MCA and Metabolic Engineering

C1net Workshop 2; Day 4

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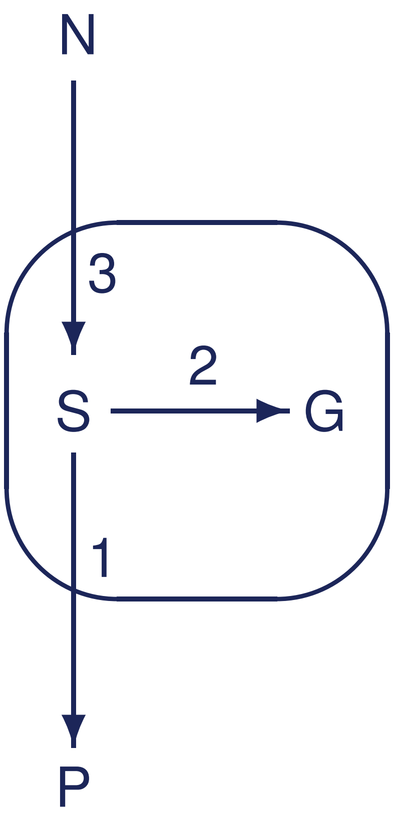
Engineering: multiple enzyme changes

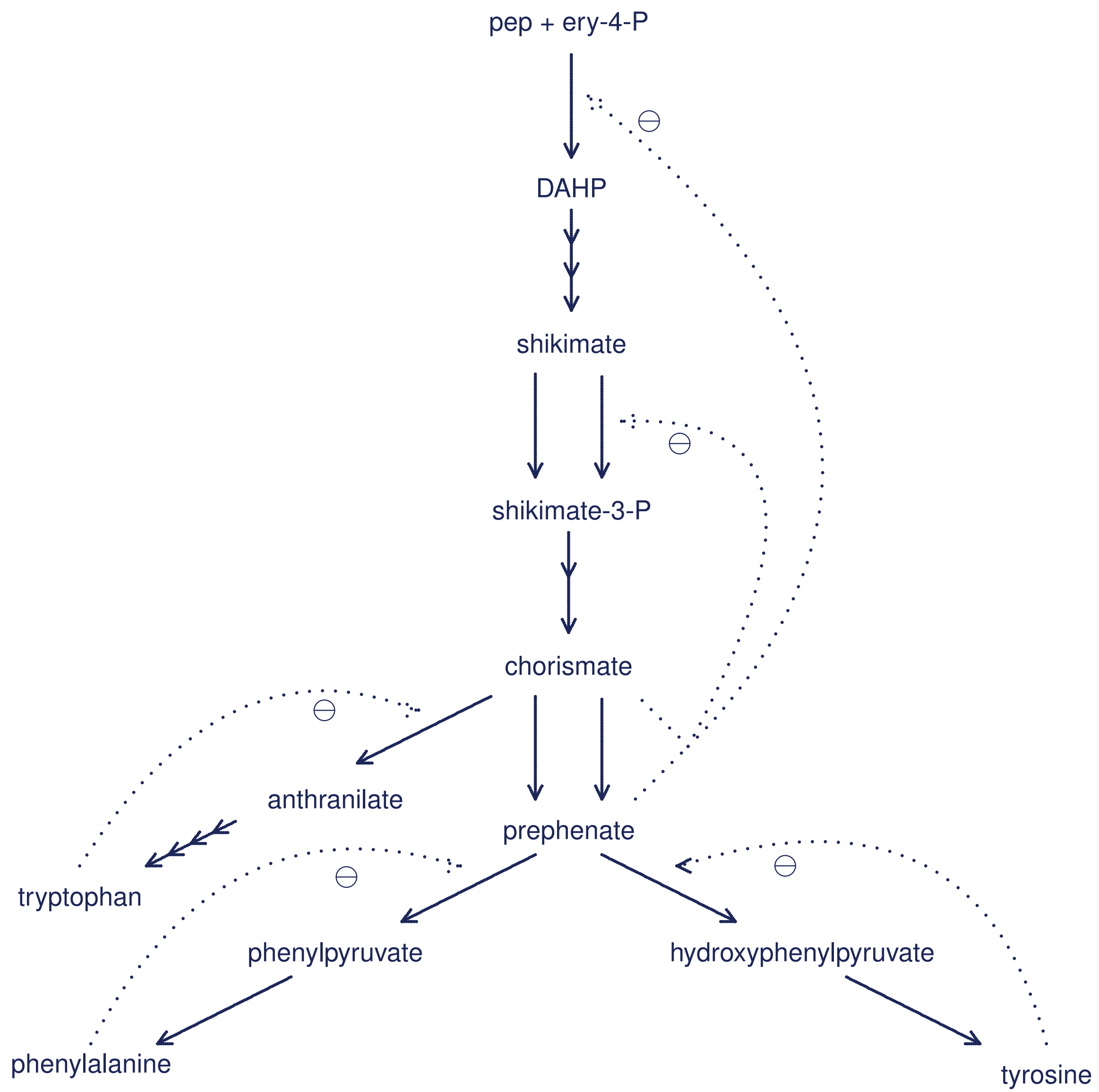
Kacser & Acerenza's Universal Method



To increase J_1 α–fold, enzymes are given:

- an increase in branch 1 of α–fold;
- a zero increase in branch 2, and
- an increase in branch 3 of $\alpha J_1/J_3$–fold

Aromatic amino acid synthesis



The example of yeast Trp biosynthesis

Genes overexpressed					Mean	Relative
2	4	1	3	5	change	trp flux
-	-	-	-	-	1	1.0
-	-	+	+	-	58	2.0
+	+	-	+	-	35	2.4
+*	-	+	+	-	34	1.2
+*	+	+	+	-	30	2.1
+	+	-	+	+	19	8.2
+*	+	+	+	+	23	8.8

'+' indicates the enzyme was overexpressed; '-' indicates wild–type level. The mean change column gives the average fold over–expression.

P. Niederberger et al, *Biochem. J. 287*, 473–479 (1992)

Penicillin synthesis — 1

There are 3 enzymes on the main route from amino acids to penicillin V.

aminoadipate + cys + val	
↓	ACVS
LLD–ACV	
↓	IPNS
isopenicillin N	
↓	IAT
penicillin V	

Penicillin synthesis — 2

Computer modelling and Control Analysis of the pathway in *Penicillium chrysogenum* show:

- control is distributed between the first two enzymes;
- the distribution changes with growth phase and O_2, and
- significantly increased flux will require activation of both enzymes.

