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Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modelling, metabolic control and experimental analysis

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Everyone who has ever tried to radically change metabolic fluxes knows that it is often harder to determine which enzymes have to be modified than it is to actually implement these changes. In the more traditional genetic engineering approaches 'bottle-necks' are pinpointed using qualitative, intuitive approaches, but the alleviation of suspected 'rate-limiting' steps has not often been successful. Here the authors demonstrate that a model of pyruvate distribution in *Lactococcus lactis* based on enzyme kinetics in combination with metabolic control analysis clearly indicates the key control points in the flux to acetoin and diacetyl, important flavour compounds. The model presented here (available at http://jjj.biochem.sun.ac.za/wcfs.html) showed that the enzymes with the greatest effect on this flux resided outside the acetolactate synthase branch itself. Experiments confirmed the predictions of the model, i.e. knocking out lactate dehydrogenase and overexpressing NADH oxidase increased the flux through the acetolactate synthase branch from 0 to 75% of measured product formation rates.

Keywords: Metabolic control analysis, *in silico* modelling, *Lactococcus lactis*, pyruvate distribution

INTRODUCTION

Micro-organisms are not naturally optimized for maximal production rates of biotechnologically important compounds. Flavour compounds, such as diacetyl and acetaldehyde, are often secondary metabolites and are produced in insignificant amounts with respect to total carbon metabolism (Hugenholtz & Starrenburg, 1992). Redirecting the carbon flux towards such products without disturbing the overall cell physiology is a complex task. Metabolism is a highly branched system connected not only via carbon metabolites but also via redox [NAD(P)H] and Gibbs free energy (ATP) carriers. An intuitive approach to the optimization of fluxes towards the desired end products is not infallible in such a complex network and such an approach alone is unable to come with unexpected strategies. Several theoretical frameworks, such as biological system theory (Savageau, 1991), metabolic control analysis (MCA) (Kacser & Burns, 1973) and metabolic design (Kholodenko *et al.*, 1998), have been developed to analyse multi-enzyme systems predictively and quantitatively. In MCA, so-called flux-control coefficients, which quantify the importance of an enzyme for the magnitude of a flux, are defined as the percentage change in the flux caused by a 1% modulation of the enzyme activity. Consequently, MCA points at the enzymes that will have the largest effect on the desired flux upon a very small change in any enzyme activity, but it also has the disadvantage that it does not deal with the more relevant larger changes. Upon modulation of an enzyme with a high control coefficient, kinetic modelling can be used to integrate the system and to study the effect of a more substantial perturbation *in silico*. Because larger increases in flux are important in bioengineering, we propose to use an integrated approach between kinetic modelling, MCA and experimentation to come to a rational strategy for genetic

Abbreviations: ALS, acetolactate synthase; LDH, L-lactate dehydrogenase; MCA, metabolic control analysis; NOX, NADH oxidase; (abbreviations used in rate reactions and equations are defined in Table 1).

The GenBank accession number for the sequence reported in this paper is AY046926.

engineering. Here we illustrate this approach for the optimization of the metabolic flux through the acetolactate synthase (ALS) branch in lactic acid bacteria.

Lactic acid bacteria are used in milk fermentation and the major product from this process is lactate. For some dairy products, like butter, diacetyl produced by *Lactococcus lactis* is an important flavour component. Diacetyl is produced in only small amounts and various groups have tried to optimize its production (Monnet *et al*., 1994b; Platteeuw *et al*., 1995; Swindell *et al*., 1996; Lopez de Felipe & Hugenholtz, 1999). Their experimental strategies have involved the overexpression and deletion of genes intuited to be important for the regulation of the carbon fluxes in *L. lactis*. Here, we use a more rational combination strategy consisting of (i) a detailed kinetic model of the branches around pyruvate metabolism, (ii) MCA and (iii) experiments to describe the various mutations and to illustrate the use of (i) and (ii) in a more successful metabolic engineering strategy.

METHODS

Construction of an *ldh* deficient mutant of *L. lactis* NZ9000. To construct an *ldh*::*ery* replacement variant of *L. lactis* NZ9000 (Kuipers *et al*., 1997), a plasmid was constructed that allowed the direct selection of the desired mutant obtained by the occurrence of a double cross-over event. The upstream flanking region of the *ldh* gene of the *las* operon was amplified by PCR using MG1363 genomic DNA as a template and the primers PYKECO-5 (5′-CTCGT**GAATTC**GAAGTTGAAGC-3′) and LDHREV-6 (5′-ATCAG**CCATGG**TTTTCTTTAATTCC-3′) (*Eco*RI and *Nco*I sites in bold, respectively). Similarly, the downstream flanking region of the *ldh* gene of the *las* operon was amplified by PCR using the primers LDHDOW-4 (5′-GACA**CCATGG**CCGC**GGATCC**AAATAAAAAAGAGTTAGTAGAAAATTTCAAC-3′) and 317REV-12 (5′-CGCA**TCTAGA**CAACACCTCTAATCTTTAC-3′) (*Nco*I, *Bam*HI and *Xba*I sites in bold, respectively). Both the up- and downstream fragments, of approximately 1·1 kb each, were cloned as *Eco*RI–*Nco*I and *Nco*I–*Xba*I fragments, respectively, into similarly digested pUCNCO. The sequence of the cloned fragment was verified by automated sequence analysis with an ALF DNA sequencer (Pharmacia Biotech). Sequence reactions were performed according to the manufacturer's protocols, using the autoread sequencing kit and fluorescein-labelled universal M13 primers. Both of the *ldh* flanking regions were re-isolated as *Eco*RI–*Nco*I (5′) and *Nco*I–*Xba*I (3′) fragments and cloned into *Eco*RI–*Xba*I-digested pUC19 in a three-point ligation. A *Bam*HI–*Xba*I fragment of pUC19ERY (Kuipers *et al*., 1995), containing the erythromycin-resistance gene, was introduced into the *Bam*HI restriction site of the resulting pUCAB plasmid after the cohesive ends had been filled using the Klenow fragment of DNA polymerase I of *Escherichia coli*. The resulting plasmid, in which the orientation of the erythromycin gene was the same as the preceding *pyk* gene, was designated pUCAeryB. Finally, a *Swa*I–*Ecl*136II fragment of pGhost8 (Chopin *et al*., 1984), containing the tetracycline-resistance gene, was cloned into the *Xba*I site of pUCAeryB after filling in the *Xba*I cohesive ends using Klenow. The resulting plasmid, designated pUCAeryBTc, was transformed into competent *L. lactis* NZ9000 cells and erythromycin-resistant colonies were selected; the resulting transformants were analysed for tetracycline resistance by replica plating. Colonies displaying erythromycin resistance and tetracycline sensitivity should have been the result of a one-step double-cross-over event on both flanking regions of the *ldh* gene of the *las* operon and should have contained the desired *ldh*::*ery* replacement mutation. The anticipated genetic organization of the *ldh*::*ery* mutant candidates was verified by PCR and Southern analysis; the resulting *ldh*::*ery* variant of *L. lactis* NZ9000 was designated NZ9010.

