

Expression of the *Escherichia coli* *pntAB* genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation

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Abstract A critical factor in the biotechnological production of L-lysine with *Corynebacterium glutamicum* is the sufficient supply of NADPH. The membrane-integral nicotinamide nucleotide transhydrogenase PntAB of *Escherichia coli* can use the electrochemical proton gradient across the cytoplasmic membrane to drive the reduction of NADP⁺ via the oxidation of NADH. As *C. glutamicum* does not possess such an enzyme, we expressed the *E. coli* *pntAB* genes in the genetically defined *C. glutamicum* lysine-producing strain DM1730, resulting in membrane-associated transhydrogenase activity of 0.7 U/mg protein. When cultivated in minimal medium with 10% (w/v) carbon source, the presence of transhydrogenase slightly reduced glucose consumption, whereas the consumption of fructose, glucose plus fructose, and, in particular, sucrose was stimulated. Biomass was increased by *pntAB* expression between 10 and 30% on all carbon sources tested. Most importantly, the lysine concentration was increased in the presence of transhydrogenase by ~10% on glucose, ~70% on fructose, ~50% on glucose plus fructose, and even by ~300% on sucrose. Thus, the presence of a proton-coupled transhydrogenase was shown to be an efficient way to improve lysine production by *C. glutamicum*. In contrast, *pntAB* expression had a negative effect on growth and glutamate production of *C. glutamicum* wild type.

Keywords *Corynebacterium glutamicum* · *Escherichia coli* · Lysine production · Glutamate production · Glucose · Fructose · Sucrose · Nicotinamide nucleotide transhydrogenase · *pntAB*

Introduction

The Gram-positive aerobic soil bacterium *Corynebacterium glutamicum* is of major importance in the industrial production of amino acids, in particular, L-glutamate (Kimura 2005) and L-lysine (Hermann 2003; Kelle et al. 2005). A number of molecular targets essential for efficient lysine production have been identified in the lysine biosynthesis pathway, in pathways competing with lysine formation for common intermediates and in pathways providing the precursor oxaloacetate (Eggeling 1994; Hermann 2003; Pfefferle et al. 2003; Sahm et al. 2000). Based on that knowledge, introduction of favorable point mutations in the aspartokinase gene *lysC*, in the homoserine dehydrogenase gene *hom* and in the pyruvate carboxylase gene *pyc*, which had been uncovered in a classically obtained lysine producer, into the *C. glutamicum* wild-type genome resulted in a very efficient lysine production strain (Ohnishi et al. 2002).

In *C. glutamicum*, lysine biosynthesis involves a split pathway in which L,L-diaminopimelate is formed from tetrahydrodipicolinate either via the energetically less expensive diaminopimelate dehydrogenase when ammonium is in excess or via the energetically more costly tetrahydrodipicolinate succinylase pathway when the ammonium concentration is low (Eggeling and Sahm 1999). Both pathways require 4 mol NADPH per mol lysine starting from oxaloacetate. In principle, NADPH can be synthesized in *C. glutamicum* by the enzymes of the

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oxidative pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Moritz et al. 2000), by isocitrate dehydrogenase (Eikmanns et al. 1995) and by malic enzyme (Gourdon et al. 2000). Carbon flux analysis revealed that during growth on glucose, NADPH for lysine biosynthesis is provided by the PPP, whereas isocitrate dehydrogenase and malic enzyme are of minor importance (Marx et al. 1996, 1997, 1999, 2003). During growth of *C. glutamicum* on fructose or on fructose/glucose mixtures, a low carbon flux through the oxidative PPP was demonstrated (Dominguez et al. 1998; Kiefer et al. 2004; Pons et al. 1996). It was proposed that to meet the NADPH requirement for growth and lysine production on fructose or on fructose/glucose mixtures, malic enzyme activity has to be increased (Dominguez et al. 1998). As fructose is transported into the cell as fructose-1-phosphate by a PEP-dependent phosphotransferase system and enters glycolysis after phosphorylation to fructose-1,6-bisphosphate (Kotrba et al. 2001; Parche et al. 2001), overexpression of the fructose-1,6-bisphosphatase gene *fbp* (Rittmann et al. 2003) was alternatively proposed to increase the PPP flux as well as lysine production on fructose (Kiefer et al. 2004; Pons et al. 1996). We could recently show that overexpression of the *malE* gene encoding malic enzyme in the genetically defined *C. glutamicum* strain DM1730 has no positive effect on lysine production on glucose, fructose, glucose/fructose, or sucrose (Georgi et al. 2005). Whereas overexpression of *fbp* in this strain did not effect lysine production on glucose, fructose, or a glucose/fructose mixture, the lysine yield on sucrose was increased twofold (Georgi et al. 2005).