P. N. Pissara et al,*Biotech. Bioeng. 51* 168–176, (1996).

Penicillin synthesis — 3

The pH–regulatory gene *pacC* induces expression of penicillin synthesis in *Aspergillus nidulans* in alkaline conditions.

Mutations in *pacC* that cause simultaneous increased expression of all 3 pathway enzymes stimulates penicillin production more than engineered over–expression of any one or pair of enzymes. E. A. Espeso et al. , *EMBO J. 12*, 3947–3956, (1993); M. A. Peñalva, ESF meeting abstract 1997.

In a low–productivity strain of *Penicillium chrysogenum*, simultaneous over-expression of all three enzymes results in the largest increase in penicilling productivity (up to 176%). (Theilgaard et al, Biotech. Bioeng. (2001) 72:379-88.)

Aphids, bacteria and amino acids

The pea aphid

Photo: Phil Sloderbeck, Kansas State University, Department of Entomology

Buchnera : amino acid factory

- Most aphids are dependent for survival on endosymbionts of the *Buchnera* genus that are present in the cells of the bacteriome.
- The *Buchnera* spp diverged from their nearest known relative *E. coli* 200 -250 million years ago.
- The bacteria cannot be cultured outside the aphids, but make essential amino acids.
- *Buchnera* contain plasmids. One of these, present in different copy numbers (1 – 21) in different aphids, contains the four genes of the whole leucine operon.

