bacterial genomes, 61complete archaeal genomes and 229 eukaryotic genomes. Growing at a many per month.
- ≈10^7 ORF's can be searched by name or homology.

Rapid - can search, design primers, clone and express in a week or two. Can be automated.

- Can now synthesise genes found by bioinformatics - for optimal codons and expression in *E. coli* or other desired chassis

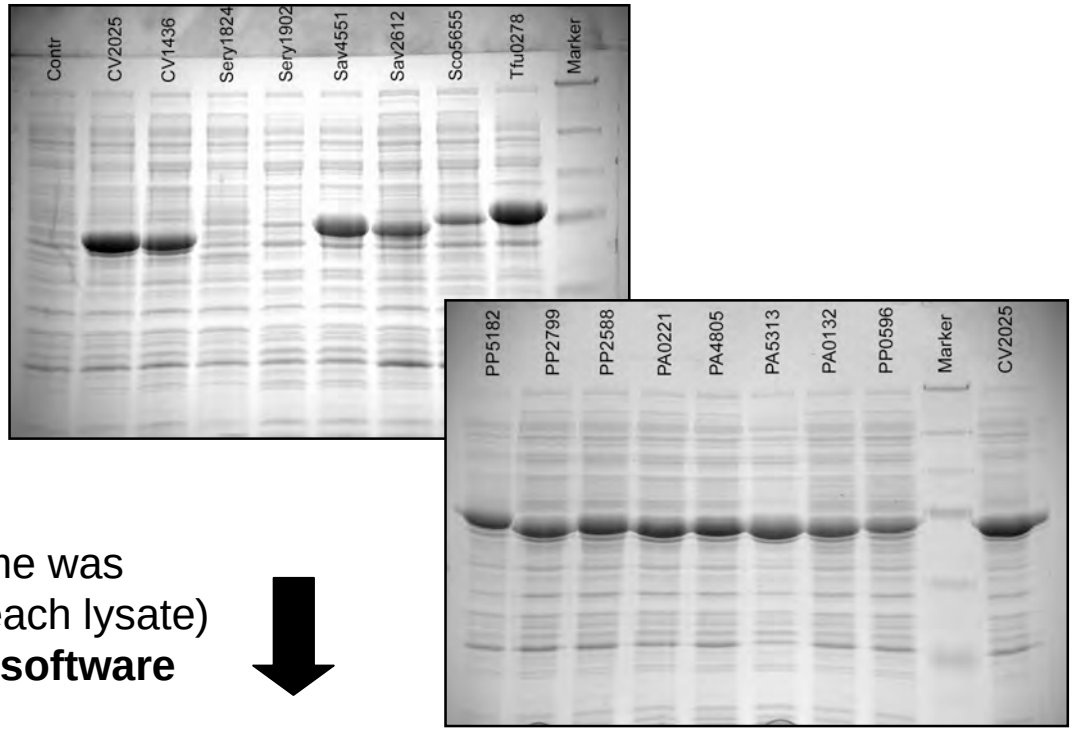
New ω-TAms obtained

- **BLAST search using the *V. fluvialis* sequence**



8 MBA:pyr TAms (highlighted in yellow) and **3 β-ala:pyr TAms** (highlighted in green) were tested for ketodiol conversion.

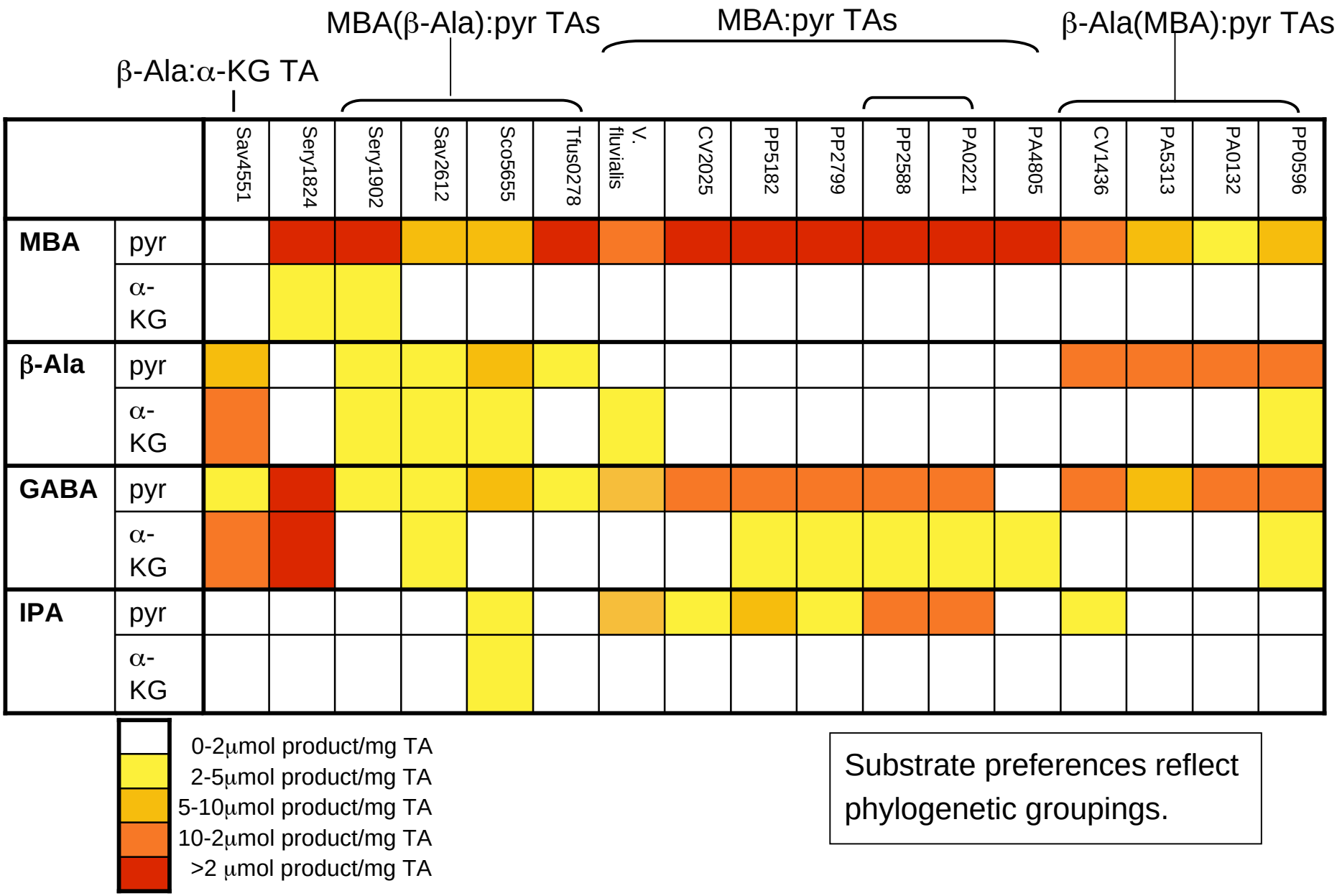
Quantification of TA in clarified cell lysates



