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Metabolic pathway analysis for rational design of L-methionine production by *Escherichia coli* and *Corynebacterium glutamicum*

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Abstract

Metabolic pathway analysis was carried out to predict the metabolic potential of *Corynebacterium glutamicum* and *Escherichia coli* for the production of L-methionine. Based on detailed stoichiometric models for these organisms, this allowed the calculation of the theoretically optimal methionine yield and related metabolic fluxes for various scenarios involving different mutants and process conditions. The theoretical optimal methionine yield on the substrates glucose, sulfate and ammonia for the wildtype of *C. glutamicum* is 0.49 (C-mol) $(\text{C-mol})^{-1}$, whereas the *E. coli* wildtype exhibits an even higher potential of 0.52 (C-mol) $(\text{C-mol})^{-1}$. Both strains showed completely different optimal flux distributions. *C. glutamicum* has a high flux through the pentose phosphate pathway (PPP), whereas the TCA cycle flux is very low. Additionally, it recruits a metabolic cycle, which involves 2-oxoglutarate and glutamate. In contrast, *E. coli* does minimize the flux through the PPP, and the flux through the TCA cycle is high. The improved potential of the *E. coli* wildtype is due to its membrane-bound transhydrogenase and its glycine cleavage system as shown by additional simulations with theoretical mutants. A key point for maximizing methionine yield is the choice of the sulfur source. Replacing sulfate by thiosulfate or sulfide increased the maximal theoretical yield in *C. glutamicum* up to 0.68 (C-mol) $(\text{C-mol})^{-1}$. A further increase is possible by the application of additional C_1 sources. The highest theoretical potential was obtained for *C. glutamicum* applying methanethiol as combined source for C_1 carbon and sulfur (0.91 (C-mol) $(\text{C-mol})^{-1}$). Substrate requirement for maintenance purposes reduces theoretical methionine yields. In the case of sulfide used as sulfur source a maintenance requirement of 9.2 mmol ATP $g^{-1} h^{-1}$, as was observed under stress conditions, would reduce the maximum theoretical yield from 67.8% to 47% at a methionine production rate of 0.65 mmol $g^{-1} h^{-1}$. The enormous capability of both organisms encourages the development of biotechnological methionine production, whereby the use of metabolic pathway analysis, as shown, provides valuable advice for future strategies in strain and process improvement.

Keywords: Elementary modes; Methionine; *Corynebacterium glutamicum*; *E. coli*; Sulfur; Methanethiol; Thiosulfate

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-16-BP, fructose-1, 6-bisphosphate; ASP, aspartic acid; ASP-P, aspartyl-phosphate; ASP-SA, aspartate-semialdehyde; HOM, homoserine; O-AC-HOM, O-acetyl-homoserine; HOMOCYS, homocysteine; 3-PHP, 3-phosphonooxypyruvate; SER-P, 3-phosphoserine; SER, serine; O-AC-SER, *O*-acetyl-serine; CYS, cysteine; CYSTA, cystathionine; GA3P, glyceraldehyde 3-phosphate; DAHP, dihydroxyacetone phosphate; 13-PG, 1, 3-bisphospho-glycerate; 3-PG, 3-phospho-glycerate; 2-PG, 2-phospho-glycerate; AC-CoA, acetyl coenzyme A; PYR, pyruvate; PEP, phosphoenol-pyruvate; CIT, citric acid; OAA, oxaloacetate; *Cis*-ACO, *cis*-aconitate; ICI, iso-citric acid; 2-OXO, 2-oxoglutarate; GLU, glutamate; SUCC-CoA, succinyl coenzyme A; SUCC, succinate; FUM, fumarate; MAL, malate; GLYOXY, glyoxylate; H_2SO_3, sulfite; H_2S, hydrogen-sulfide; 6-P-gluconate, 6-Phospho-gluconate; GLC-LAC, 6-phospho-glucono-1, 5-lactone; RIB-5P, ribulose 5-phosphate; RIBO-5P, ribose 5-phosphate; XYL-5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E-4P, erythrose 4-phosphate; MET, L-methionine; NADP, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ACETAT, acetate; H-CoA, coenzyme A; FAD, oxidized flavin adenine dinucleotide; FADH, reduced flavin adenine dinucleotide; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; GLC, glucose; METex, excreted methionine; O_2, oxygen; NH_3, ammonia; CO_2, carbon dioxide; SO_4, sulfate; GLYCIN, glycine; HPL, H-protein-lipoyllysine; methyl-HPL, H-protein-S-aminomethyldihydrolipoyllysine