Escherichia coli and a variety of other bacteria, but not *C. glutamicum*, possess a membrane-bound nicotinamide nucleotide transhydrogenase encoded by the *pntAB* genes which uses the electrochemical proton gradient as driving force for the reduction of NADP^+ to NADPH by oxidation of NADH to NAD^+ according to the following equation: $\text{NADH} + \text{NADP}^+ + \text{H}_{\text{out}}^+ \rightarrow \text{NAD}^+ + \text{NADPH} + \text{H}_{\text{in}}^+$ (Jackson 2003). The *E. coli* transhydrogenase PntAB is composed of two membrane-spanning subunits, α (50 kDa) and β (47 kDa), arranged as an $\alpha_2\beta_2$ heterotetramer (Clarke and Bragg 1985; for review, see Jackson 2003). Sauer et al. (2004) reported that during aerobic batch cultivation of *E. coli* in minimal medium, glucose catabolism alone generated less NADPH than was required for biosynthesis and that the membrane-bound transhydrogenase contributed 35–45% of the total anabolic demand for NADPH under these conditions, although it was not essential for growth on glucose.

In this paper, we report on the expression of the *E. coli* *pntAB* genes in *C. glutamicum*. Our objective was to determine if a membrane-bound transhydrogenase could

serve as an alternative source of NADPH. In contrast to the oxidative PPP, transhydrogenase activity is not accompanied by a loss of substrate carbon as carbon dioxide and, thus, might allow for increased lysine yields.

Materials and methods

Bacterial strains and culture conditions

The *C. glutamicum* strains and plasmids used in this work are listed in Table 1. For analysing lysine or glutamate production, a brain–heart infusion preculture was inoculated from a fresh Luria–Bertani (LB) plate and cultivated overnight. After washing cells in CGXII medium (as described by Keilhauer et al. 1993, but with 30 mg/l 3,4-dihydroxybenzoic acid as iron chelator) without carbon source, the main culture with CGXII medium was inoculated to an OD_{600} of 1. For lysine production, 10% (w/v) glucose, 10% (w/v) fructose, 10% (w/v) sucrose, or 5% (w/v) glucose plus 5% (w/v) fructose were used as carbon source, whereas for glutamate production, 4% (w/v) glucose, 4% (w/v) fructose, 4% (w/v) sucrose or 2% (w/v) glucose + 2% (w/v) fructose were used. To trigger glutamate production, 300 $\mu\text{g/ml}$ ethambutol was added to the minimal medium (Radmacher et al. 2005). After 72 h in the case of lysine production or after 27 h in the case of glutamate production, samples were withdrawn from the cultures for the determination of the amino acid and sugar concentrations in the medium. *C. glutamicum* cultivations were performed in baffled 500-ml Erlenmeyer flasks with 60 ml medium at 30°C and 120 rpm. *E. coli* DH5 α , which was used as host for cloning, was cultivated in LB medium or on LB agar plates at 37°C. When appropriate, kanamycin was used at a concentration of 25–50 $\mu\text{g/ml}$ and isopropyl β -D-1-thiogalactopyranoside (IPTG) at a concentration of 1 mM.

Preparation of DNA and transformation

Chromosomal DNA from *E. coli* was isolated as described by Ausubel et al. (1992). Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Transformation of *E. coli* was performed using the rubidium chloride method (Hanahan 1985), whereas *C. glutamicum* was transformed by electroporation as described by van der Rest et al. (1999).