Cloning of the lactococcal *nox* gene and construction of the *nox* overexpression plasmid. The *nox* gene of *Streptococcus mutans*, derived from pNZ2600 (Lopez de Felipe *et al*., 1998), was used as a heterologous probe in Southern blotting experiments to clone the NADH-oxidase(NOX)-encoding gene of *L. lactis* MG1363. A 1·8 kb fragment of *Eco*RI-digested chromosomal DNA of *L. lactis* MG1363 hybridized with this probe. The 1·8 kb fragment was cloned into *Eco*RI-digested pUC19 and colonies containing the hybridizing fragment were selected by colony blotting, again using the *nox* gene of *S. mutans* as a probe. Sequence analysis of the 1·8 kb fragment revealed that it contained the 3′ end of a gene that had high similarity with the *nox* gene of *S. mutans*, putatively representing the 3′ end of the lactococcal *nox* gene. Moreover, downstream of the 3′ end of this putative *nox* gene, ORFs were found that shared a high level of similarity (43·8 % at the protein sequence level) with the *ssb* gene of *B. subtilis*, encoding a putative single-stranded DNA-binding protein (Meyer & Laine, 1990). Following the *ssb* gene, the 5′ end of the previously cloned *groES* operon of *L. lactis* was found (Kim & Batt, 1993).

The 5′ end of the lactococcal *nox* gene was amplified by PCR using *L. lactis* MG1363 chromosomal DNA as a template and the fully degenerated primer NOX2F (5′-ACNGGNACYGAYCANGCNGCNGGYATHGC-3′; N = A/C/G/T, Y = C/T and H = A/C/T), based on the previously determined N-terminal sequence of the lactococcal NOX protein (Lopez de Felipe & Hugenholtz, 2001), combined with primer NOXR (5′-TGAC**CTGCAG**TTCTGCGTCAATTGCTTGACC-3′), which was based on the 3′ region of the cloned *nox* sequence, and was extended with a *Pst*I-generating clamp sequence (indicated in bold). The approximately 400 bp fragment obtained was cloned into pGEMT (Promega) and sequenced; the sequence revealed that the fragment contained the 5′ region of *nox*. This fragment (the *Nco*I–*Pst*I fragment from the pGEMT clone) was used as a probe in the cloning of a hybridizing 1·4 kb *Eco*RI *L. lactis* MG1363 chromosomal fragment. Sequence analysis of this fragment revealed that it contained the entire 5′ region of the lactococcal *nox* gene. Upstream of *nox*, an ORF encoding a hypothetical protein displaying local similarity with a phenylalanine tRNA ligase (78 % identity with the YdjD protein of *L. lactis* IL1403) was found. The sequence of the combined *Eco*RI fragments, containing the entire *noxE* sequence (93 % identity with the NoxE protein of *L. lactis* IL1403), has been deposited in the GenBank database under accession number AY046926.

The water-forming NOX-encoding *nox* gene of *L. lactis* was amplified by PCR using *L. lactis* MG1363 chromosomal DNA as a template and the primers NOXF (5′-CGTA**CCATGG**AAATCGTAGTTATCGGTAC-3′) and NOXR (5′-CGTA**TCTAGA**TTCAAAAGCCTGCCTACTGTGC-3′). The PCR fragment obtained was cloned as a *Nco*I–*Xba*I fragment (restriction sites were introduced into the primers and are indicated in bold) into similarly digested pNZ8048 (de Ruyter *et al*., 1996; Kuipers *et al*., 1997). The resulting plasmid was designated pNZ2610 and contained the lactococcal *nox* gene translationally fused to the *nisA* promoter. Transcription of *nox* in this construct is dependent on the activity of the nisin-

Table 1. Mathematical symbols and abbreviations for equations and kinetic models used in this study

