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Metabolic engineering of cellular transport for overproduction of the platform chemical 1,5-diaminopentane in *Corynebacterium glutamicum*

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ABSTRACT

The present work describes the development of a superior strain of *Corynebacterium glutamicum* for diaminopentane (cadaverine) production via metabolic engineering of cellular transport processes. In *C. glutamicum* DAP-3c, a tailor-made producer, the diaminopentane forming enzyme, lysine decarboxylase, was inhibited *in vivo* by its end-product, suggesting a potential bottleneck at the level of the export. The previously proposed lysine exporter *lysE* was shown not to be involved in diaminopentane export. Its deletion did not reduce diaminopentane secretion and could therefore be exploited to completely eliminate the export of lysine, an undesired by-product. Genome-wide transcription profiling revealed the up-regulation of 35 candidate genes as response to diaminopentane overproduction, including several transporters. The highest expression increase (2.6-fold) was observed for a permease, encoded by *cg2893*. Targeted gene deletion in the producer resulted in a 90% reduced diaminopentane secretion. Genome-based overexpression of the exporter, however, revealed a 20% increased yield, a 75% reduced formation of the undesired by-product N-acetyl-diaminopentane and a substantially higher viability, reflected by increased specific rates for growth, glucose uptake and product formation. Similarly, deletion of *cg2894*, TetR type repressor neighboring the permease gene, resulted in improved production properties. The discovery and amplification of the permease, as presented here, displays a key contribution towards superior *C. glutamicum* strains for production of the platform chemical diaminopentane. The exact function of the permease remained unclear. Its genetic modification had pronounced effects on various intracellular pools of the biosynthetic pathway, which did not allow a final conclusion on its physiological role, although a direct contribution to diaminopentane export appears possible.

1. Introduction

The shortage of fossil resources and the global warming are major drivers for a new bio-economy. As bio-based platform chemical, 1.5-diaminopentane (cadaverine) has recently come into focus (Kind and Wittmann, in press). It displays an interesting building block for bio-polyamides (Mimitsuka et al., 2007). Polymerization of this di-amine from microbial biosynthesis with bio-based di-carboxylic acids such as succinate or sebacic acid provides completely bio-based products such as PA 5.4 or PA 5.10, which exhibit excellent, long-known material properties (Carothers, 1938). This opens novel markets, e.g. in the automotive industry or high value consumer end products, beyond current applications of existing low performance biopolymers such as poly-lactic acid or poly-hydroxyalkanoates. Pioneering studies successfully demonstrated bio-based diaminopentane production by heterologous expression of genes encoding lysine decarboxylase such as *cadA* (Tateno et al., 2007) or *ldcC* (Kind et al., 2010a) in *Corynebacterium glutamicum*. Based on the meanwhile available genome scale model of *C. glutamicum* (Kjeldsen and Nielsen, 2009) and sophisticated methods for global strain profiling (Yuan et al., 2010; Sawada et al., 2010; Becker et al., 2011), systems metabolic engineering of the biosynthetic pathway and supporting reactions in *C. glutamicum* (Kind et al., 2010a) as well as identification and elimination of the undesired, competing pathway towards N-acetyl-diaminopentane (Kind et al., 2010b) recently resulted in efficient production strains with molar conversion yields up to 30%. The extension of the substrate spectrum of *C. glutamicum* towards pentose sugars now even allows the production of diaminopentane from hemicellulose feed stocks (Buschke et al., 2011). In all producing strains reported so far, 1,5-diaminopentane is secreted into the production medium. For *C. glutamicum*, the obviously existing transport system, however, has not been identified to date. Based on the charged nature of the product and previous findings for other compounds, an active transport system appears very likely. As example, active transport in *C. glutamicum* was demonstrated for

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L-glutamate (Hoischen and Krämer, 1989), L-lysine (Broer and Krämer, 1991; Vrljic et al., 1995; Bellmann et al., 2001), L-isoleucine (Zittrich and Krämer, 1994; Kennerknecht et al., 2002) and L-threonine (Palmieri et al., 1996; Simic et al., 2001). Most strikingly, metabolic engineering of the export was successfully used to increase the secretion of various of these products, including glutamate (Lapujade et al., 1999), lysine (Kelle et al., 1996), isoleucine (Morbach et al., 1996) or threonine (Reinscheid et al., 1994). In the case of lysine, overexpression of *lysE*, encoding the lysine exporter, even resulted in a five times higher lysine export rate as compared to the wild type (Vrljic et al., 1996). In this regard, it appeared highly important to unravel the unknown export mechanisms for diaminopentane in *C. glutamicum* as potential target for strain optimization. The present work comprised the identification of potential transporters for product export by global transcription profiling, the examination of protein function of one of the candidates by targeted gene deletion and subsequent physiological analysis of the corresponding mutant. Subsequently, the effect of targeted overexpression of the exporter on the production of diaminopentane was examined. In addition, the lysine exporter LysE was deleted in different strain backgrounds to eliminate the undesired secretion of lysine as by-product, observed occasionally (Tateno et al., 2007). With regard to the proposed contribution of LysE in the transport of diaminopentane (Kind et al., 2010b; Stäbler et al., 2011) this was also relevant concerning secretion of the target product.