Construction of a *pntAB* expression plasmid

For *pntAB* overexpression in *C. glutamicum*, the genes were cloned into the vector pEKEx2 (Eikmanns et al. 1991) under the control of an IPTG-inducible *tac* promoter.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference/source
Strains		
<i>C. glutamicum</i> ATCC13032	Wild-type, biotin-auxotrophic	Abe et al. (1967)
<i>C. glutamicum</i> DM1730	<i>pyc</i> ^{P458S} ; <i>hom</i> ^{V59A} ; <i>lys</i> ^{CT3111} ; <i>zwf</i> ^{A243T} derived from ATCC13032	B. Bathe; Degussa AG
<i>E. coli</i> DH5 α	F [−] Φ 80dlac Δ (<i>lacZ</i>)M15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (r_K^- , m_K^+) <i>deoR thi-1 phoA supE44</i> λ^- <i>gyrA96 relA1</i>	Invitrogen (Karlsruhe, Germany)
Plasmids		
pEKEx2	Kan ^R ; <i>E. coli</i> — <i>C. glutamicum</i> shuttle vector for regulated gene expression (<i>P</i> _{<i>lac</i>} , <i>lacI</i> ^q , pBL1 <i>oriV</i> _{C.g.} , pUC18 <i>oriV</i> _{E.c.})	Eikmanns et al. (1991)
pEKEx2- <i>pntAB</i>	Kan ^R ; pEKEx2 derivative containing the <i>E. coli pntAB</i> genes under the control of an IPTG-inducible <i>tac</i> promoter	This work

To this end, the *pntAB* genes including the *pntA* ribosome binding site were amplified by polymerase chain reaction (PCR) from chromosomal DNA of *E. coli* MG1655 with the primers *pntAB*-for (5'-CAG CTG CAG TCA TCA ATA AAA CCG) and *pntAB*-rev (5'-CGT GAG CTC TTA CAG AGC TTT CAG). After restriction of the 2961-bp PCR product with *Pst*I and *Sac*I, the fragment was ligated into pEKEx2 cut with the same restriction enzymes, transferred into *E. coli* DH5 α , and plated on LB plates containing 50 μ g/ml kanamycin. Plasmids from kanamycin-resistant clones were isolated and analysed by restriction with *Pst*I and *Sac*I. One of the plasmids containing the *pntAB* fragment was used for transformation of *C. glutamicum* strains ATCC13032 and DM1730 by electroporation.

Membrane isolation and determination of protein concentration

For isolation of membranes, cells from a 60-ml culture at an OD₆₀₀ of 8 were harvested by centrifugation for 10 min at 5,000 \times g, washed with one culture volume of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and centrifuged again. After resuspension in 1 ml TE buffer containing 40 μ l of complete protease inhibitor (Roche Diagnostics) and addition of 100 μ l RNase/DNase I solution (1 mg/ml DNase I, 0.25 mg/ml RNase A in 50 mM MgCl₂), cells were disrupted by bead beating [3 \times 30 s using a Silamat S5 (Vivadent, Erlangen, Germany)]. Intact cells and cell debris were removed by centrifugation for 20 min at 5,000 \times g and 4°C. The resulting cell-free extract was subjected to ultracentrifugation for 90 min at 150,000 \times g and 4°C. The sedimented membranes were washed twice in 1 ml 10 mM Tris–HCl and finally resuspended in 0.5 ml of the same buffer, resulting in a protein concentration of about 0.2 mg/ml. Protein concentrations were determined with the bicin-

chonic acid protein assay (Smith et al. 1985) using bovine serum albumin as the standard.