Mathematical symbol/abbrevation	Definition
Mathematical symbols	
K_{eq}	Equilibrium constant
K_i	Inhibition constant
K_m	Affinity constant
N	Hill coefficient
v	Predicted enzyme activities
V^+	Maximal enzyme activities under saturating substrate and activator conditions, and in the absence of inhibitors
$\boldsymbol{k}$	Rate constant
Abbreviations	
AC	Acetate
ACAL	Acetaldehyde
ACALDH	Acetaldehyde dehydrogenase
ACCOA	Acetyl coenzyme A
ACET	Acetoin
ACETDH	Acetoin dehydrogenase
ACETEFF	Acetoin efflux
ACK	Acetate kinase
ACLAC	Acetolactate
ACP	Acetyl phosphate
ADH	Alcohol dehydrogenase
ALDC	Acetolactate decarboxylase
ALS	Acetolactate synthase
BUT	2,3-Butanediol
COA	Coenzyme A
ETOH	Ethanol
GLC	Glucose
GLYC	Glycolysis
LAC	Lactate
LDH	Lactate dehydrogenase
NEALC	Non-enzymic acetolactate decarboxylase
NOX	NADH oxidase
O	Oxygen
P	Inorganic phosphate
PDH	Pyruvate dehydrogenase
PTA	Phosphotransacetylase
PYR	Pyruvate

inducible *nisA* promoter, whereas translation of the *nox* transcript depends on the *nisA*-derived RBS (de Ruyter *et al*., 1996).

Control strain. *L. lactis* NZ9000(pNZ8048) was used as a control during this study.

Fermentation. Cultures were grown at 30 °C in M17 medium (Merck) supplemented with 1% (w/v) glucose. Chloramphenicol and erythromycin were used at 10 and 5 μg ml^{-1}, respectively. Nisin was used at 1 ng ml^{-1}. A 1 l bioreactor (Applikon Dependable Instruments) was inoculated with cells from an overnight culture to an initial OD_{600} of about 0·1 in 700 ml medium. A pH of 6·5 was maintained by the addition of 2 M NaOH and the stirrer speed was set at 500 r.p.m. Air was bubbled through with a flow rate of 520 ml min^{-1}. The batch cultures used showed exponential growth between about 2 and 5·5 h post-inoculation. The flux distribution was calculated during this exponential (pseudo steady-state) phase.

Analysis of fermentation products. Glucose, lactate, acetate, formate, ethanol, acetoin and 2,3-butanediol were analysed by HPLC, as described previously (Starrenburg & Hugenholtz, 1991). In contrast to the wild-type, the carbon recovery for the mutant strains was incomplete (between 70 and 85% recovery). This may have been due to activity of the pentose phosphate pathway, which leads to CO_2 release. However, this can not be confirmed as this pathway was not monitored in this study.

Growth and enzyme assays. The OD_{600} was determined and corrected for the optical density of the growth medium. When the OD_{600} value was too high ($>0·75$) the sample was diluted with medium.

Table 2. Rate equations used in this study

The reaction numbers correspond to those depicted in Fig. 1.

Reaction no.	Rate equation
1	$$v_{\mathrm{GLYC}}=\frac{V^{+}\left(\frac{\mathrm{GLC}}{K_{\mathrm{m,GLC}}}\right)\times\left(\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right)\times\left(\frac{\mathrm{ADP}}{K_{\mathrm{m,ADP}}}\right)}{\left(1+\frac{\mathrm{GLC}}{K_{\mathrm{m,GLC}}}+\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}\right)\times\left(1+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}\right)\times\left(1+\frac{\mathrm{ADP}}{K_{\mathrm{m,ADP}}}+\frac{\mathrm{ATP}}{K_{\mathrm{m,ATP}}}\right)}$$
2	$$v_{\mathrm{LDH}}=\frac{V^{+}\left(\frac{1}{K_{\mathrm{m,PYR}}\times K_{\mathrm{m,NADH}}}\right)\times\left(\mathrm{PYR}\times\mathrm{NADH}-\frac{\mathrm{LAC}\times\mathrm{NAD}}{K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}+\frac{\mathrm{LAC}}{K_{\mathrm{m,LAC}}}\right)\times\left(1+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right)}$$
3	$$v_{\mathrm{PDH}}=\frac{V^{+}\left(\frac{1}{1+K_{\mathrm{i}}\times\frac{\mathrm{NADH}}{\mathrm{NAD}}}\right)\times\left(\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}\right)\times\left(\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right)\times\left(\frac{\mathrm{COA}}{K_{\mathrm{m,COA}}}\right)}{\left(1+\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}\right)\times\left(1+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}\right)\times\left(1+\frac{\mathrm{COA}}{K_{\mathrm{m,COA}}}+\frac{\mathrm{ACCOA}}{K_{\mathrm{m,ACCOA}}}\right)}$$
4	$$v_{\mathrm{PTA}}=\frac{V^{+}\left(\frac{1}{K_{\mathrm{i,ACCOA}}\times K_{\mathrm{m,P}}}\right)\times\left(\mathrm{ACCOA}\times\mathrm{P}-\frac{\mathrm{ACP}\times\mathrm{COA}}{K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{ACCOA}}{K_{\mathrm{i,ACCOA}}}+\frac{\mathrm{P}}{K_{\mathrm{i,P}}}+\frac{\mathrm{ACP}}{K_{\mathrm{i,ACP}}}+\frac{\mathrm{COA}}{K_{\mathrm{i,COA}}}+((\mathrm{ACCOA}\times\mathrm{P})/(K_{\mathrm{i,ACCOA}}\times K_{\mathrm{m,P}})+((\mathrm{ACP}\times\mathrm{CAO})/(K_{\mathrm{m,ACP}}\times K_{\mathrm{i,COA}}))\right)}$$
5	$$v_{\mathrm{ACK}}=\frac{V^{+}\left(\frac{1}{K_{\mathrm{m,ADP}}\times K_{\mathrm{m,ACP}}}\right)\times\left(\mathrm{ACP}\times\mathrm{ADP}-\frac{\mathrm{AC}\times\mathrm{ATP}}{K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{ACP}}{K_{\mathrm{m,ACP}}}+\frac{\mathrm{AC}}{K_{\mathrm{m,AC}}}\right)\times\left(1+\frac{\mathrm{ADP}}{K_{\mathrm{m,ADP}}}+\frac{\mathrm{ATP}}{K_{\mathrm{m,ATP}}}\right)}$$
6	$$v_{\mathrm{ACALDH}}=\frac{V^{+}\left(\frac{1}{K_{\mathrm{m,ACCOA}}\times K_{\mathrm{m,NADH}}}\right)\times\left(\mathrm{ACCOA}\times\mathrm{NADH}-\frac{\mathrm{COA}\times\mathrm{NAD}\times\mathrm{ACAL}}{K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}\right)\times\left(1+\frac{\mathrm{ACCOA}}{K_{\mathrm{m,ACCOA}}}+\frac{\mathrm{COA}}{K_{\mathrm{m,COA}}}+\frac{\mathrm{ACAL}}{K_{\mathrm{m,ACAL}}}+\frac{\mathrm{ACAL}\times\mathrm{COA}}{K_{\mathrm{m,ACAL}}\times K_{\mathrm{m,COA}}}\right)}$$
7	$$v_{\mathrm{ADH}}=\frac{V^{+}\left(\frac{1}{K_{\mathrm{m,ACAL}}\times K_{\mathrm{m,NADH}}}\right)\times\left(\mathrm{ACAL}\times\mathrm{NADH}-\frac{\mathrm{ETOH}\times\mathrm{NAD}}{K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}\right)\times\left(1+\frac{\mathrm{ACAL}}{K_{\mathrm{m,ACAL}}}+\frac{\mathrm{ETOH}}{K_{\mathrm{m,ETOH}}}\right)}$$
8	$$v_{\mathrm{ALS}}=\frac{V^{+}\left(\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}\right)\times\left(1-\frac{\mathrm{ACLAC}}{\mathrm{PYR}\times K_{\mathrm{eq}}}\right)\times\left(\left(\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}+\frac{\mathrm{ACLAC}}{K_{\mathrm{m,ACLAC}}}\right)^{N-1}\right)}{\left(1+\left(\frac{\mathrm{PYR}}{K_{\mathrm{m,PYR}}}+\frac{\mathrm{ACLAC}}{K_{\mathrm{m,ACLAC}}}\right)^{N}\right)}$$
9	$$v_{\mathrm{ALDC}}=\frac{V^{+}\left(\frac{\mathrm{ACLAC}}{K_{\mathrm{m,ACLAC}}}\right)}{1+\frac{\mathrm{ACLAC}}{K_{\mathrm{m,ACLAC}}}+\frac{\mathrm{ACET}}{K_{\mathrm{m,ACET}}}}$$

