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ScienceDirect
Metabolic Engineering 9 (2007) 177–192
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Determining *Actinobacillus succinogenes* metabolic pathways and fluxes by NMR and GC-MS analyses of ^{13}C-labeled metabolic product isotopomers

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Received 31 July 2006; received in revised form 12 October 2006; accepted 31 October 2006
Available online 17 November 2006

Abstract

Actinobacillus succinogenes is a promising candidate for industrial succinate production. However, in addition to producing succinate, it also produces formate and acetate. To understand carbon flux distribution to succinate and alternative products we fed *A. succinogenes* [1-^{13}C]glucose and analyzed the resulting isotopomers of excreted organic acids, proteinaceous amino acids, and glycogen monomers by gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. The isotopomer data, together with the glucose consumption and product formation rates and the *A. succinogenes* biomass composition, were supplied to a metabolic flux model. Oxidative pentose phosphate pathway flux supplied, at most, 20% of the estimated NADPH requirement for cell growth. The model indicated that NADPH was instead produced primarily by the conversion of NADH to NADPH by transhydrogenase and/or by NADP-dependent malic enzyme. Transhydrogenase activity was detected in *A. succinogenes* cell extracts, as were formate and pyruvate dehydrogenases, which the model suggested were contributing to NADH production. Malic enzyme activity was also detected in cell extracts, consistent with the flux analysis results. Labeling patterns in amino acids and organic acids showed that oxaloacetate and malate were being decarboxylated to pyruvate. These are the first in vivo experiments to show that the partitioning of flux between succinate and alternative fermentation products can occur at multiple nodes in *A. succinogenes*. The implications for designing effective metabolic engineering strategies to increase *A. succinogenes* succinate production are discussed.

Keywords: *Actinobacillus succinogenes*; Succinate; Metabolic flux analysis; Biomass composition; Oxaloacetate decarboxylase; Malic enzyme; Transhydrogenase

1. Introduction

Declining oil reserves, rising petrochemical prices, and the environmental impact of oil-based industries have prompted the development of bio-based processes for fuel and chemical production (Wilke, 1995, 1999). Industrial-scale microbial processes are meeting the global demands for a variety of amino acids, organic acids, vitamins, and antibiotics (Wilke, 1999). Succinate is produced petrochemically from butane to satisfy a specialty chemical market, but it can also be produced from microbial fermentations (Zeikus et al., 1999). More importantly, bio-based succinate could replace a large petrochemical-based commodity chemical market for making bulk chemicals including 1,4-butanediol (a precursor to "stronger-than-steel" and biodegradable plastics), ethylene diamine disuccinate (a biodegradable chelator), diethyl succinate (a "green" solvent replacement for methylene chloride), and adipic acid (a nylon precursor) (Zeikus et al., 1999). In addition to being based on renewable resources, bio-based succinate production has the environmental

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doi:10.1016/j.ymben.2006.10.006

benefit of using CO_2, a greenhouse gas, as a substrate. Developing a more cost-effective industrial succinate fermentation to replace the butane-based commodity maleic anhydride will require advances on several fronts. In particular, it will require developing organisms that produce high succinate concentrations at high rates.

The capnophilic bacterium, *Actinobacillus succinogenes*, is a promising biocatalyst for industrial succinate production. While *A. succinogenes* produces some of the highest succinate concentrations ever reported (Guettler et al., 1996a, b), it does so as part of a mixed acid fermentation, producing high concentrations of formate and acetate as well. Therefore, it is desirable to genetically engineer *A. succinogenes* to produce succinate as the sole fermentation end product.

Metabolic engineering is most effective when it is based on an understanding of the pathways involved and of how the fluxes through those pathways are controlled (Fell, 1997; Stephanopoulos et al., 1998). Knowledge of *A. succinogenes* metabolism comes from fermentation balances, from in vitro enzyme assays (McKinlay et al., 2005; van der Werf et al., 1997), and, more recently, from its genome sequence (McKinlay et al., manuscript in preparation., http://genome.ornl.gov/microbial/asuc/). A simplified map of *A. succinogenes* central metabolism is depicted in Fig. 1. Previous data suggest that *A. succinogenes* ferments glucose to phosphoenolpyruvate (PEP) by glycolysis and the oxidative pentose phosphate pathway (OPPP) (van der Werf et al., 1997). PEP is thought to serve as the branchpoint between the formate-, acetate-, and ethanol-producing pathway (C_3 pathway), and the succinate-producing (C_4) pathway. Malic enzyme and oxaloacetate (OAA) decarboxylase activities measured in vitro (van der Werf et al., 1997) suggest that malate and OAA can also serve as branch points between the C_3 and C_4 pathways. The presence of these activities and others in vivo, such as those of the Entner–Doudoroff (ED) and glyoxylate pathways (for which questionable in vitro activities were detected [van der Werf et al., 1997]), must be determined to develop effective *A. succinogenes* engineering strategies.