Measurement of transhydrogenase activity

To measure pyridine nucleotide transhydrogenase activity, 5 and 10 μ g membrane protein was added to a 1-ml assay mixture (prewarmed to 30°C) containing 50 mM potassium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM NADPH, 0.1 mM 3-acetylpyridine-NAD⁺ and 10 mM β -mercaptoethanol, and the increase in absorbance at 375 nm was used to follow reduction of 3-acetylpyridine-NAD⁺ by NADPH. The assay was carried out with a Jasco V560 spectrophotometer at 30°C using 10-mm light path cuvettes. For calculation of the specific activity (U/mg protein), an extinction coefficient for 3-acetylpyridine-NADH at 375 nm of 6.1 mM^{−1} cm^{−1} (Venning and Jackson 1999) was used. One unit corresponds to the formation of 1 μ mol/min 3-acetylpyridine-NADH.

Quantification of sugars and amino acids

D-Glucose and D-fructose were quantified by high-performance liquid chromatography (HPLC) using a LaChrom LC system (Merck-Hitachi, Darmstadt, Germany) with a Bio-Rad HPX-87C 300 \times 7.8-mm column (Bio-Rad Laboratories GmbH, München, Germany) at 70°C using isocratic elution with H₂O at a flow rate of 0.6 ml/min. Substances were detected with a refraction index detector. Sucrose was quantified enzymatically with a sucrose/D-glucose kit as described by the manufacturer (R-Biopharm, Darmstadt, Germany). Sample concentrations were determined by comparison with external standards.

Quantitative determination of L-lysine and L-glutamate in supernatants was carried out by reversed phase HPLC at 40°C after pre-column derivatization with a mixture of *o*-

phthalaldehyde and β -mercaptoethanol (Pierce Biotechnology, Rockford, USA) according to Lindroth and Mopper (1979) using an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) with an ODS Hypersil 120 \times 4-mm column (CS Chromatographie Service GmbH, Langerwehe, Germany) with 5- μ m particle size and a guard cartridge (40 \times 4 mm). L-Ornithine and L-asparagine at final concentrations of 1 mM and 50 μ M, respectively, were used as internal standards in each sample. Substances were eluted with a flow rate of 0.35 ml min⁻¹ within the first minute and 0.6 ml min⁻¹ for the following 15 min with a gradient of 0.1 M sodium acetate (pH 7.2) as the polar phase and methanol as the non-polar phase. Fluorescence of the amino acid isoindol derivatives was detected at an emission wavelength of 450 nm after excitation at 230 nm using a fluorescence detector.

Determination of physiological parameters

Growth was followed by measuring the OD₆₀₀ with an Ultrospec 500-pro spectrophotometer (Amersham Biosciences). The biomass concentration was calculated from OD₆₀₀ values using an experimentally determined correlation factor of 0.25 g cell dry weight (cdw) l⁻¹ for OD₆₀₀=1. The carbon content of cells grown in CGXII glucose medium was previously determined to be 40% of the cell dry weight (Liebl 2005).

Results

Effect of *pntAB* expression in *C. glutamicum* DM1730 on growth and lysine production with PTS sugars

As the NADPH supply is a critical factor in lysine production by *C. glutamicum*, we tested whether the membrane-bound nicotinamide nucleotide transhydrogenase PntAB from *E. coli* can be used to improve NADPH provision and, thereby, also lysine production. For this purpose, we expressed the *E. coli pntAB* genes under the control of an IPTG-inducible *tac* promoter in the defined lysine-producing strain *C. glutamicum* DM1730 using the plasmid pEKEx2-*pntAB*. For measuring membrane-associated transhydrogenase activity, *C. glutamicum* DM1730 carrying either the pEKEx2-*pntAB* plasmid or the expression vector pEKEx2 (negative control) was cultivated in CGXII minimal medium with 4% glucose in the presence and in the absence of IPTG. The specific transhydrogenase activity, which was measured as the reduction of 3-acetylpyridine-NAD⁺ by NADPH, was below the detection limit of the assay (approximately 0.01 U/mg protein) in strain DM1730/pEKEx2 cultivated without or with IPTG and in DM1730/pEKEx2-*pntAB* grown without IPTG. In

contrast, DM1730/pEKEx2-*pntAB* cultivated in the presence of IPTG showed a transhydrogenase activity of 0.7 ± 0.1 μ mol min⁻¹ (mg membrane protein)⁻¹. This result indicated that the expression of the *E. coli pntAB* genes in *C. glutamicum* led to the synthesis of an active transhydrogenase.