Table 2 (*cont.*)

Reaction no.	Rate equation
10	$v_{\mathrm{ACETEFF}} = \dfrac{V^{+}\left(\dfrac{\mathrm{ACET}}{K_{\mathrm{m,ACET}}}\right)}{1+\left(\dfrac{\mathrm{ACET}}{K_{\mathrm{m,ACET}}}\right)}$
11	$v_{\mathrm{ACETDH}} = \dfrac{V^{+}\left(\dfrac{1}{K_{\mathrm{m,ACET}} \times K_{\mathrm{m,NADH}}}\right) \times \left(\mathrm{ACET} \times \mathrm{NADH} - \dfrac{\mathrm{BUT} \times \mathrm{NAD}}{K_{\mathrm{eq}}}\right)}{\left(1+\dfrac{\mathrm{ACET}}{K_{\mathrm{m,ACET}}}+\dfrac{\mathrm{BUT}}{K_{\mathrm{m,BUT}}}\right) \times \left(1+\dfrac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}+\dfrac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right)}$
12	$v_{\mathrm{ATPase}} = \dfrac{V^{+}\left(\dfrac{\mathrm{ATP}}{\mathrm{ADP} \times K_{\mathrm{m,ATP}}}\right)^{N}}{1+\left(\dfrac{\mathrm{ATP}}{\mathrm{ADP} \times K_{\mathrm{m,ATP}}}\right)^{N}}$
13	$v_{\mathrm{NOX}} = \dfrac{V^{+}\left(\dfrac{\mathrm{NADH} \times \mathrm{O}}{K_{\mathrm{m,NADH}} \times K_{\mathrm{m,O}}}\right)}{\left(1+\dfrac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}+\dfrac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right) \times \left(1+\dfrac{\mathrm{O}}{K_{\mathrm{m,O}}}\right)}$
14	$v_{\mathrm{NEALC}} = \boldsymbol{k} \times \mathrm{ACLAC}$

