Journal of Biotechnology 143 (2009) 124–129

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Metabolic flux analysis of a phenol producing mutant of *Pseudomonas putida* S12: Verification and complementation of hypotheses derived from transcriptomics

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ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 17 June 2009
Accepted 18 June 2009

Keywords:
Pseudomonas putida S12
Solvent tolerance
Metabolic flux analysis
Phenol
Metabolic engineering

ABSTRACT

The physiological effects of genetic and transcriptional changes observed in a phenol producing mutant of the solvent-tolerant *Pseudomonas putida* S12 were assessed with metabolic flux analysis. The upregulation of a malate/lactate dehydrogenase encoding gene could be connected to a flux increase from malate to oxaloacetate. A mutation in the *pykA* gene decreased *in vitro* pyruvate kinase activity, which is consistent with a lower flux from phosphoenolpyruvate to pyruvate. Changes in the *oprB-1*, *gntP* and *gnuK* genes, encoding a glucose-selective porin, gluconokinase and a gluconate transporter respectively, altered the substrate uptake profile. Metabolic flux analysis furthermore revealed cellular events not predicted by the transcriptome analysis. Gluconeogenic formation of glucose-6-phosphate from triose-3-phosphate was abolished, in favour of increased phosphoenolpyruvate production. An increased pentose phosphate pathway flux resulted in higher erythrose-4-phosphate production. Thus, the availability of these two central phenol precursors was improved. Furthermore, metabolic fluxes were redistributed such that the overall TCA cycle flux was unaffected and energy production increased. Engineering *P. putida* S12 for phenol production has yielded a strain that channels carbon fluxes to previously unfavourable routes to reconcile the drain on metabolites required for phenol production, while maintaining basal flux levels through central carbon metabolism.

1. Introduction

The species *Pseudomonas putida* has become well established in biotechnology (Wackett, 2003). Among this species, a well studied group exists consisting of organic solvent-tolerant strains, which have found a solid foothold in biocatalysis (Nijkamp et al., 2006; Park et al., 2007; Ramos-Gonzalez et al., 2003; Rojas et al., 2004; Verhoef et al., 2007).

Previously, a mutant of the solvent-tolerant *P. putida* strain S12, designated *P. putida* S12TPL3, was constructed that converts glucose into phenol (Wierckx et al., 2005). The basis for enhanced phenol production was investigated by transcriptomics, sequencing, and targeted mutagenesis, providing insight into several key changes relating to the metabolic routes leading directly to phenol (Wierckx et al., 2008). In addition, a number of genes related to primary metabolism, specifically the tricarboxylic acid (TCA) cycle, were found to be differentially expressed in the phenol producing strain, compared to a control strain. Moreover, the *oprB-1* gene, encoding a glucose-selective porin, was disrupted by transposon insertion, whereas genes responsible for gluconate uptake and phosphorylation (*gnuK* and *gntP*) were upregulated. Also, a point mutation was found in the *pykA* gene, encoding pyruvate kinase A. These results gave clues about changes in the primary metabolism of *P. putida* S12TPL3, but the effects on operation of the metabolic network remained speculative.

Metabolic flux analysis has been used successfully to gain insight into the activity of metabolic routes in microorganisms (Blank et al., 2005; del Castillo et al., 2007; Fischer and Sauer, 2003; Sauer et al., 1999). The method allows quantification of the intracellular reaction rates of cellular metabolism by balancing the fluxes of substrate consumption, product secretion, and biomass synthesis within a stoichiometric model. Since most metabolic networks of interest are underdetermined (i.e. have a degree of freedom higher

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doi:10.1016/j.jbiotec.2009.06.023

than zero), additional constraints are necessary to estimate a unique flux solution. These additional constraints can be relative contributions of alternative pathways to the synthesis of intracellular metabolites, which can be inferred from ^{13}C-labelling patterns of proteinogenic amino acids. In more detail, microbial cultures are fed with ^{13}C-labelled substrate. The distribution of ^{13}C atoms within proteinogenic amino acids from these cultures can be measured by advanced gas chromatography–mass spectrometry (GC–MS) techniques, and is used to infer the labelling pattern of the primary metabolism intermediates from which the amino acids were synthesized. These patterns can then be used to determine the relative fluxes through the parallel pathways of cellular metabolism. By determining physiological parameters such as substrate uptake rates, product formation rates, and growth rate, absolute fluxes for most reactions in the primary metabolism can be calculated (Nanchen et al., 2007; Sauer, 2006).

The goal of this study is to investigate the metabolic fluxes through the primary metabolism of the optimized phenol producing strain *P. putida* S12TPL3 by ^{13}C metabolic flux analysis. The analysis is used to evaluate the effects of the observed transcriptional and genetic changes on metabolic events. In addition, insight is gained into those metabolic events that are not reflected in transcriptional changes.

2. Materials and methods

2.1. Strains and culture conditions

P. putida S12TPL3 (Wierckx et al., 2005) is a phenol producing mutant of *P. putida* S12 (Hartmans et al., 1989). *P. putida* S12JNNmcs(t) (Wierckx et al., 2008) is a control strain transformed with the empty expression vector pJNNmcs(t).