For analysing the effect of *pntAB* expression on growth and lysine formation, *C. glutamicum* DM1730/pEKEx2-*pntAB* and the reference strain DM1730/pEKEx2 were cultivated in CGXII minimal medium containing 50 μ g/ml kanamycin, 1 mM IPTG, and as carbon source, 10% glucose or 10% fructose or 5% glucose plus 5% fructose or 10% sucrose. In Table 2, carbon source consumption, biomass formation and lysine production, as measured after 72 h, are summarized. The reference strain consumed much more glucose than fructose, glucose plus fructose or sucrose. In the *pntAB*-expressing strain, glucose consumption was slightly decreased, whereas the consumption of fructose, glucose plus fructose, and sucrose was increased, in particular, that of sucrose about twofold. With respect to biomass synthesis, *pntAB* expression led to increases between 10 and 30% on all carbon sources. The lysine concentration was increased in the presence of transhydrogenase on all carbon sources tested, but to a highly varying extent: about 10% on glucose, 70% on fructose, 50% on glucose plus fructose and 300% on sucrose. The lysine yield (mol-C lysine/mol-C carbon source consumed) was increased on all carbon sources, most strongly on sucrose (Fig. 1a). The biomass yield (mol-C biomass formed/mol-C carbon source consumed) was increased in the case of glucose, unchanged in the case of fructose, and decreased on glucose plus fructose and on sucrose (Fig. 1b).

Effect of *pntAB* expression in *C. glutamicum* ATCC13032 on growth and glutamate production with PTS sugars

In contrast to lysine formation, glutamate production requires only 1 mol NADPH per mole glutamate. In *C. glutamicum*, NADPH for the glutamate dehydrogenase-catalyzed reductive amination of 2-oxoglutarate is provided by the isocitrate dehydrogenase reaction in the tricarboxylic acid cycle (Eikmanns et al. 1995). The possibility for NADPH formation by a membrane-bound transhydrogenase should consequently be much less relevant for glutamate formation than for lysine formation. To test this proposal, the *C. glutamicum* wild-type strain ATCC13032 was transformed with pEKEx2-*pntAB* or the control vector pEKEx2 and analysed with respect to growth and glutamate formation. The two strains were cultivated for 27 h in CGXII medium with 50 μ g/ml kanamycin, 1 mM IPTG, and either 4% (w/v) glucose, or 4% (w/v) fructose, or 2% (w/v) glucose plus 2% (w/v) fructose, or 4% (w/v) sucrose. Glutamate excretion was triggered by adding 300 μ g/ml ethambutol to the medium before inoculation (Radmacher

Table 2 Influence of *pntAB* expression in the defined lysine-producing strain *C. glutamicum* DM1730 on biomass and lysine formation from glucose, fructose, glucose plus fructose or sucrose (mean values from two independent cultures measured after 72 h of cultivation; deviation was below 5%)

Carbon source	10% Glucose		10% Fructose		5% Glucose + 5% fructose		10% Sucrose	
	+	–	+	–	+	–	+	–
<i>PntAB</i> plasmid								
Carbon source consumed (mM)	474	540	287	236	190	163	267	126
Mol-C carbon source consumed (mM)	2,844	3,240	1,722	1,416	1,890	1,614	3,204	1,512
Cell dry weight (g/l)	15.2	14.3	6.5	5.7	9.9	9.0	9.8	7.5
C in biomass (g/l)	6.1	5.7	2.6	2.3	4.0	3.6	3.9	3.0
Mol-C in biomass (mM)	508	477	217	190	330	300	327	250
Lysine formed (mM)	87	79	22	13	26	17	69	17
Mol-C lysine (mM)	522	474	132	78	156	102	414	102