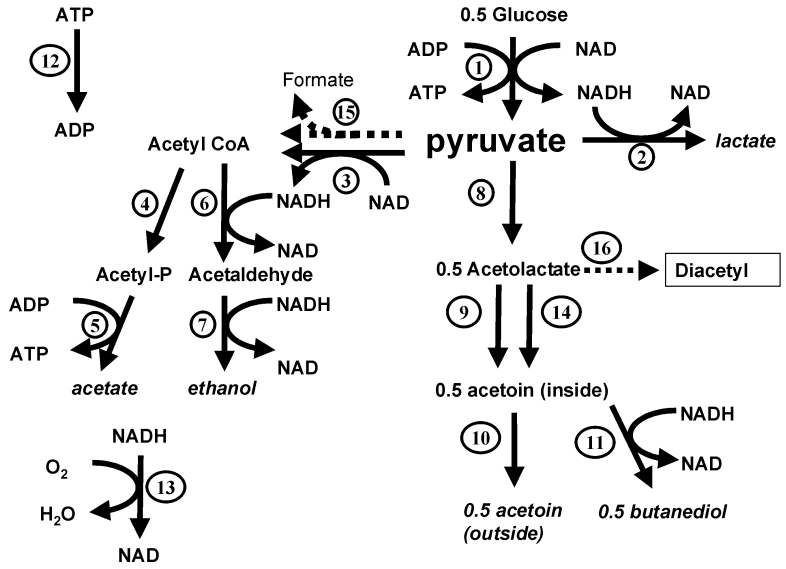

Fig. 1. Reactions included in the model to describe the distribution of carbon from pyruvate in *L. lactis*. Numbers in circles indicate the following enzymes or steps: 1, 'lumped' glycolysis; 2, LDH; 3, pyruvate dehydrogenase; 4, phosphotransacetylase; 5, acetate kinase; 6, acetaldehyde dehydrogenase; 7, alcohol dehydrogenase; 8, ALS; 9, acetolactate decarboxylase; 10, acetoin efflux; 11, acetoin dehydrogenase; 12, ATPase; 13, NOX; 14, non-enzymic acetolactate decarboxylation; 15, pyruvate formate lyase, which is considered not to be active under aerobic conditions (see Table 3); 16, chemical conversion to diacetyl, not included in the model. Substrates and products that were clamped in the model are indicated in italics.

L-Lactate dehydrogenase (LDH) activity was determined by the method of Hillier & Jago (1982) and the NOX and ALS activities were determined according to Lopez de Felipe *et al*. (1998) and Platteeuw *et al*. (1995), respectively. Protein concentrations were determined according to the Bradford method (Bradford, 1976), with BSA as a standard. Preliminary experiments along these lines have been published previously (Platteeuw *et al*., 1995; Lopez de Felipe *et al*., 1998), but they were not performed in an isogenic background and did not contain precise measurements of catabolic fluxes. Instead, they consisted merely of end-point determinations of product concentrations. This made them unsuitable for a proper demonstration of the rational engineering procedure we propose here.

Table 3. Kinetic parameters used for the enzymes in reactions 1–15

Unless indicated otherwise, the data are from *L. lactis*. The numbers of the reactions correspond with those depicted in Fig. 1 and the rate equations in Table 2.

Reaction no.	Kinetic parameter	Value*	Reference/derivation†
1, v_{GLYC}	V^+	2397	Obtained by 'fitting'
	$K_{m,\ PYR}$	25	Set in this study
	$K_{m,\ GLC}$	0·1	Set in this study
	$K_{m,\ NAD}$	0·14	Obtained by 'fitting'
	$K_{m,\ NADH}$	0·09	Obtained by 'fitting'
	$K_{m,\ ADP}$	0·047	Obtained by 'fitting'
	$K_{m,\ ATP}$	0·019	Obtained by 'fitting'
2, v_{LDH}	V^+	5118	This study and in agreement with Hugenholtz & Starrenburg (1992)
	K_{eq}	21120·7	Thauer *et al.* (1977)
	$K_{m,\ NADH}$	0·08	
	$K_{m,\ PYR}$	1·5	
	$K_{m,\ NAD}$	2·4	
	$K_{m,\ LAC}$	100	Crow & Pritchard (1977)
3, v_{PDH}	V^+	259	
	$K_{m,\ PYR}$	1	Snoep *et al.* (1992a)
	$K_{m,\ NAD}$	0·4	Snoep *et al.* (1992b)
	$K_{m,\ COA}$	0·014	
	$K_{m,\ ACCOA}$	0·008	*Azotobacter*; Bresters *et al.* (1975)
	$K_{m,\ NADH}$	0·1	
	K_i	46·4	Estimated from Snoep *et al.* (1993)
4, v_{PTA}	V^+	42	Abbe *et al.* (1982)
	$K_{i,\ ACCOA}$	0·2	
	$K_{m,\ P}$	2·6	
	$K_{i,\ P}$	2·6	
	$K_{m,\ ACP}$	0·7	
	$K_{i,\ ACP}$	0·2	
	$K_{i,\ COA}$	0·029	
	$K_{m,\ COA}$	0·12	*Clostridium kluyverii*; Henkin & Abeles (1976)
	K_{eq}	0·0281	Thauer *et al.* (1977)
5, v_{ACK}	V^+	2700	*S. mutans*; Abbe *et al.* (1982)
	$K_{m,\ ACP}$	0·16	
	$K_{m,\ ADP}$	0·5	
	$K_{m,\ AC}$	7	
	$K_{m,\ ATP}$	0·07	*E. coli*; Fox & Roseman (1986)
	K_{eq}	174·2	Thauer *et al.* (1977)
6, v_{ACALDH}	V^+	97	*S. mutans*; Abbe *et al.* (1982)
	$K_{m,\ ACCOA}$	0·007	
	$K_{m,\ NADH}$	0·025	
	$K_{m,\ NAD}$	0·08	
	$K_{m,\ COA}$	0·008	
	$K_{m,\ ACAL}$	10	*E. coli*; Shone & Fromm (1981)
	K_{eq}	1	Thauer *et al.* (1977)
7, v_{ADH}	V^+	162	Cachon & Divies (1993)
	$K_{m,\ ACAL}$	0·03	
	$K_{m,\ NADH}$	0·05	
	$K_{m,\ NAD}$	0·08	
	$K_{m,\ ETOH}$	1	*Zymomonas*; Wills *et al.* (1981)
	K_{eq}	12354·9	Thauer *et al.* (1977)

Table 3 (*cont.*)