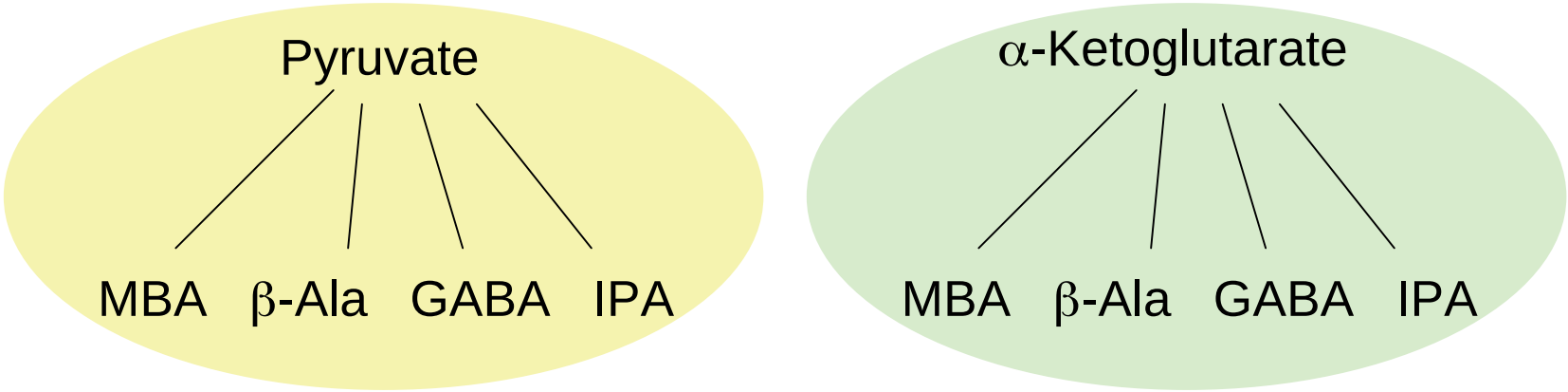
SDS-PAGE
(same volume was applied for each lysate)
GeneTools software

	contr	CV 2025	CV 1436	Sery 1824	Sery 1902	Sav 4551	Sav 2612	SCO 5655	Tfu 0278	PP 5182	PP 2799	PP 2588	PA 0221	PA 4805	PA 5313	PA 0132	PP 0596	Vfl
[protein] of lysate	11.9	14.8	17.3	10.6	16.4	15.7	17.7	13.2	15.1	13.5	17.6	17.7	14.5	16.0	14.4	15.9	15.4	20.1
% TA in lysate	0	48	35	8	7	26	28	22	54	32	35	34	38	36	51	34	29	46
[TA] of lysate	0	7.1	6.1	0.8	1.1	4.1	5.0	2.9	8.1	4.3	6.2	6.0	5.5	5.8	7.4	5.4	4.5	9.3

Substrate screen using HPLC Results from 2h conversions

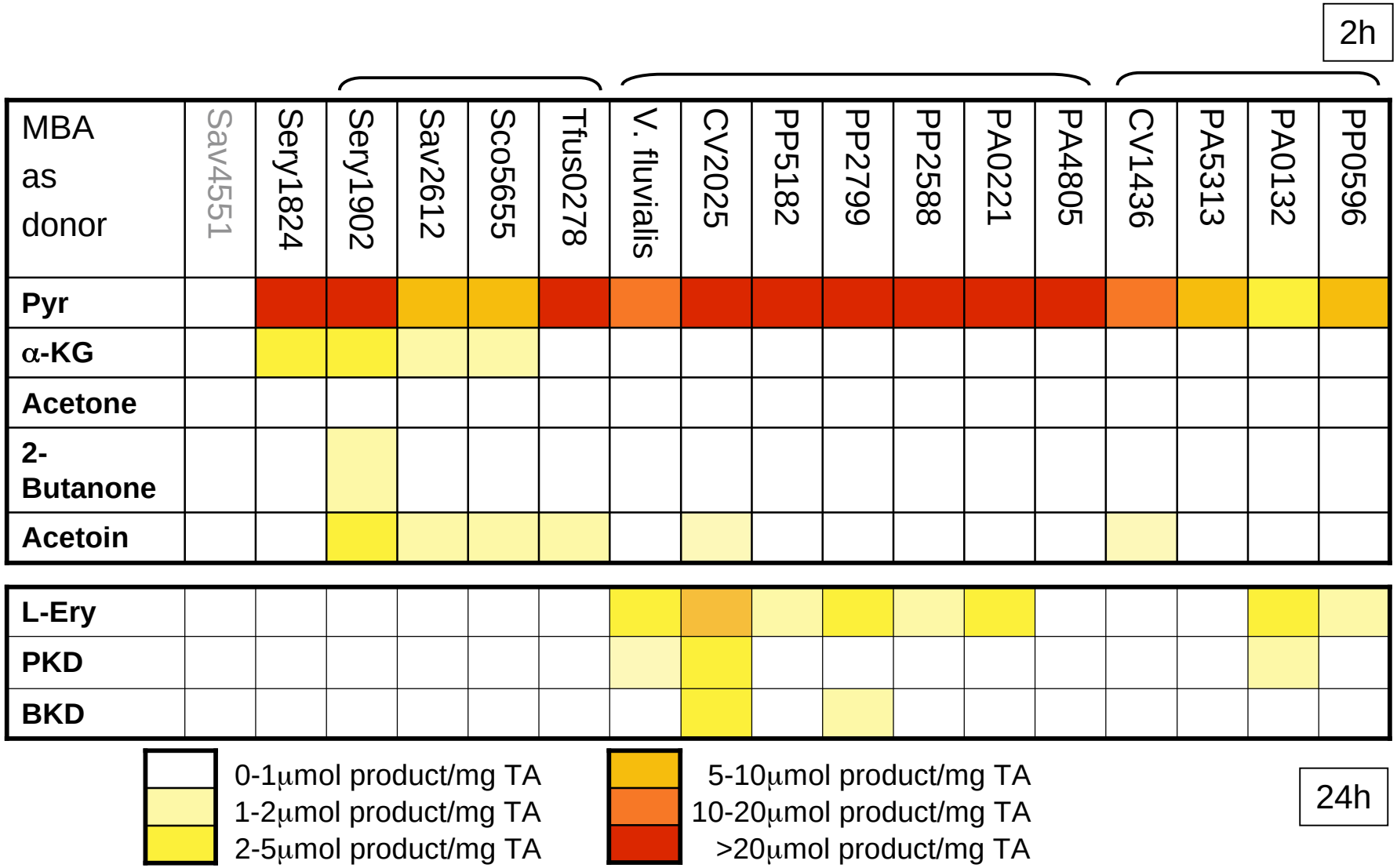


Testing ‘key’ donors and acceptors for grouping of the ω-TA toolbox



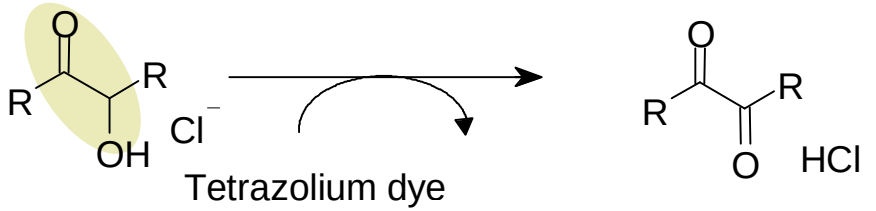
Comparison with sequence alignment leads to conclusions for the molecular basis of the observed substrate preferences.