2. Materials and methods

2.1. Strains and plasmids

The wild type *Corynebacterium glutamicum* ATCC 13032 was obtained from the American Type Strain and Culture Collection (Manassas, USA). The diaminopentane-producer *Corynebacterium glutamicum* DAP-3c has been rationally derived from *C. glutamicum* 11424 by codon optimized expression of lysine decarboxylase (Kind et al., 2010a). For strain construction, the *Escherichia coli* strains DH5α and NM522 (Invitrogen, Karlsruhe, Germany) and the plasmids pTc and pClik int *sacB* were applied as described previously (Kind et al., 2010a).

2.2. Media

Pre-culture of cells was performed as described previously (Kind et al., 2010a). For main cultures a minimal medium with glucose as sole source of carbon was applied (Kind et al., 2010a). In tolerance studies, it was additionally supplemented with 0.25 M (25.5 g L^{-1}), 0.5 M (51 g L^{-1}) or 1 M (102 g L^{-1}) diaminopentane di-hydrochloride, respectively. In addition, the production of diaminopentane was investigated for selected strains on complex medium, which contained per liter: 50 g glucose, 50 g yeast extract, 50 g $CaCO_3$, 25 g $(NH_4)_2SO_4$, 1.25 g $MgSO_4$, 2.5 g K_2HPO_4, 200 mg citrate, 3 mg biotin, 5 mg thiamine.HCl, 20 mg Ca pantothenic acid, 6 mg nicotinamide, and 20 mg $FeSO_4.7H_2O$.

2.3. Cultivation

All cultivations were performed at 30 °C and 230 rpm on a rotary shaker (shaking diameter 5 cm, Multitron, Infors AG, Bottmingen, Switzerland). For the first pre-culture, single colonies were used as inoculum. After an incubation for about 8 h, the cells were harvested by centrifugation (8800 × g, 2 min, 4 °C), washed twice with sterile 5% NaCl solution and then used as inoculum for the second pre-cultivation (50 mL in 500 mL baffled flasks). The cells were harvested in the exponential growth phase under the same conditions as described above and used as inoculum for the main cultures, which were performed in triplicate (50 mL in 500 mL baffled flasks). During the cultivations in minimal medium, the pH remained constant at 7.0 ± 0.2. In the complex medium, the pH was maintained in the range of 6.0–7.2 by manual addition of 2 M NaOH.

2.4. Chemicals

Tryptone, beef extract, yeast extract and agar were obtained from Difco Laboratories (Detroit, USA). All other chemicals were of analytical grade and obtained from Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

2.5. Genetic engineering of C. glutamicum

The construction, the purification and the analysis of plasmid DNA and the transformation of *E. coli* and *C. glutamicum* were performed as described previously (Kind et al., 2010a). The targeted deletion of genes and the exchange of native *C. glutamicum* promoters were carried out as described recently (Bolten et al., 2010; Dickschat et al., 2010). Shortly, genes were deleted by replacement of the coding region by a shortened gene fragment. For genome-based amplification of expression, the native promoter of the corresponding gene was replaced by the strong promoter of the *sod* gene (NCgl2826) (Becker et al., 2007). For this purpose, the integrative plasmid pClik int *sacB*, which cannot replicate in *C. glutamicum*, was used (Becker et al., 2005). The genetic modifications were verified by PCR. The primers used for construction and verification of the genetic changes are listed in Table 1.

2.6. Analysis of substrates and products

The concentration of glucose was quantified in 1:10 diluted cultivation supernatants by a glucose analyzer (2300 STAT Plus, Yellow Springs Instrument, Ohio). Trehalose and organic acids were quantified in 1:10 diluted culture supernatants by HPLC (LaChrom Elite, VWR Hitachi, West Chester, PA, USA), employing an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) as stationary phase and 12.5 mM H_2SO_4 as mobile phase at 0.5 mL min^{-1} and 40 °C (Becker et al., 2008). The detection was performed using UV light at 220 nm (organic acids) and refractive index (trehalose), respectively. The determination of the cell concentration as optical density at 660 nm and as cell dry mass was performed as described previously (Kiefer et al., 2004). Amino acid quantification in culture supernatants (1:10 diluted) and in cell extracts was carried out by HPLC (Krömer et al., 2005). The same method was adapted employing a modified elution gradient to the quantification of 1,5-diaminopentane and N-acetyl-diaminopentane (Kind et al., 2010a).

2.7. Intracellular metabolite analysis

Sampling for intracellular amino acids and related compounds was performed via fast filtration and subsequent extraction in boiling water, displaying a protocol, which has been previously validated for such analyses in *C. glutamicum* (Wittmann et al., 2004; Bolten et al., 2007). The sample pre-treatment included a washing step of the cells on the filter with 15 ml 5% NaCl solution, matching the ionic strength of the cultivation medium, to avoid metabolite leakage (Bolten et al., 2007). The intracellular level of metabolites was calculated considering a cytoplasmic volume of 1.95 μL per mg of dry cells as described previously (Krömer et al., 2004).