Reaction no.	Kinetic parameter	Value*	Reference/derivation†
8, v_{ALS}	V^+	600	Snoep *et al.* (1992a); Platteeuw *et al.* (1995)
	$K_{m,\ PYR}$	50	
	N	2·4	Snoep *et al.* (1992a)
	$K_{m,\ ACLAC}$	100	Guessed; introduced to represent product sensitivity of the reaction
	K_{eq}	9×10^{12}	Overexpressed
	V^+	6764	Platteeuw *et al.* (1995)
9, v_{ALDC}	V^+	106	Swindell *et al.* (1996)
	$K_{m,\ ACLAC}$	10	Monnet *et al.* (1994a)
	$K_{m,\ ACET}$	100	Guessed
10, $v_{ACETEFF}$	V^+	200	
	$K_{m,\ ACET}$	5	Guessed
11, v_{ACETDH}	V^+	105	Petit *et al.* (1989)
	$K_{m,\ ACET}$	0·06	
	$K_{m,\ NADH}$	0·02	Gibson *et al.* (1991)
	K_{eq}	1400	Strecker & Harary (1954)
	$K_{m,\ BUT}$	2·6	
	$K_{m,\ NAD}$	0·16	*Enterobacter*; Carballo *et al.* (1991)
12, v_{ATPase}	V^+	900	Obtained by 'fitting'
	$K_{m,\ ATP}$	6·2	Set in this study
	N	2·6	Set in this study
13, v_{NOX}	V^+	118	Wild-type; NOX overexpressed
	V^+	4117	This study
	$K_{m,\ NADH}$	0·041	*S. faecalis*; Schmidt *et al.* (1986)
	$K_{m,\ NAD}$	1·0	Guessed
	$K_{m,\ O}$	0·01	Guessed
14, v_{NEALC}	$\boldsymbol{k}$	0·0003	Monnet *et al.* (1994b)
15			

* Units for each parameter are: V^+, mmol (l internal vol.)$^{-1}$ min^{-1}; K_m, mM; K_{eq}, mM; $\boldsymbol{k}$, min^{-1}.

† The kinetic parameters of the glycolytic step (reaction 1) and the ATP consumption (reaction 12) were estimated by fitting the model to the glucose fluxes of oxygenated and anaerobically [160 and 190 mmol (l internal vol.)$^{-1}$ min^{-1}, respectively] grown wild-type *L. lactis* (strain NZ9000). For this estimation and MCA we used GEPASI (Mendes, 1993). The kinetic parameters of the other enzymes were derived from the literature. Under aerobic conditions pyruvate formate lyase is considered not to be active (Abbe *et al.*, 1982); therefore, reaction 15 was not included in the model.

The kinetic model. A set of ordinary differential equations was used to describe the time dependence of the metabolite concentrations. Table 1 gives definitions of the mathematical symbols and abbreviations used in the equations and kinetic models in this study. Please note that the flux of glycolysis is given in C_3 units.

$$d[PYR]/dt = v_{GLYC} - v_{LDH} - v_{PDH} - 2v_{ALS}$$
$$d[ACP]/dt = v_{PTA} - v_{ACK}$$
$$d[ACAL]/dt = v_{ACALDH} - v_{ADH}$$
$$d[ACLAC]/dt = v_{ALS} - v_{ALDC} - v_{NEALC}$$
$$d[ACET]/dt = v_{ALDC} + v_{NEALC} - v_{ACETDH} - v_{ACETEFF}$$
$$d[ATP]/dt = v_{GLYC} + v_{ACK} - v_{ATPase}$$
$$[ADP] + [ATP] = \text{constant}$$
$$d[NADH]/dt = v_{GLYC} + v_{PDH} - v_{LDH} - v_{ACALDH} - v_{ADH} - v_{ACETDH} - v_{NOX}$$
$$[NAD] + [NADH] = \text{constant}$$
$$d[ACCOA]/dt = v_{PDH} - v_{ACALDH} - v_{PTA}$$
$$[COA] + [ACCOA] = \text{constant}$$

Most of the enzymes were modelled using a reversible Michaelis–Menten equation with noncompeting substrate–product couples. When the specific enzyme has been characterized with respect to the kinetic type, then this type was used. For v_{PDH} (reaction 3) an inhibition by a high NADH:NAD ratio has been described and these kinetics have been included in the equation. For v_{PTA} (reaction 4) the equation was derived from Henkin & Abeles (1976). For v_{ALS} (reaction 8) the reversible Hill equation, as derived by Hofmeyr & Cornish-Bowden (1997), was used. For v_{ATPase} (reaction 12) an irreversible Hill equation, sensitive to the ATP:ADP ratio, was used. For reaction 9 and 12 kinetics for an irreversible reacion were used. Simple Michaelis–Menten kinetics were used for reaction 10. Reaction 14 represents non-enzymic decarboxylation of acetolactate. The rate equations shown in Table 2 were used.

The concentrations of the following metabolites were assumed to be constant: glucose, 15 mM; lactate, 1 mM; inorganic phosphate, 10 mM; ethanol, 1 mM; butanediol, 0·01 mM; oxygen, 0·2 mM; and acetate, 1 mM. The sums of

[ATP]+[ADP], [NAD]+[NADH] and [coenzyme A]+[acetyl coenzyme A] were assumed to be constant at the respective values of 5, 10 and 1 mM.

Kashket & Wilson (1973) found that in *L. lactis* the internal cell volume is 1·5 μl (mg dry weight)$^{-1}$. We assumed that protein makes up 50% of the dry weight, as it does in *E. coli* (Neidhardt & Umbarger, 1996), and that an OD_{600} of 1 is equal to 0·445 mg dry weight ml^{-1}.

Kinetic parameters. The kinetic parameters used for the enzymes in reactions 1–15 (Fig. 1) and the rate equations in Table 2 can be found in Table 3.