Results with (*S*)-α-MBA as the donor

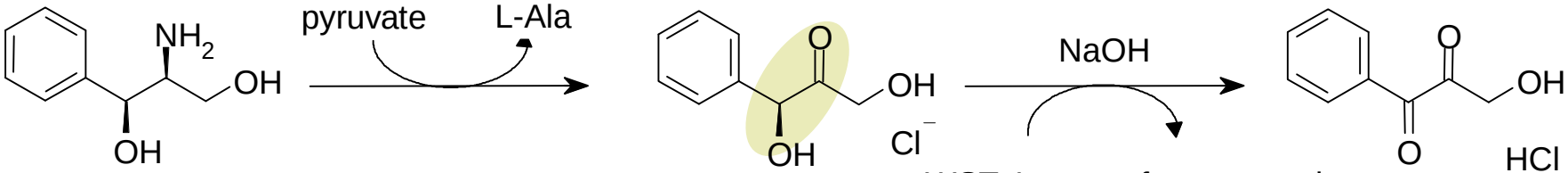


Colour assays for aminoalcohol conversions

General Scheme:



Aminoalcohol assay:



(1S,2S)-2-amino-1-phenyl-1,3-propandiol (APP)

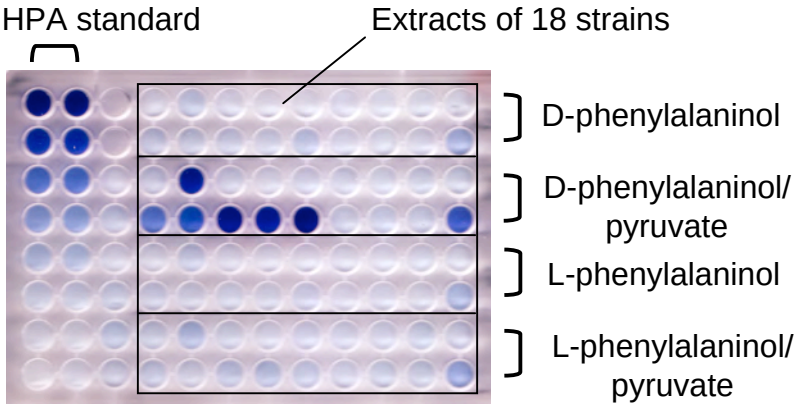
Tetrazolium Assay for detection of ketol groups:

> Applied in plate tests for ketodiol production via TK

> Useful for testing ketol production from aminoalcohols via TAm

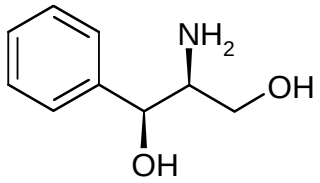
Aminoalcohol conversions

Example:

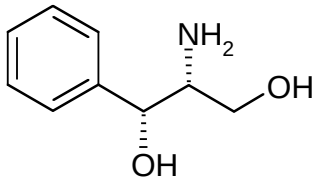


The conversion was done in 100mM HEPES pH7.5 with 10mM of donor, 10mM of pyruvate, 5% extract, 37C, 2h, then addition of WST-1/NaOH and A600 measurement.

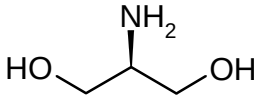
Tested substrates



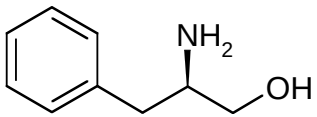
(1R,2R)-2-amino-1-phenyl-1,3-propandiol (APP)



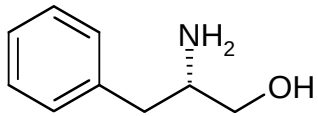
(1S,2S)-2-amino-1-phenyl-1,3-propandiol (APP)



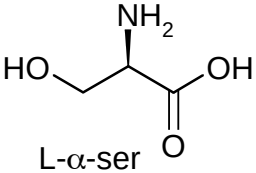
serinol



D-phenylalaninol



L-phenylalaninol



L-α-ser

Results with pyruvate as the acceptor

MBA:pyr TAs

β-Ala:α-KG TA | MBA(β-Ala):pyr TAs | β-Ala(MBA):pyr TAs

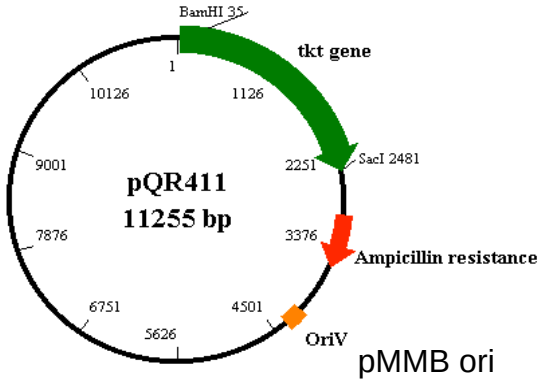
	Sav4551	Sery1824	Sery1902	Sav2612	Sco5655	Tfus0278	V. fluvialis	CV2025	PP5182	PP2799	PP2588	PA0221	PA4805	CV1436	PA5313	PA0132	PP0596
(1S,2S)-APP																	
(1R,2R)-APP																	
Serinol																	
L-Ser												n.d.					
D-Phe-Alaninol																	
L-Phe-Alaninol																	

0-1μmol ketol/mg TA
1-2μmol ketol/mg TA
2-5μmol ketol/mg TA

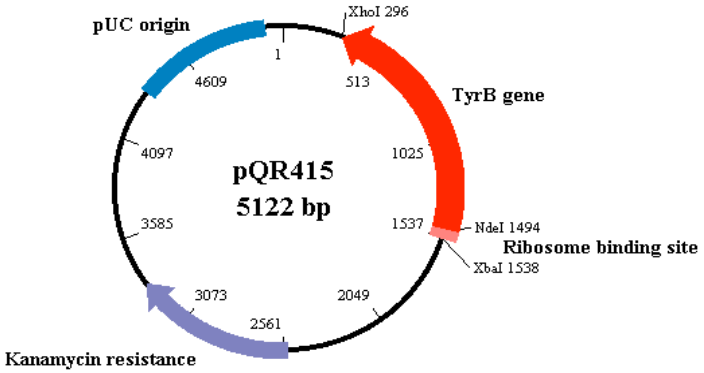
5-10μmol ketol/mg TA
10-20μmol ketol/mg TA