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1. Introduction

The essential sulfur-containing amino acid methionine is one of the most important industrial amino acids. It has a worldwide annual production of about 500.000 tons. Methionine is the most limiting amino acid in usual poultry feed (Moran, 1994; Murillo and Jensen, 1976; Sekiz et al., 1975) and, due to this, mainly applied as feed supplement. In contrast to other industrial amino acids, methionine is almost exclusively applied as a racemate produced by chemical synthesis (Wagner et al., 1970). Animals can basically metabolize both stereo isomers of methionine which allows direct feed of the chemically produced racemic mixture (D'Mello and Lewis, 1978). However, there is a large interest to replace the existing chemical production by a biotechnological process. This is due to the fact that, at lower levels of supplementation, L-methionine is a better source of sulfur amino acids than D-methionine (Katz and Baker, 1975). Moreover, the chemical process uses rather hazardous chemicals and produces substantial waste streams. This could be avoided developing a sustainable biotechnological process. Despite many attempts in the past, however, no industrially competitive methionine over-producing organism has been discovered so far (Mondal et al., 1996). In the light of the new powerful tools of metabolic engineering, the quest for targeted development of methionine-producing strains is strongly revived (Lee and Hwang, 2003; Nakamori et al., 1999; Rückert et al., 2003). Hereby, the two most promising future candidates for methionine over-production are *Corynebacterium glutamicum* and *Escherichia coli*, the dominating organisms for industrial amino acid production (Leuchtenberger, 1996). Of central importance for rational development of methionine over-producers is the identification of promising targets. Two alternative approaches for metabolic pathway analysis, elementary flux mode analysis and extreme pathway analysis, are novel powerful tools to study metabolic properties of cellular systems (Papin et al., 2004; Schilling et al., 2000; Schuster et al., 1999). Elementary flux mode analysis allows the calculation of a solution space that contains all possible steady-state flux distributions of a network. Hereby the stoichiometry of the metabolic network studied, including carbon as well as cofactor requirements, is fully considered. Several genome scale studies have been published recently dealing with, e.g., the production of succinate (Cox et al., 2006) and of polyhydroxybutanoate in yeast (Carlson et al., 2002) as well as growth-related aspects in *Saccharomyces cerevisiae* (Duarte et al., 2004; Liao and Oh, 1999) and *E. coli* (Carlson and Srienc, 2004a; Ibarra et al., 2003; Liao and Oh, 1999; Vijayasankaran et al., 2005). Metabolic pathway analysis is also very useful in rational strain development. It allows determining the overall capacity, i.e., theoretical maximum yield, of a cellular system and studying effects of any genetic modification. Based on such studies, valuable advices can be given to genetic engineers. Moreover, knowledge of the theoretical maximum yield allows estimating the potential economic efficiency of a process. In the case of methionine, a central question surely is the competitiveness with current chemical synthesis.

In the present work, elementary flux mode analysis was carried out for methionine production by comparing the metabolic networks of two major industrial amino acid producers, *C. glutamicum* and *E. coli*. Thus important questions for future development of biotechnological methionine production processes could be addressed. Here, we investigate which pathways are involved in the flux scenario representing optimal methionine production. We identify most promising genes for improving the methionine production yield. We study the influence of carbon and sulfur sources to find out the maximum potential of a biotechnological production process. Finally, we investigate the influence of maintenance on maximum theoretical yields.

2. Metabolic reaction networks

Biochemical reaction models were constructed for *C. glutamicum* (Fig. 1) and *E. coli* (Fig. 2). The models comprise all relevant routes of central carbon and sulfur metabolism involving all pathways linked to methionine production. Both models were constructed from current biochemical knowledge of the organisms investigated. Detailed lists showing all reactions considered for *C. glutamicum* and *E. coli,* respectively, are given in the appendix. It is assumed that water, protons and phosphate are ubiquitous in the cells and not limiting.

C. glutamicum network: The basic metabolic network of the *C. glutamicum* wildtype (Fig. 1) was set up for utilization of glucose and sulfate as carbon and sulfur sources, respectively (http://www.genome.jp/kegg/metabolism.html). It includes glucose uptake via a phosphotransferase system (PTS), glycolysis (EMP), pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, anaplerosis and respiratory chain as depicted in Fig. 1. During the analysis of the *C. glutamicum* network, several additional reactions were introduced. These comprise the transhydrogenases and the glycine cleavage system of the *E. coli* network, the formic acid metabolism and the use of sulfide, thiosulfate and methanethiol as sulfur sources. All reactions are defined in the appendix. The assimilation of sulfate comprises uptake and subsequent conversion into hydrogensulfide (Schiff, 1979). For the uptake an ATP-dependent ABC-transporter was assumed (Lee, 2005). In the stoichiometric model, the sulfate assimilation pathway is lumped into two reactions: the reduction of sulfate to sulfite requiring 2 ATP and 1 NADPH and the reduction of sulfite to sulfide demanding for 3 NADPH. The complete model consists of 59 internal and eight external metabolites, 62 reactions and 19 of them reversible. The external metabolites comprise substrates (glucose, sulfate, ammonia, oxygen) and products (biomass, CO_2, methionine, glycine). Glycine is considered as external metabolite, because once formed as by-product it cannot be re-utilized