et al. 2005). In Table 3, carbon source consumption, biomass formation, and glutamate production, as measured after 27 h, are summarized. The biomass formed by strain 13032/pEKEx-*pntAB* was reduced compared to the reference strain 13032/pEKEx2 on all carbon sources tested by 13–26%. At the same time, the glutamate concentration was also reduced in the presence of transhydrogenase by 4–36%. Thus, expression of the transhydrogenase genes *pntAB* had a negative impact on growth and glutamate formation. As both strains had consumed all of the carbon source, the glutamate yields and the biomass yields were decreased in the *pntAB*-expressing strain (Table 3), indicating that transhydrogenase caused a shift in carbon source usage from biomass and glutamate formation to CO₂ or byproduct synthesis.

Discussion

Hitherto, an increased flux through the oxidative PPP was identified as the almost exclusive way to improve the NADPH supply for lysine biosynthesis by *C. glutamicum*. In this study, we tested another possibility of NADPH provision based on the activity of the membrane-bound

nicotinamide nucleotide transhydrogenase PntAB from *E. coli*. This enzyme uses the proton-motive force to drive the reduction of NADP⁺ to NADPH by oxidation of NADH to NAD⁺ (for a recent review, see Jackson 2003). In *C. glutamicum*, the proton-motive force is generated mainly by the cytochrome *bc*₁–*aa*₃ supercomplex (Niebisch and Bott 2003) of the respiratory chain or less efficiently by cytochrome *bd* oxidase (Bott and Niebisch 2003, 2005). The transhydrogenase activity of 0.7 μmol min^{−1} (mg membrane protein)^{−1} measured in *C. glutamicum* carrying the pEKEx2-*pntAB* plasmid was in the same range as that determined for *Saccharomyces cerevisiae* expressing the *E. coli pntAB* genes (Anderlund et al. 1999) and about 40-fold higher than the intrinsic PntAB-catalyzed transhydrogenase activity of *E. coli* (Sauer et al. 2004).

In the defined lysine-producing strain DM1730, the presence of an active transhydrogenase stimulated the consumption of fructose, glucose plus fructose and sucrose, indicating that an insufficient NADPH supply might be responsible for the restricted utilization of these carbon sources. The most drastic effect was observed on sucrose, whose consumption was doubled by *pntAB* expression. Sucrose is taken up into the cell as sucrose-6-phosphate by a PEP-dependent phosphotransferase system (*ptsS*) and

Fig. 1 Influence of *pntAB* expression in *C. glutamicum* on lysine yield (a) and biomass yield (b) after cultivation for 72 h on 10% (w/v) of glucose, fructose or sucrose or 5% (w/v) each of glucose and fructose. White columns, strain DM1730/pEKEx2; grey columns, strain DM1730/pEKEx2-*pntAB*. The data represent mean values and deviation of two independent experiments

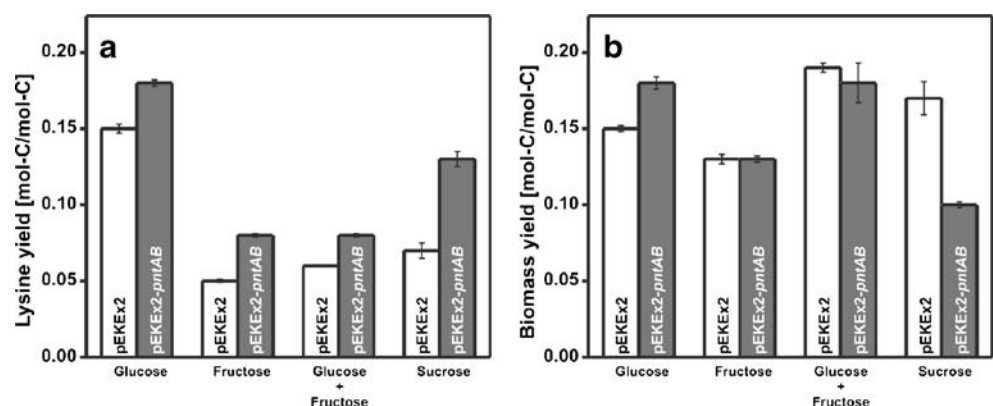


Table 3 Influence of *pntAB* expression in *C. glutamicum* ATCC13032 on biomass and ethambutol-induced glutamate formation on glucose, fructose, glucose plus fructose or sucrose (mean values from two independent cultures measured after 27 h of cultivation; deviation was below 5%)