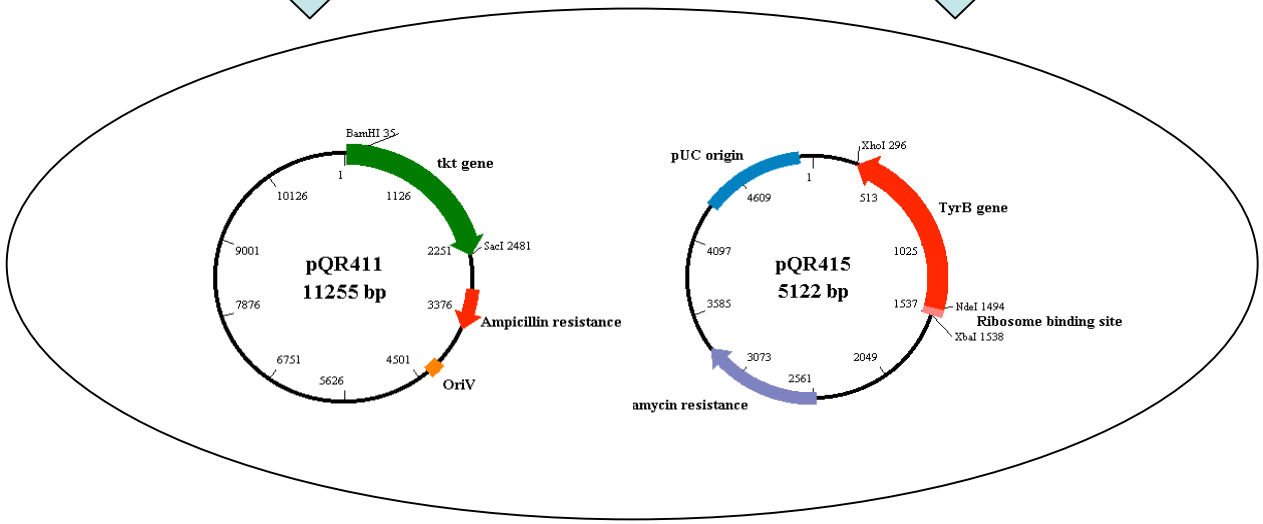
de novo pathway of TK and TAm

Broad host range IncQ group replicon

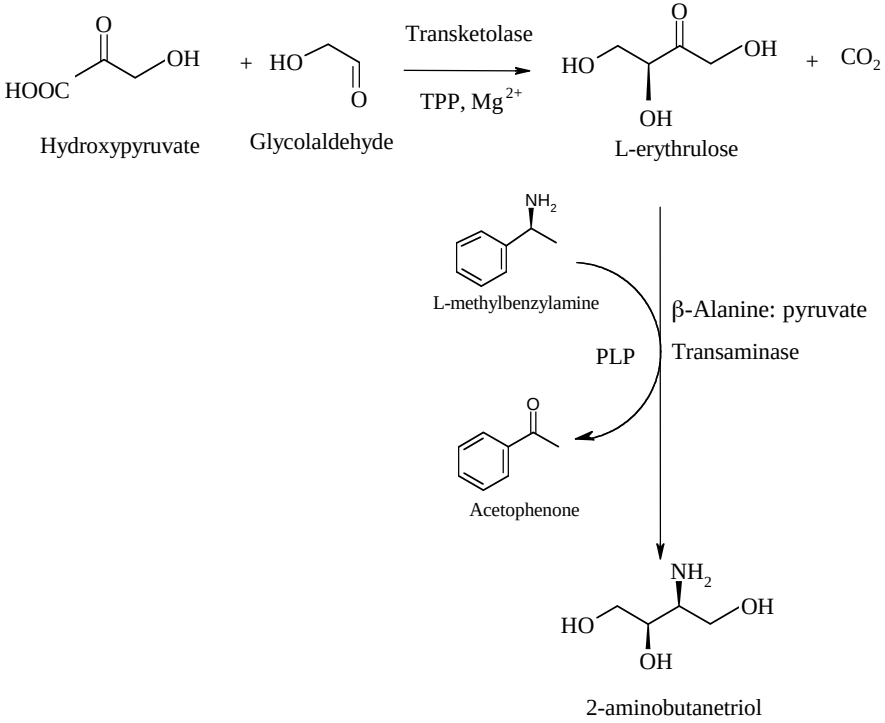


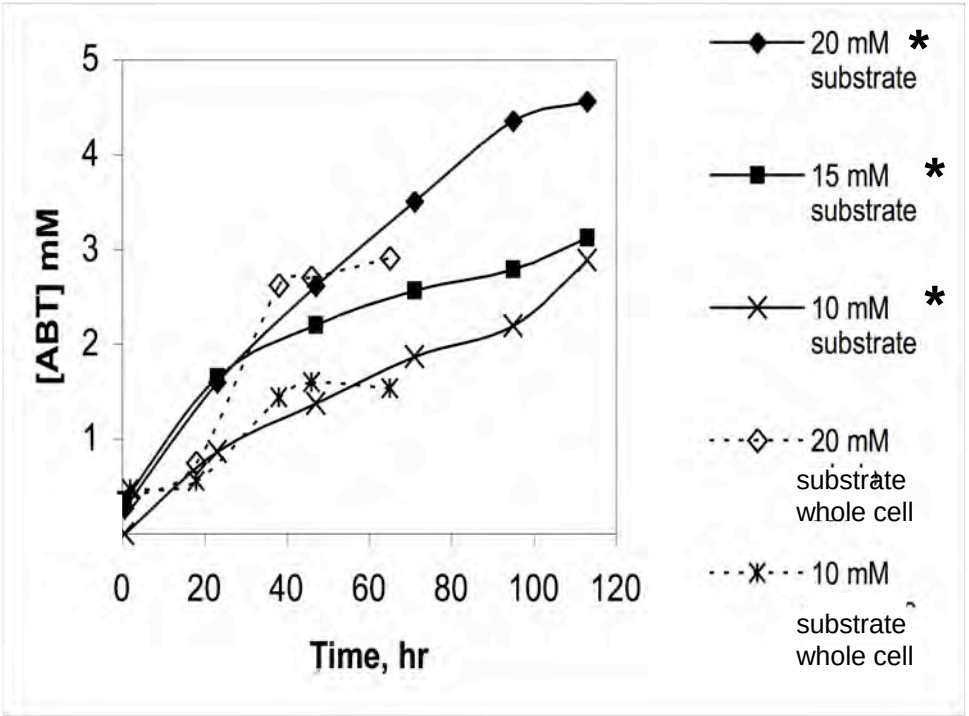
pMMB ori





E. coli BL21





TK works fast and gives 95% conversion, Tam is slower on this substrate

* Dual plasmid strain lysed cells

Modelling the reactions

Table I. TK reaction kinetic parameters.