RESULTS

Modelling the pyruvate branches in *L. lactis*

We first constructed a kinetic model describing the flux distribution over the pyruvate branches in *L. lactis* (Fig. 1). Glycolysis was modelled as a single irreversible step (Fig. 1, 1) with product inhibition; the ATP-consuming reactions (Fig. 1, 12) were grouped in a single module. The kinetic parameters of these two modules were estimated on the basis of 'fits' to *in vivo* measurements of fluxes (see Methods and Table 3). In contrast to these unstructured modules, all of the kinetic steps in the pyruvate branches were modelled in detail. *In vitro* kinetic data from the literature were used as input. A detailed description of the model is given in Methods and Table 3. The model prediction of the flux distribution in the wild-type strain (white lettering) as compared to the experimental data (black lettering) is shown in Fig. 2(a). The model predicted an essentially (97%) homolactic fermentation, which is in agreement with experimental observations (Fig. 2), where between 90 and 100% of the glucose is converted to lactate (Hugenholtz & Starrenburg, 1992; Platteeuw *et al*., 1995). The flux through the acetolactate branch was predicted to be less than 0·1% of the glucose influx, in agreement with what was observed experimentally (Fig. 2a). Thus, in the wild-type strain only a negligible flux was directed towards the branch of interest and this can be understood on the basis of the known enzyme kinetics (i.e. the model gives a good description of this condition). What strategy can now be followed to optimize the flux through this branch?

MCA quantifies the importance of each of the enzymes in controlling the flux through the acetolactate branch. Thus, the enzyme with the highest flux-control coefficient is the enzyme that will have the biggest effect upon a small modulation around its wild-type level, suggesting that MCA is a good approach for genetic engineering. But is it?

MCA versus the intuitive approach

In an intuitive approach ALS would be the enzyme of choice for genetic engineering, as it is the first dedicated enzyme in the branch and it is far from equilibrium. But what does MCA say? At first sight it might seem to say the same, i.e. in the wild-type situation the ALS has a flux-control coefficient of 1·0 for the flux to acetoin (Table 4). This qualifies as a high flux-control coefficient, as flux-control coefficients always sum up to 1. However, flux-control coefficients can be negative and the complete MCA analysis shows how relevant this is for this branched pathway – the highest flux-control coefficients for the acetolactate branch reside in enzymes outside this branch! (Namely LDH and NOX; Table 4). This corresponds to an early MCA observation for branched pathways, in which the fluxes over the different branches differ by orders of magnitude (Kacser, 1983). With hindsight, this can be understood also intuitively: a minor decrease in any branch carrying a major flux from pyruvate might increase the pyruvate concentration more than proportionately provided that the concentration exceeded the apparent Michaelis constant for that major pathway. This could then result in a more than proportional increase in flux through the ALS branch.

MCA suggested that ALS should have a substantial control on the ALS flux, implying that a doubling of the activity of the enzyme could at most double the very small flux towards acetoin. However, we were seeking much more substantial increases. Moreover, the overexpression of enzymes with high control coefficients is often not successful because the control coefficient tends to decrease strongly as the enzyme is overexpressed (i.e. the enzyme stops being limiting). A calculation with the kinetic model (not shown) showed that increasing the amount of ALS by a factor of 20 [the overexpression level found in Platteeuw *et al*. (1995)] increased the ALS flux to only 0·02% of the glucose flux. Indeed, Platteeuw *et al*. (1995) demonstrated that in anaerobically cultured *L. lactis* (MG5267) overexpression of ALS did not increase the production of acetoin and 2,3-butanediol. Therefore, we decided that overexpression of ALS was not going to be a successful strategy to increase the flux through ALS.

LDH knockout

LDH has the highest (negative) flux-control coefficient (−2·3; Table 4). Thus, a 1% reduction in LDH activity should lead to a 2·3% increase in the flux through the acetolactate branch. After MCA has indicated the best candidate for genetic engineering, modulations substantially exceeding 1% can be test-run in the kinetic model. Such a test-run is an important part of the genetic engineering strategy, since MCA is defined at the original steady state and the size of the optimal perturbation might be limited due to kinetic restrictions in the system. For example, if the acetolactate dehydrogenase and pyruvate dehydrogenase complex branches have only a low capacity these branches might not be able to absorb all of the carbon flow from lactate upon a knockout mutation in LDH. Such an effect was observed in the model calculations, where pyruvate and NADH accumulated upon a deletion in LDH and the glycolytic flux was severely inhibited (Fig. 2b). Such a major perturbation of primary metabolism is bound to lead to a strong