Cultivations of *P. putida* S12TPL3 and *P. putida* S12JNNmcs(t) were performed at 30 °C in 500 ml Erlenmeyer flasks (no baffles) containing 50 ml minimal medium (Hartmans et al., 1989) with 10 mg l^{-1} gentamicin and 0.1 mM salicylate as an inducer for the heterologous *nagR*/P_{nagAa} promoter controlling the expression of TPL (tyrosine phenol lyase). Both *P. putida* strains were cultured on minimal medium containing 20 mM of either 100% [1-^{13}C] glucose (ARC Laboratories B.V., Apeldoorn, The Netherlands) or a mixture of 80% (w/w) unlabelled and 20% (w/w) uniformly labelled [U-^{13}C] glucose (ARC Laboratories B.V., Apeldoorn, The Netherlands), resulting in the incorporation of the ^{13}C-label in proteinogenic amino acids. The combination of these two labelling strategies was previously reported to have a high information content (Fischer et al., 2004). All cultivations were performed in triplicate. The ^{13}C-labelling cultures were started at 0.02 g l^{-1} cell dry weight (CDW) or lower to minimize dilution of labelled biomass with the unlabelled biomass from the inoculum. As inoculum, an overnight culture (stationary phase) in medium with 20 mM unlabelled glucose was used. Cells for labelling analysis were harvested at three timepoints during or just after the exponential growth phase (less than 0.7 g l^{-1}CDW) by centrifugation for 1 minute at 13,000 rpm at 4 °C. The cell-free medium was stored at −20 °C.

2.2. METAFoR analysis using amino acid mass isotopomer data and ^{13}C-constrained flux analysis

The labelling patterns of proteinogenic amino acids were determined by GC–MS as described previously (Blank et al., 2008; Fischer and Sauer, 2003). The mass isotopomer data can be found in supplemental data table S1. Metabolic flux ratios were calculated from the GC–MS data as described in detail by Nanchen et al. (2007) using Fiat Flux (Zamboni et al., 2005).

Absolute values of intracellular fluxes were calculated with a flux model comprising all major pathways of *P. putida* central carbon metabolism described by Blank et al. (2008). The model was altered by adding the heterologous phenol producing reactions of *P. putida* S12TPL3. The biomass requirements of *P. putida* were assumed to be highly similar to those published for *Escherichia coli* (Emmerling et al., 2002), as previously assumed (Blank et al., 2008; Fuhrer et al., 2005). A complete set of the reactions used in the model is listed in supplemental data S2. ^{13}C-constrained net flux analysis (Fischer et al., 2004) was applied to obtain a unique flux solution for the underdetermined *Pseudomonas* model (matrix rank of 22, 4 degrees of freedom). To uniquely solve the system for fluxes, a set of linearly independent equations that quantify flux ratios were used to obtain 6 constraints on the relative flux distribution from METAFoR analysis (see Table S2 and equations 1–6 of the supplemental data S2). The error minimization was carried out as described by Fischer et al. (2004).

The *in vivo* production rates of NADH, NADPH, PQQH (lumped as NAD(P)H) and ATP were estimated by determining the sum of all the reaction velocities that generate or consume NAD(P)H or ATP, basically as described previously by Blank et al. (2008). Phenol and 4-hydroxyphenylpyruvate biosynthesis from phosphoenolpyruvate (PEP) and erythrose-4-phosphate costs one NADPH and one ATP. It generates one NADH if synthesized via 4-hydroxyphenylpyruvate, while synthesis via phenylpyruvate does not generate NADH. The cost of 4-hydroxyphenylpyruvate and phenol production is therefore assumed to be one ATP and 0.5 NAD(P)H. Substrate uptake was assumed to require 1/3 mole ATP per mole of substrate, since three parallel uptake routes are possible, of which only direct glucose uptake consumes ATP.

2.3. Analytical methods

Cell density and phenol and 4-hydroxyphenylpyruvate concentrations were determined as described previously (Wierckx et al., 2008). Glucose, gluconic acid, and 2-ketogluconic acid were analyzed by ion chromatography (Dionex ICS3000 system), using a CarboPac PA20 column (length, 150 mm; internal diameter, 3 mm) with 10 mM NaOH at a flow rate of 0.5 ml min^{-1} as the eluent for glucose or an IonPac ICE AS6 column (length, 250 mm; internal diameter, 9 mm) with 0.4 mM heptafluorobutyric acid as the eluent at a flow rate of 1.2 ml min^{-1} for the organic acids. Carbon source concentrations were determined from two replicates of the cultures with 1-^{13}C glucose, since all replicate cultures were comparable.

2.4. Pyruvate kinase activity assays

Cell extracts were prepared from cultures in the late-exponential phase by sonication basically as described by Wierckx et al. (2008). *In vitro* pyruvate kinase activity was measured in a coupled assay with lactate dehydrogenase. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 2 mM $MgCl_2$, 2 mM ADP, 10 U lactate dehydrogenase, 0.2 mM NADH, and cell extract in a total volume of 1 ml. The reaction was started by adding 0.2 mM phosphoenolpyruvate. The decrease of NADH was monitored spectrophotometrically at 340 nm (extinction coefficient of 6220 M^{-1} cm^{-1}). One unit is defined as the amount of enzyme needed to oxidize one μmol NADH per minute.

3. Results and discussion

3.1. Growth and phenol production of P. putida S12TPL3

In order to determine metabolic fluxes, *P. putida* S12TPL3 and *P. putida* S12JNNmcs(t) were each cultivated on two different ^{13}C-labelled glucose substrates. *P. putida* S12TPL3 produced 1.5 mM