Kinetic parameters	Value	Standard deviation
Rate constant: K_{cat}	84.3 s^{-1}	1.26
Michaelis constant for HPA: K_a	13.2 mM	0.73
Michaelis constant for GA: K_b	18.3 mM	2.12
Inhibition constant for HPA: K_{ia}	39.9 mM	6.37
Inhibition constant for GA: K_{ib}	450.1 mM	14.00
Inhibition constant for ERY: K_{iq}	395.6 mM	13.67

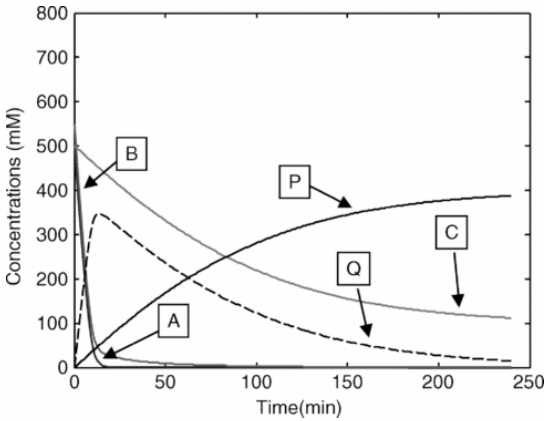
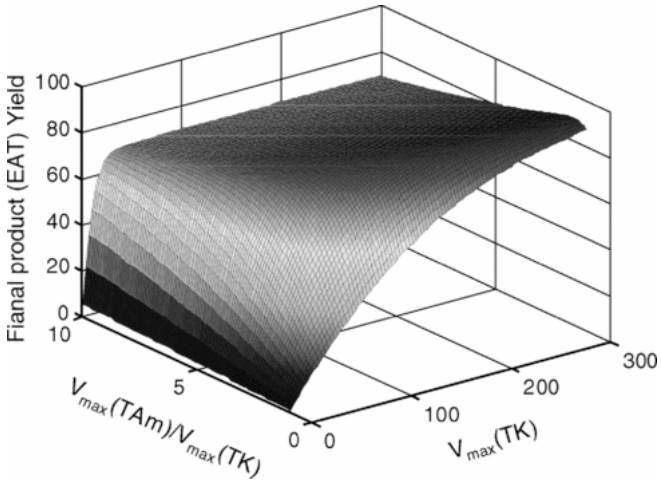


Table II. TAm reaction kinetic parameters (adapted from Shin & Kim 1998).

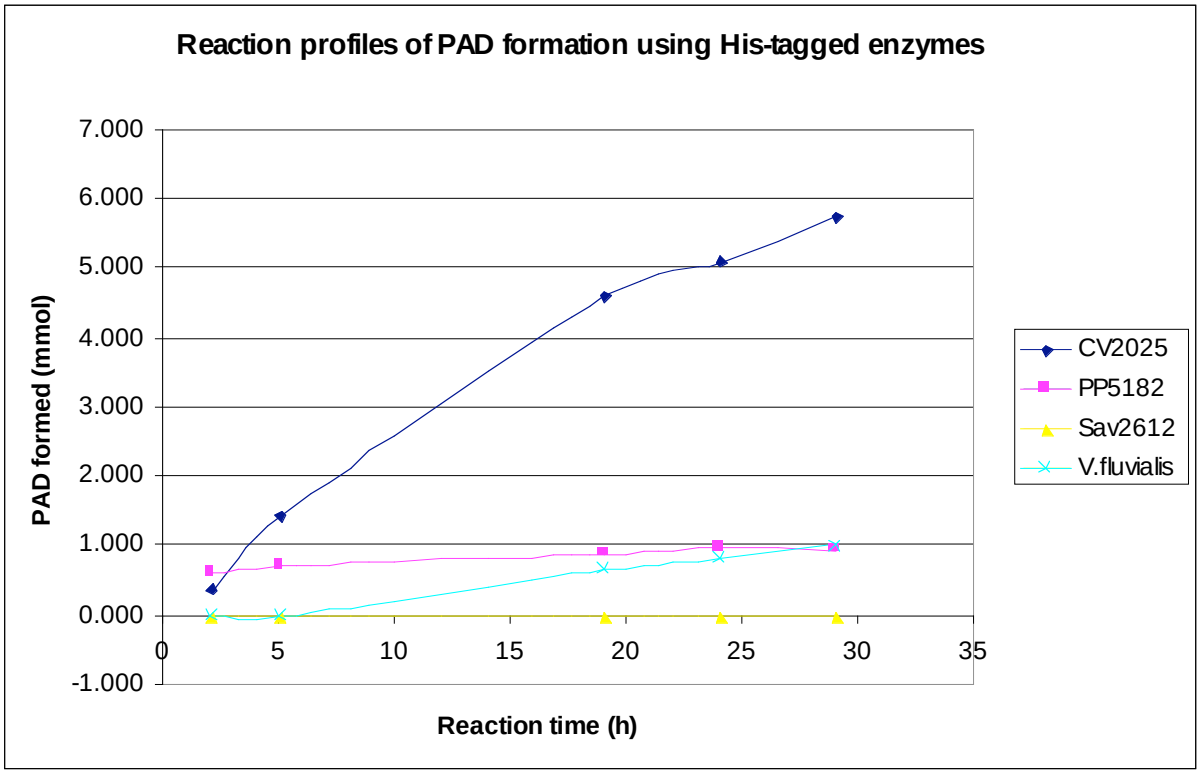
Kinetic parameters	Value
Forward velocity: V_f	0.42 mM min^{-1}
Reverse velocity: V_r	5.1810^{-4} mM min^{-1}
Michaelis constant for MBA: K_c	35.03 mM
Michaelis constant for ERY: K_q	9.85 mM
Michaelis constant for EAT: K_p	1.07 mM
Michaelis constant for ACP: K_r	0.54 mM
Inhibition constant for EAT in reverse reaction: K_{ips}	25.82 mM
Inhibition constant for ACP in reverse reaction: K_{irs}	1.24 mM
Inhibition constant for ERY: K_{iq}	1.0210^{-2} mM
Inhibition constant for MBA: K_{ic}	3.1410^{-2} mM
Inhibition constant for EAT: K_{ip}	2.85 mM
Inhibition constant for ACP: K_{iq}	0.13 mM