Bracho, A. M. et al, J. Mol. Evol. 41, 67–73 (1995)

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Examples from amino acid production

Particularly successful examples of increased production of amino acids by bacteria show features that support the 'Universal Method' analysis:

- More than one enzyme in the pathway must be activated for best results.
- Use of feedback resistant enzymes is necessary to decouple synthesis rate from utilisation rate and allow accumulation, but this is not well–tolerated as indicated by problems such as plasmid instability.

Aa examples: trp 1

Increased trp production in *E. coli* by:

- Overexpression of all trp enzymes on multicopy plasmids
- Feedback inhibition of anthranilate synthase and PRTase abolished
- Host strain used lacked trp repression and tryptophanase

but

- Anthranilate added for best yield.
- Poor growth and plasmid instability at high expression levels

S. Aiba et al, *Appl. Env. Microbiol. 43,* 289–297, (1982)

Aa examples: trp 2

Increased trp production in *Corynebacterium glutamicum* by:

- Starting with a phe, tyr auxotroph derepressed to overexpress all trp enzymes a few fold.
- Overexpressing DAHP synthase (8x) and all the trp genes (11x) after chorismate.
- Abolishing feedback on PRT to stop accumulation of toxic anthranilate caused by imbalance in ANS-PRT activities.

but

- Continuous selection pressure needed to retain plasmid.

small R. Katsumata & M. Ikeda, *Biotechnology 11*, 921–925, (1993).

Aa examples: aromatic precursors

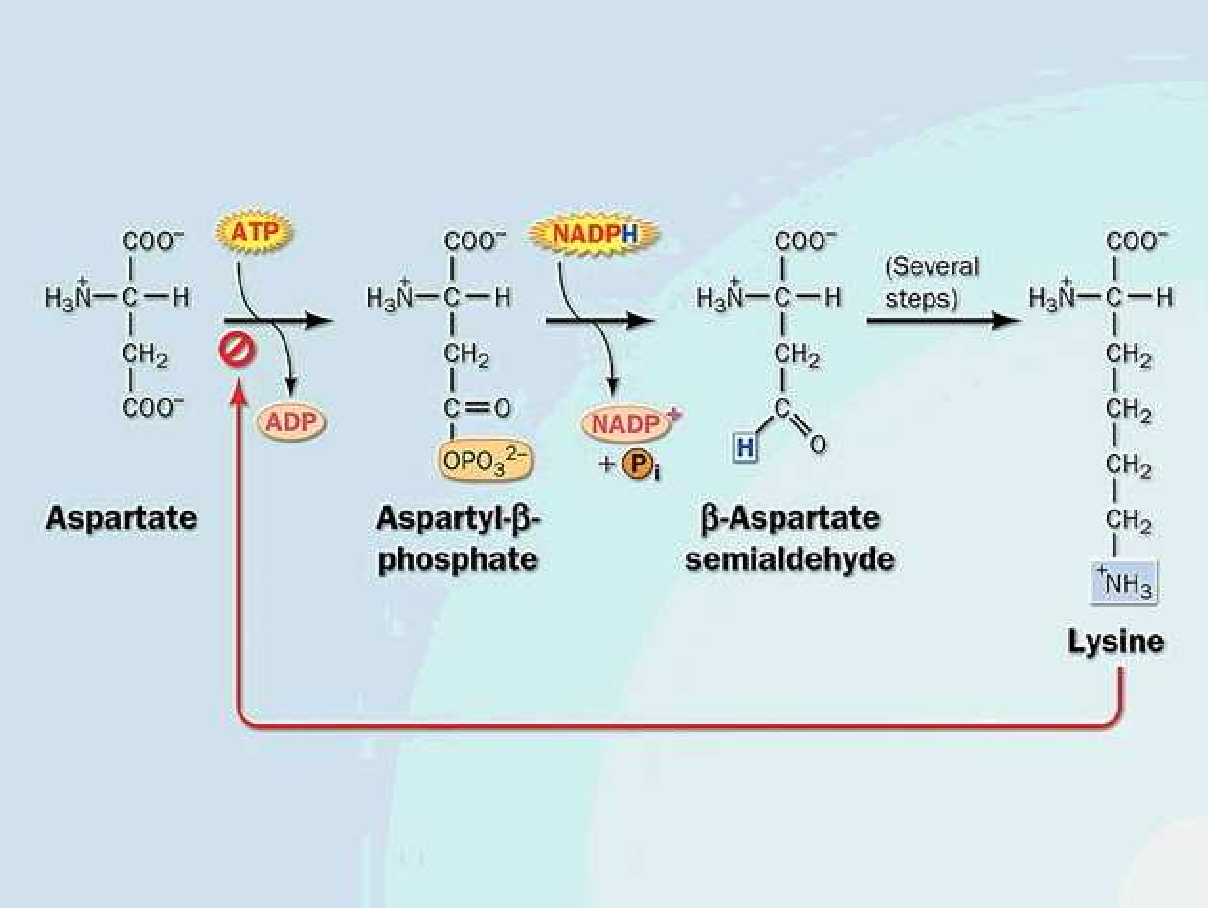
Synthesis of DAHP in *E. coli* studied in an *aroB* mutant that excretes DAHP.