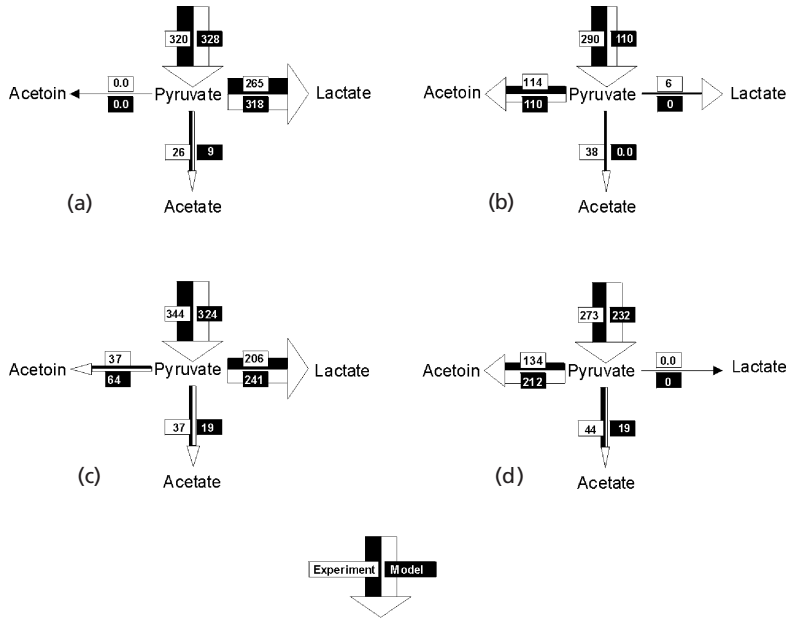

Fig. 2. Flux distribution over the pyruvate branches in *L. lactis*. Experimental results are shown in black letters on a white background, whereas model predictions are shown in white letters on a black background. The fluxes are given in mmol (l internal vol.)$^{-1}$ min^{-1} [multiply by 290 to obtain μmol (mg protein)$^{-1}$ min^{-1}]. (a) Wild-type; (b) Ldh^{-} mutant; (c) NOX overexpression mutant; (d) Ldh^{-} and NOX overexpression mutant. As can be seen from the sum of the fluxes, the carbon balance is not complete in all strains. In (b, d) pyruvate that accumulated in the medium accounted for up to 15% of the carbon flux.

Table 4. Control coefficients of the different steps/enzymes on the flux through ALS as predicted by the model for the wild-type and three mutants

The only steps included are those that have a control coefficient of at least 0·1 in any of the mutants. NOX^{++}, NOX overexpression.

Step/enzyme	Control coefficient			
	Wild-type	Ldh^{-} mutant	NOX^{++} mutant	Ldh^{-} NOX^{++} mutant
Glycolysis	1·6	0·0	1·0	0·0
ATPase	0·8	0·0	0·5	0·0
LDH	−2·3	0·0	−1·1	0·0
NOX	1·5	0·9	1·0	0·0
ALS	1·0	0·0	0·1	0·0
Acetoin decarboxylase	0·0	0·0	0·0	1·0
Phosphotransacetylase	−0·6	0·0	−0·3	0·0
Pyruvate dehydogenase	−0·8	0·0	−0·1	0·0

reduction in growth rate and possibly to deleterious effects in Ldh^{-} strains. Therefore, even though all of the remaining flux was directed into the ALS branch, this modulation seemed ill-advised (Fig. 2b).

Experimentally, the overall carbon flux was reduced by 11% but this reduction was not as severe as the model had predicted. The major product formed was acetoin (50% of the measured product formation rates, Fig. 2), which was in agreement with the model prediction.

Overexpression of NOX

In addition to LDH, NOX also had a high (positive) control coefficient (Table 4). The model predicted that about 20% of the glucose would be converted via the ALS branch if NOX was 40-fold overexpressed, whereas in the experimental set-up 13% was converted to acetoin (Fig. 2c).

The strongest effect was expected when the LDH knockout mutant was combined with NOX overexpression. The model predicted that, under these conditions, 92% of the pyruvate would be converted via the acetolactate branch, but with a much higher glycolytic flux when compared to the situation in which only LDH was deleted (Fig. 2d). Indeed, in the experimental situation acetoin was the main product (75%) of the recovered carbon.

DISCUSSION

In this study we have illustrated the combined use of MCA, kinetic modelling and experimentation to increase dramatically the flux through a pathway that is of biotechnological relevance. The predicted increase in flux was substantial enough to be of great industrial interest and was confirmed experimentally.

The model

L. lactis is an ideal organism for modelling purposes, as it is well-studied, genetically accessible and can be cultured readily. Furthermore, its anabolic reactions can be simulated as being separate from the catabolic reactions with respect to carbon metabolism: when *L. lactis* is cultured on rich medium almost all the carbon (95%) from the free-energy source (e.g. glucose) is recovered as external products (e.g. lactate) (Novak *et al*., 1998). This simplifies the modelling process considerably. The only link between anabolism and catabolism that was considered in this study was ATP and this could be modelled as a single step; all ATP-consuming reactions in anabolism were grouped into a single module. Biomass formation from glucose was assumed to be, overall, a redox-neutral process; the homolactic fermentation in the wild-type indicates that catabolism itself is redox neutral.

The accuracy of the model depends largely on the kinetic data on which it was built. These data were taken from the literature and were determined by several different groups working with different organisms and they were not always determined under physiological conditions. Also, sometimes no data were available for *L. lactis*. In these instances, data either from another streptococcal species or from other bacteria were used (see Table 3). Another limitation of the model is that a constant level of gene expression was assumed, i.e. the enzyme levels that were measured in the wild-type strain were assumed not to change during the genetic manipulation steps.

The model is available online at http://jjj.biochem.sun.ac.za/wcfs.html and it can be run from this site. As well as the standard simulations, the site also allows the user to set various parameters and to test all sorts of mutants.

Designing the optimal strain: reducing trial and error

Indeed, if the kinetic parameters for the system under study are available then the construction of a kinetic model is relatively simple and quick. Even without all of the kinetic parameters available, one can sometimes make simplifications that allow for the construction of a model that can be useful. For instance, here we have focused on the flux distribution over the pyruvate branches and have grouped all of the reactions outside these branches, thereby avoiding modelling all of the glycolytic steps in detail.

Once a kinetic model has been constructed it is simple to calculate the control coefficients on the flux (or on the concentration of a metabolite) that one wishes to optimize. On the basis of the control coefficients one can then design a genetic engineering strategy, which can again be tested in the kinetic model. This latter testing is important, as one will want to make large changes in enzyme concentrations in order to attain biotechnologically interesting improvements. If indeed the perturbation leads to an appreciable improvement of the production strain *in silico* one can then move to the experimental process of genetic engineering.

All in all, the model we used was not perfect. Therefore, our finding that it was very useful in the engineering strategy, and that it even came close to predicting its results quantitatively, may reflect the phenomenon that metabolic fluxes have limited sensitivities to many kinetic parameters. More generally, it suggests that the frequent adage that biotechnology is not yet ready for the application of mathematical models may be much too pessimistic.

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