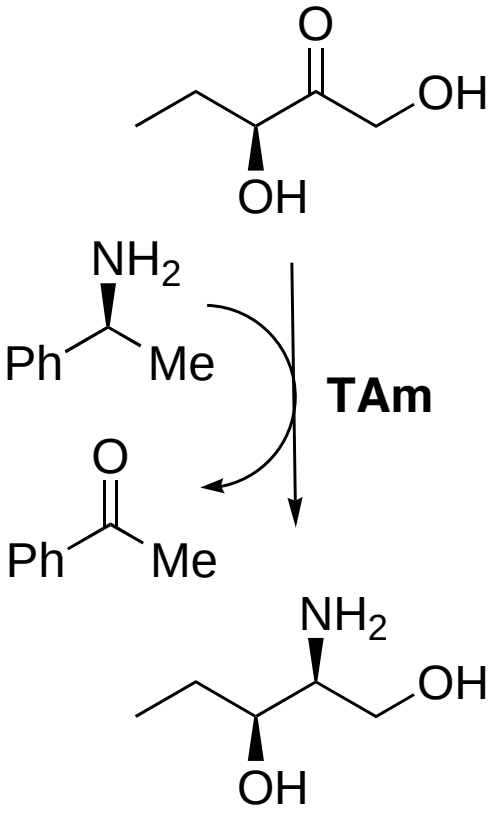


B.H.Chen, A.Sayar, U.Kaulmann, P.A.Dalby, J.M.Ward and .Woodley (2006) Biocatalysis and Biotransformation 24. 449-457

Aliphatic Ketodiol

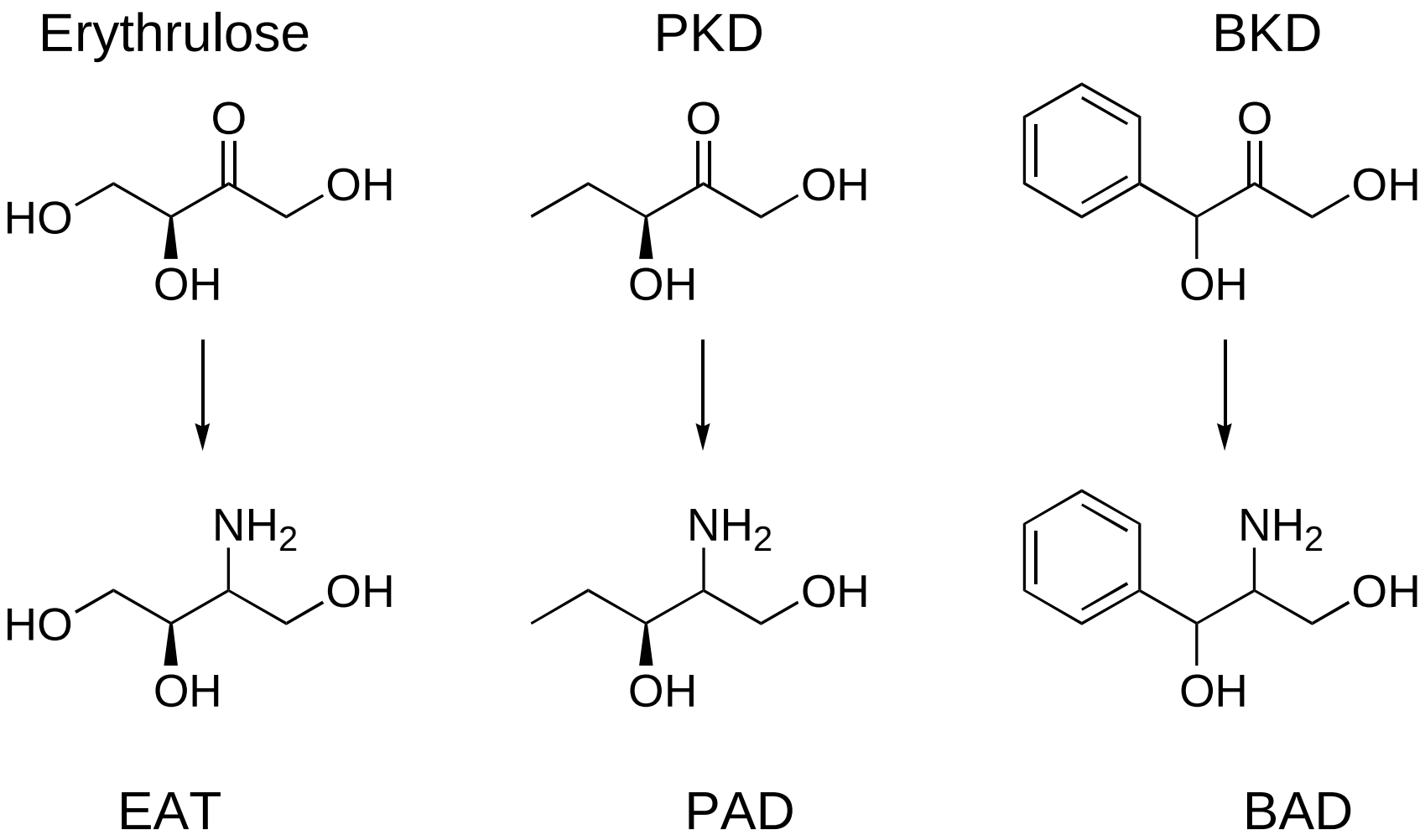
- Reaction profiles of 3 His-tagged and purified enzymes (CV2025, Sav2612 and PP5182) were measured for conversion of the ketodiols