- Synthesis increased by overproduction of a feedback resistant DAHP synthetase (AroG).
- Further increase obtained by overproduction of transketolase (yields E4P).
- Still further increase obtained overexpressing pyruvate, water dikinase to increase formation of PEP.

Pyruvate, water dikinase only has an effect when the other two enzymes are first overexpressed.

R. Patnaik et al, *Biotech. Bioeng. 46*, 361–370 (1995).

Lysine biosynthesis



small From:Lehninger Principles of Biochem. 3rd ed., 2000, CD ROM

Aa examples: lysine

In *Corynebacterium glutamicum*:,

- Of the 6 enzymes tested between asp and lys, only overexpression of feedback-resistant aspartate kinase and dihydrodipicolinate synthase leads to excretion of lysine.
- Overexpression of both these together gives higher yields than either separately.
- In aspartate kinase overexpressers, overexpression of phosphoenolpyruvate carboxylase increases lysine synthesis, though it has no effect alone.
- However, the plasmid coding the feedback resistant aspartate kinase is very unstable.

J. Cremer et al. *Appl. Env. Microbiol. 57*, 1746–1752 (1991)

In vivo evidence?

Simon Thomas and I[1] have proposed that cells achieve large increases in flux by

multisite modulation

not by activating single enzymes.

In brief, for pathways that show large changes in flux:

- Control sites are distributed throughout the pathway.
- Long–term adaptations involve coordinate induction or repression of all the enzymes.

[1] *Biochem. J. 311*, 35–39, (1995).

Engineering: increasing demand

Abolition of feedback inhibition?

Both practically and theoretically, is often ineffective. (Examples already mentioned include PFK and anthranilate synthase.)

Theoretical analysis[1,2] shows cooperativity of feedback inhibition is more important for metabolite homeostasis than for flux control. (Similarly, note that when yeast PFK is freed from control by its effector F–2,6–BP, metabolites change more than fluxes.[3])

[1] J. Hofmeyr & A. Cornish–Bowden, *Eur. J. Biochem. 200*, 223–236 (1991). [2] S. Thomas & D. Fell, *J. theor. Biol. 182,* 285–298 (1996). [3] J. Heinisch et al, *J. Biol. Chem. 271*, 15928–15933 (1996); E. Boles et al, Mol. Microbiol. 20, 65–76 (1996).