^{13}C-labeling studies are a useful approach for understanding the in vivo workings of metabolism (Ratcliffe and Shachar-Hill, 2006; Wiechert, 2001). Knowledge resulting from these techniques has been used to guide the metabolic engineering of industrial microbes, such as vitamin-producing *Bacillus subtilis* (Dauner et al., 2002; Sauer et al., 1997; Zamboni et al., 2003) and amino acid-producing *Corynebacterium glutamicum* (Koffas et al., 2003; Park et al., 1997; Petersen et al., 2000). ^{13}C-labeling studies can distinguish fluxes through different pathways when these fluxes result in different positional isotopic enrichments in metabolic intermediates. These labeling patterns are imprinted on metabolic products (e.g., proteinaceous amino acids and excreted organic acids) and can be analyzed by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). We recently developed a chemically defined growth medium that makes ^{13}C-labeling experiments with *A. succinogenes* possible (McKinlay et al., 2005). Here we describe the use of [1-^{13}C]glucose to obtain an *A. succinogenes* metabolic flux map based on analyses of its cell composition, extracellular fluxes, and isotopomers of amino acids, organic acids, and glycogen monomers.

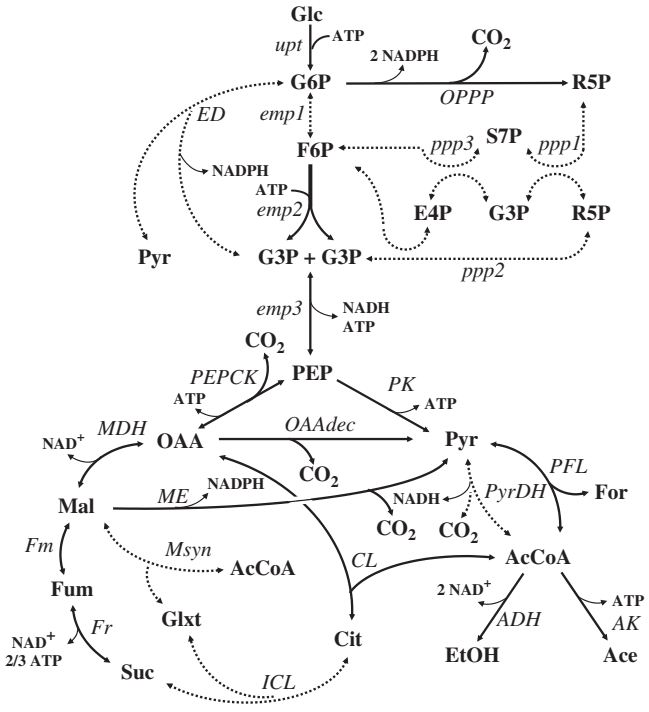

Fig. 1. *A. succinogenes* metabolic pathways addressed in this study. Solid lines: pathways or reactions for which enzyme activity was detected in vitro; dotted lines: pathways or reactions where no activity or uncertain activity was detected in vitro (van der Werf et al., 1997). Unidirectional arrows: fluxes considered to be unidirectional (all other fluxes are considered to be reversible). The C_4 pathway is defined as: PEP→OAA→Mal→Fum→Suc. The C_3 pathway is defined as: PEP→Pyr→AcCoA→Ace + EtOH. Alternative PPP reactions, cysteine and methionine degradation pathways, and amino acid synthesis pathways are not shown (Supplementary material). It was assumed that 0.67 ATP is produced per fumarate reductase reaction based on data from *Wolinella succinogenes* (Kroger et al., 2002). Metabolites: AcCoA, acetyl-coenzymeA; Ace, acetate; Cit, citrate; EtOH, ethanol; E4P, erythrose-4-phosphate; For, formate; Fum, fumarate; F6P, fructose-6-phosphate; Glc, glucose; Glxt, glyoxylate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; R5P, pentose-phosphates, Suc, succinate; S7P, sedoheptulose-7-phosphate. Pathways and reactions: *ADH*, alcohol dehydrogenase; *AK*, acetate kinase; *CL*, citrate lyase; *ED*, Entner–Doudoroff pathway; *emp1*, 2, and 3, Embden–Meyerhoff–Parnas (EMP) or glycolytic reactions; *Fm*, fumarase; *FR*, fumarate reductase; *ICL*, isocitrate lyase and aconitase; *MDH*, malate dehydrogenase; *ME*, malic enzyme; *Msyn*, malate synthase; *OAAdec*, oxaloacetate decarboxylase; *OPPP*, oxidative pentose phosphate pathway; *PEPCK*, PEP carboxykinase; *PFL*, pyruvate formate-lyase; *PK*, pyruvate kinase and PEP:glucose phosphotransferase system (PTS); *ppp1* and 2, transketolase; *ppp3*, transaldolase; *PyrDH*, pyruvate dehydrogenase or PFL coupled with formate dehydrogenase; *upt*, glucose phosphorylation by hexokinase and PTS.