Carbon source	4% Glucose		4% Fructose		2% Glucose + 2% fructose		4% Sucrose	
	+	–	+	–	+	–	+	–
<i>PntAB</i> plasmid								
Carbon source consumed (mM)	222	222	222	222	111	111	117	117
Mol-C carbon source consumed (mM)	1,332	1,332	1,332	1,332	1,332	1,332	1,404	1,404
Cell dry weight (g/l)	8.85	103	6.25	7.05	7.9	9.15	7.53	10.15
C in biomass (g/l)	3.54	4.12	2.5	2.82	3.16	3.66	3.01	4.06
Mol-C in biomass (mM)	295	343	208	235	263	305	251	338
Biomass yield (mol-C/mol-C)	0.22	0.26	0.16	0.18	0.20	0.23	0.18	0.24
Glutamate formed (mM)	44	53	51	53	41	52	36	56
Mol-C glutamate (mM)	220	265	255	265	205	260	180	280
Glutamate yield (mol-C/mol-C)	0.17	0.20	0.19	0.20	0.15	0.20	0.13	0.20

subsequently hydrolyzed to glucose-6-phosphate and fructose by sucrose-6-phosphate hydrolase (Moon et al. 2005). As *C. glutamicum* lacks a fructokinase enzyme, the free fructose is transported out of the cell and subsequently taken up as fructose-1-phosphate by the fructose-PTS (*ptsF*) and, to a minor proportion, as fructose-6-phosphate by the glucose-PTS (*ptsG*; Dominguez and Lindley 1996; Dominguez et al. 1998; Moon et al. 2005). As glucose-6-phosphate generated from sucrose can be directly metabolized in the oxidative pentose phosphate pathway, it is unclear why there should be a deficiency in NADPH supply during growth on sucrose that is relieved by transhydrogenase. Free fructose or fructose-1-phosphate can possibly act as an allosteric effector that inhibits the operation of the oxidative pentose phosphate pathway.

As shown in Table 2, the presence of transhydrogenase stimulated lysine formation on all carbon sources tested. The smallest increase was observed on glucose (~10%), probably due to the fact that the NADPH supply is almost sufficient due to a high carbon flux through the oxidative PPP. Mid or large increases in lysine concentration were observed on fructose (~70%), glucose plus fructose (~50%), and sucrose (~300%), suggesting that on these carbon sources the NADPH supply significantly limits lysine synthesis. However, the absolute lysine concentration (and also lysine and biomass yield) was still higher on glucose than on fructose or sucrose. Whereas the lysine yields were increased on all carbon sources in the presence of transhydrogenase, the biomass yield was increased on glucose, unchanged on fructose, and decreased on glucose plus fructose and on sucrose. This indicates that the increased lysine yield on sucrose and glucose plus fructose is obtained at the expense of a reduced biomass yield, whereas the increased lysine yield on glucose and on fructose is achieved at the expense of reduced CO₂ (or byproduct) formation. Reduced CO₂ formation might be

caused by a reduced flux through the oxidative PPP due to the inhibition of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by high NADPH concentrations (Moritz et al. 2000).

In contrast to the positive effects of *pntAB* expression in the lysine-producing strain DM1730, both growth and glutamate formation were negatively influenced by *pntAB* expression in the wild-type strain ATCC13032 cultivated in the presence of ethambutol. The reason for this inhibitory effect is not yet clear and requires further experimentation. In summary, our data have shown that the proton-coupled transhydrogenase PntAB of *E. coli* is a valuable metabolic engineering tool for increasing lysine synthesis by *C. glutamicum*, in particular, on carbon sources containing fructose and sucrose.

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