A simpler alternative:

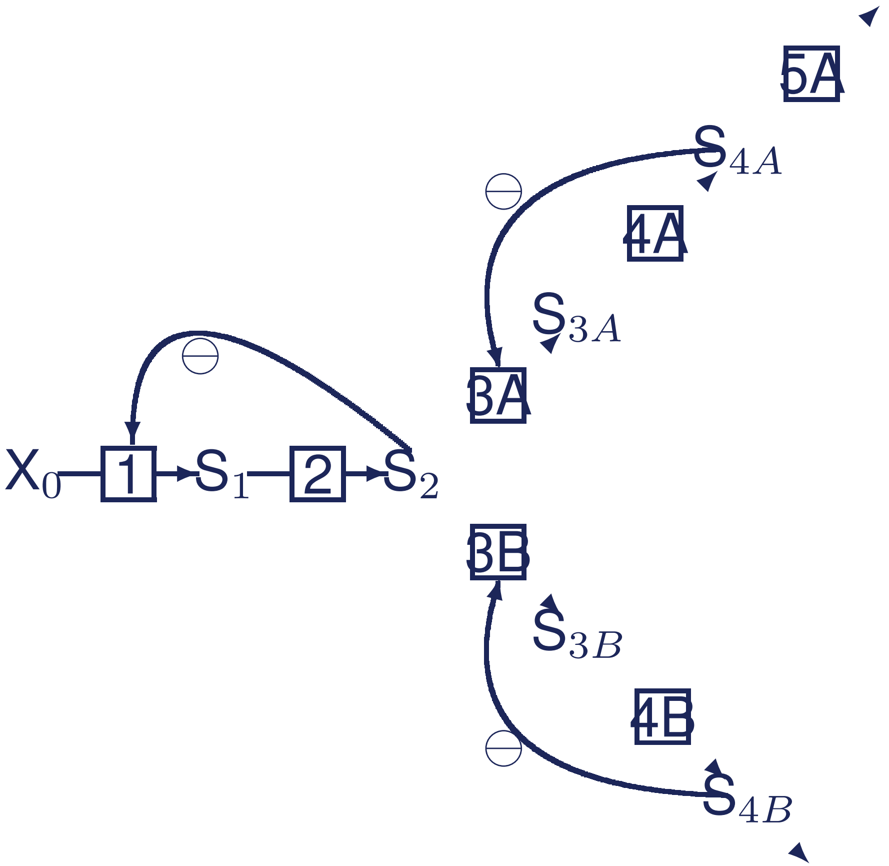
Increasing demand after a feedback loop.

In theory, this ‘subversion’ strategy might be almost as good as the Universal method.[1]

Paradoxically, this is probably why abolition of feedback inhibition in amino–acid producing strains of *C. glutamicum* **is** effective: the increased concentrations induce its specific excretion systems, creating increased demand.[2]

[1] Cornish–Bowden, A. *Biotechnology*, H–J Rehm et al (eds), Vol. 3, 121–136 (1995); Cornish–Bowden, A. et al, *Bioinorganic Chem. 23*, 439–449, (1995). [2] H. Sahm et al, *FEMS Micro. Rev. 16*, 243–252, (1995); R. Kelle et al, *Biotechnol. Bioeng. 51*, 40–50 (1996).

Pathway simulated by Cornish–Bowden et al.



Co–responses obtained on simulated engine

Enzyme change	ΔJ	Met:flux co–response			
		S_1:J	S_2:J	S_{3A}:J	S_{4A}:J
5e1	1.02	20.5	20.8	5.3	5.3
5e3A	1.08	0.3	-0.1	5.2	5.0
5(e1+e3A)	1.10	4.4	4.2	5.3	5.3
3(e1–e2)+ 5(e3A–e5A)	5.00	0.0	0.0	0.0	0.0
(e1+e3A)fr	1.31	14.3	13.9	24.7	24.7
5e5A	4.13	0.4	-0.2	0.9	-0.4

(fr = feedback resistant)

Calculated from Cornish–Bowden, A. et al, *Bioorganic Chem. 23*, 439–449, (1995).