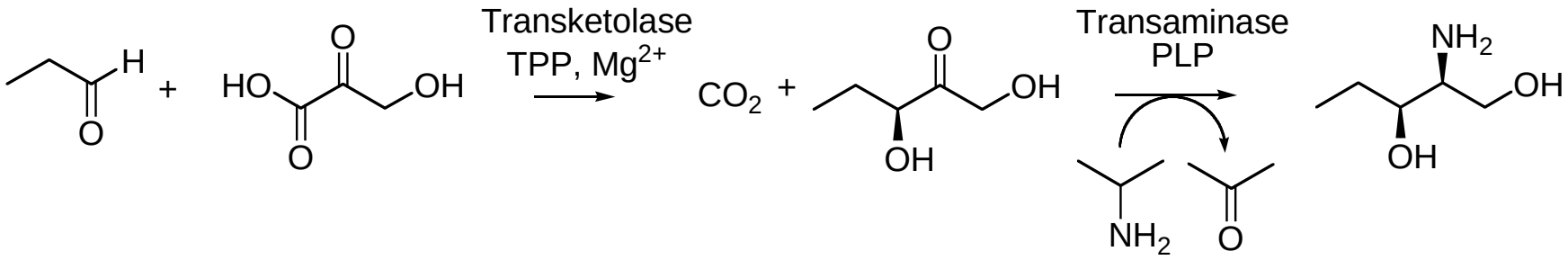


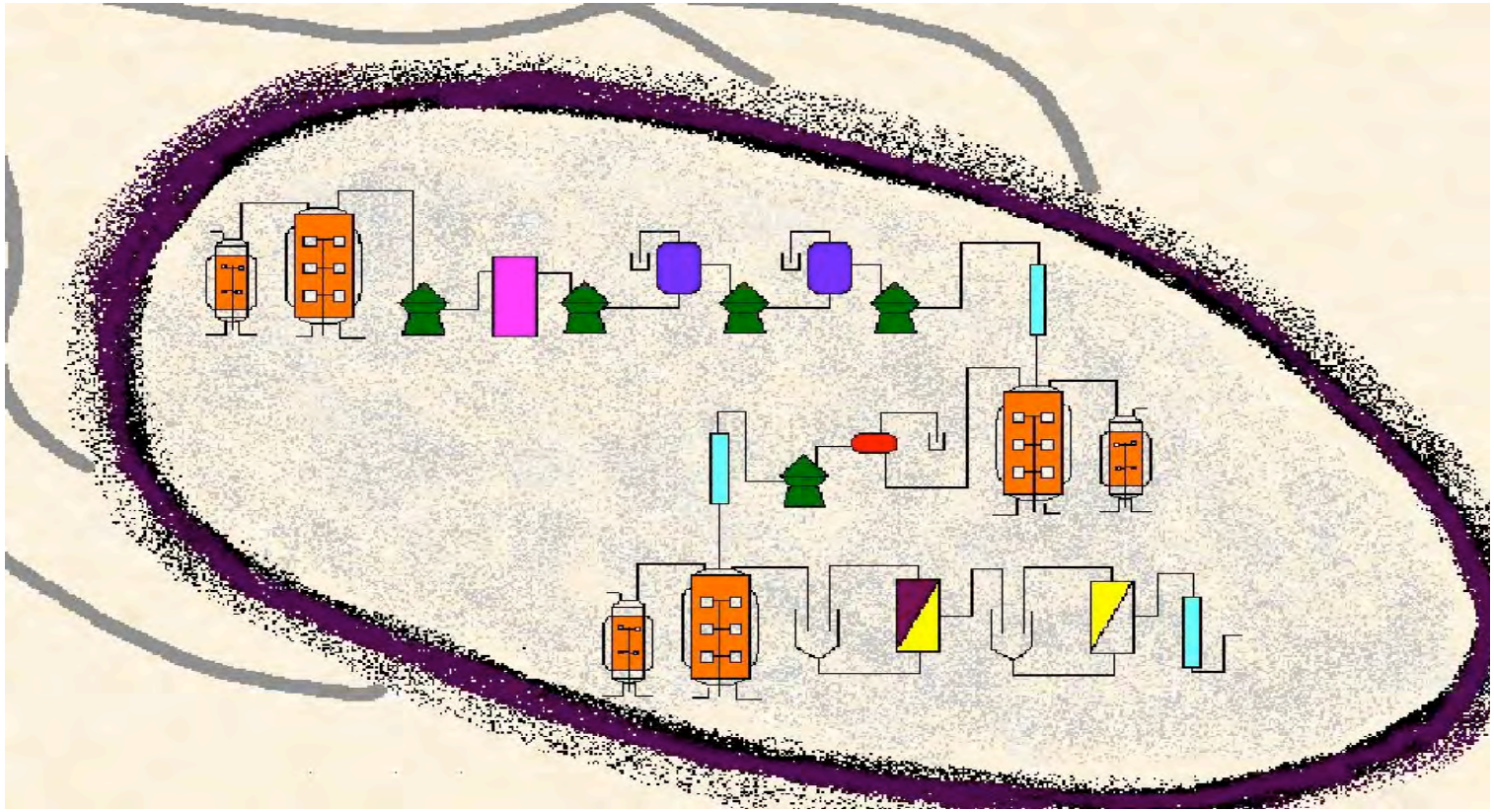
U. Kaulmann, K. Smithies, M. E. B. Smith, H. C. Hailes, and J. M. Ward, *Enz. Microb. Tech.*, **2007**, *41*, 628

Key Transaminase Reactions

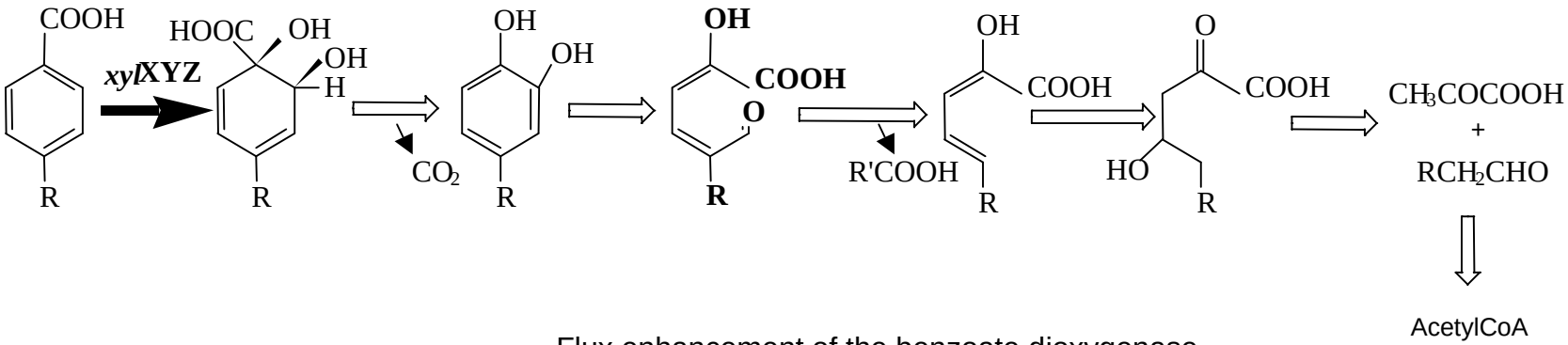


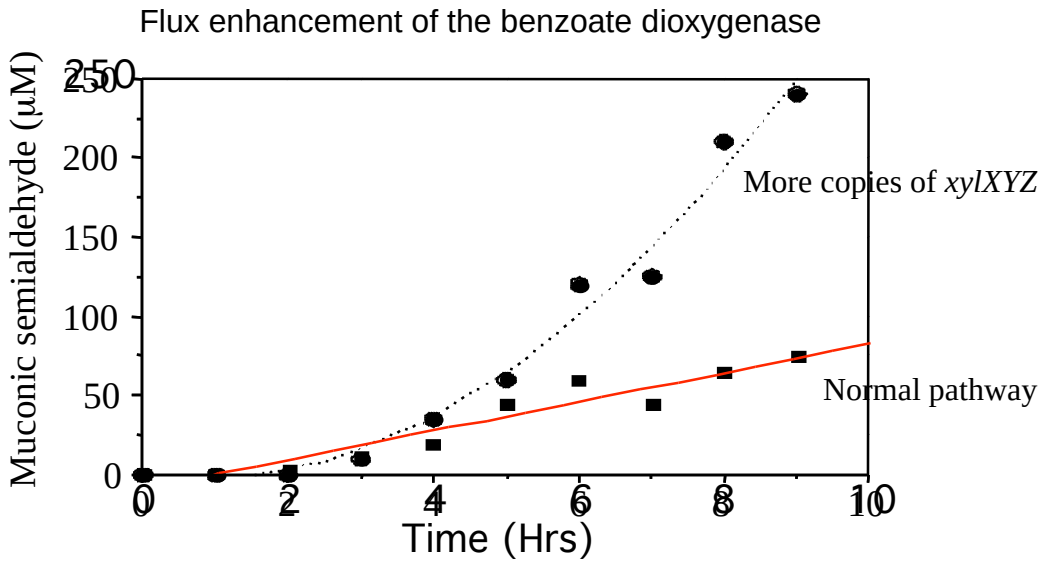
EAT: C. U.Ingram, M. Bommer, M. E. B. Smith, P. A. Dalby, J. M. Ward, H. C. Hailes, and G. J. Lye, *Biotech. Bioeng.* **2007**, *96,* 559–569.


Transketolase
TPP, Mg2+
CO2 +
Transaminase
PLP
NH2
OH
HO
O



Linkage Of Modelling To Genetics - Aromatic Oxidation (TOL Pathway)





Sheridan, R., Jackson, G.A., Regan, L., Ward, J.M. and Dunnill, P. (1998) Biotech. Bioeng. 58, 240-249

Biochemical Engineers

Synthetic biology allows creativity

Take what nature has given us, understand its workings

For enzymes this can be K_m, substrate preference, stability etc, then use as a discrete element in creating new pathways.

Use of sequenced genomes as a vast resource of enzymes.

Acknowledgements

At UCL:

Helen Hailes, Paul Dalby, Gary Lye, Frank Baganz, Ursula Kaulmann, Martina Micheletti, Bing Chen, Martin Bommer, John Woodley, Ed Hibbert, Mark Smith, Armando Cázares-Robles, James Galman, Haitham Hussain

At Exeter:

TK and TAm structures: Jenny Littlechild, Chris Sayer, Misha Isupov

BiCE collaborating companies (aminodiol project)

BBSRC LINK collaborating companies (P450 project)

EPSRC Engineering and Physical Sciences Research Council

BBSRC

REFERENCES

Hobbs, G.R., Lilly, M.D., Turner, N.J., Ward, J.M., Woodley, J.M., 1994. Development of transketolase as a catalyst for carbon-carbon bond formation. Progress Biotechnol. **9**, 463-466.

Lilly M. D., Chauhan R., French C., Gyeraha M., Hobbs G. R., Humphrey A., Isupov I., Littlechild J. A., Mitra R. K., Morris K.G., Rupprecht M., Turner N. J., Ward J. M., Willetts A. J. and Woodley J. M. (1996) Ann. New York Acad. Sci 782, 513-525 鼎arbon-carbon bond synthesis: The impact of rDNA technology on the production and use of *E. coli* transketolase'

French C., and Ward J. M. (1995) Biotech. Lett. 17, 247-252. 'Improved production and stability of *E. coli* recombinants expressing transketolase for large scale biotransformations.'

French C., and Ward J.M. (1996) Annal. New York Acad. Sci. 799, 11-18 Enzyme Engineering XIII 'Production and modification of E. coli transketolase for large-scale biocatalysis'

Sheridan, R., Jackson, G.A., Regan, L., Ward, J.M. and Dunnill, P. (1998) Biotech. Bioeng. 58, 240-249 'Rational engineering of the TOL *meta*-cleavage pathway.'

H. Hussain and J. M. Ward. (2003). Enhanced Heterologous Expression of two *Streptomyces griseolus* cytochrome P450s and *Streptomyces coelicolor* Ferredoxin Reductase as potentially efficient hydroxylation catalysts. Appl. Env. Microbiol. 69. 373-382

H. Hussain and J. M. Ward. (2003) Ferredoxin reductase enhances heterologously expressed cytochrome CYP105D1 in *Escherichia coli* and *Streptomyces lividans* Enzyme and Microbial Technology 32. 790-800

E. G. Hibbert, F. Baganz, H. C. Hailes, J. M Ward, G. J Lye, J. M Woodley and P. A Dalby (2005) 'Directed evolution of biocatalytic processes' BioMol Eng 22. 11-19

Ingram CU, Bommer M, Smith ME, Dalby PA, Ward JM, Hailes HC, Lye GJ. (2006) One-pot synthesis of amino-alcohols using a de-novo transketolase and beta-alanine:pyruvate transaminase pathway in Escherichia coli. Biotechnol Bioeng. 96:559-569

Smith ME, Kaulmann U, Ward JM, Hailes HC.(2006) A colorimetric assay for screening transketolase activity. Bioorg Med Chem. 2006 Oct 15;14(20):7062-5. Epub 2006 Jun 19.
PMID: 16784864

B.H.Chen, A.Sayar, U.Kaulmann, P.A.Dalby, J.M.Ward and J.M.Woodley (2006) 'Reaction modelling and simulation to assess the integrated use of transketolase and w-transaminase for the synthesis of an aminotriol' Biocatalysis and Biotransformation 24. 449-457

Kaulmann, U., Smithies, K., Smith, M.E.B., Hailes, H.C., and Ward, JM., (2007) Substrate spectrum of w-transaminase from *Chromobacterium violaceum* DSM30191 and its potential for biocatalysis. Enzyme and Microbial Technology. 41. 628-637

REFERENCES CONTD.

Hibbert, E.G., Senussi, T., Costelloe, S.J., Lei, W., Smith, M.E.B., Ward, J.M., Hailes, H.C., and Dalby, P.A. (2007) Journal of Biotechnology. 'Directed evolution of transketolase activity on non-phosphorylated substrates. 131: 425-432.

Costelloe, S.J., Ward, J.M., and Dalby, P.A. (2008) Evolution of the TPP-dependent enzyme family. Journal of Molecular Evolution. 66:36-49.

Hibbert, E.G., Senussi, T., Smith, M.E.B., Costelloe, S.J., Ward, J.M., Hailes, H.C., and Dalby, P.A. (2008) Directed evolution of transketolase substrate specificity towards an aliphatic aldehyde. Journal of Biotechnology, 134: 240-245

Hilker I, Guti驊rez MC, Furstoss R, Ward J, Wohlgemuth R, Alphand V. (2008) Preparative scale Baeyer-Villiger biooxidation at high concentration using recombinant Escherichia coli and *in situ* substrate feeding and product removal process. Nat Protoc. 3:546-554.

Ursula Schell, Roland Wohlgemuth and John M. Ward (2009) Synthesis of pyridoxamine 5'-phosphate using an MBA:pyruvate transaminase as biocatalyst. J. Mol. Catal. B: Enzymatic. (in press) doi:10.1016/j.molcatb.2008.10.005