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Increased lysine production by flux coupling of the tricarboxylic acid cycle and the lysine biosynthetic pathway—Metabolic engineering of the availability of succinyl-CoA in *Corynebacterium glutamicum*

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ABSTRACT

In this study, we demonstrate increased lysine production by flux coupling using the industrial work horse bacterium *Corynebacterium glutamicum*, which was mediated by the targeted interruption of the tricarboxylic acid (TCA) cycle at the level of succinyl-CoA synthetase. The succinylase branch of the lysine production pathway functions as the bridging reaction to convert succinyl-CoA to succinate in this aerobic bacterium. The mutant *C. glutamicum* Δ*sucCD* showed a 60% increase in the yield of lysine when compared to the advanced lysine producer which was used as parent strain. This mutant was highly vital and exhibited only a slightly reduced specific growth rate. Metabolic flux analysis with ^{13}C isotope studies confirmed that the increase in lysine production was mediated by pathway coupling. The novel strain exhibited an exceptional flux profile, which was closer to the optimum performance predicted by in silico pathway analysis than to the large set of lysine-producing strains analyzed thus far. Fluxomics and transcriptomics were applied as further targets for next-level strain engineering to identify the back-up mechanisms that were activated upon deletion of the enzyme in the mutant strain. It seemed likely that the cells partly recruited the glyoxylate shunt as a by-pass route. Additionally, the α-ketoglutarate decarboxylase pathway emerged as the potential compensation mechanism. This novel strategy appears equally promising for *Escherichia coli*, which is used in the industrial production of lysine, wherein this bacterium synthesizes lysine exclusively by succinyl-CoA activation of pathway intermediates. The channeling of a high flux pathway into a production pathway by pathway coupling is an interesting metabolic engineering strategy that can be explored to optimize bio-production in the future.

1. Introduction

With a continuously increasing market production volume of 1.5 million tons per year, lysine is an important industrial amino acid. Since only the L-isomer of lysine is bioactive as a feed supplement, lysine production utilizes the fermentative production by the Gram-positive soil bacterium *Corynebacterium glutamicum* (Becker and Wittmann, 2012a). The high industrial relevance of *C. glutamicum* has constantly stimulated efforts to analyze and modify the underlying metabolic and regulatory networks for improved lysine yield, titer and productivity in a targeted manner. In recent years, sophisticated experimental and computational tools in systems biology have provided an excellent platform to overcome the limitations of classical strain engineering and have initiated the era of metabolic engineering, and more recently, systems metabolic engineering (Atsumi et al., 2008; Becker and Wittmann, 2012b; Kind et al., 2010a; Lee et al., 2005; Ohnishi et al., 2002). *C. glutamicum* possesses a functionally split pathway for the biosynthesis of lysine (Schrumpf et al., 1991), which is closely connected to the carbon core pathways (Fig. 1). Metabolic engineering of the terminal lysine production pathway was previously shown to be relatively straightforward (Kelle et al., 2005). However, despite extensive modification of the various catalytic steps involved, the various mutant strains revealed insufficient production performance, suggesting the need for global engineering of the metabolic pathway. With regard to carbon building blocks, the synthesis of lysine demands the use of oxaloacetate and pyruvate. Lysine production was increased by enhancing the supply of oxaloacetate either by the amplification of the enzyme pyruvate carboxylase forming oxaloacetate (Peters-Wendisch et al., 2001) or by the deletion of the enzyme phosphoenolpyruvate carboxykinase withdrawing oxaloacetate (Petersen et al., 2001). Furthermore, lysine biosynthesis requires high redox power. In this context, the metabolic

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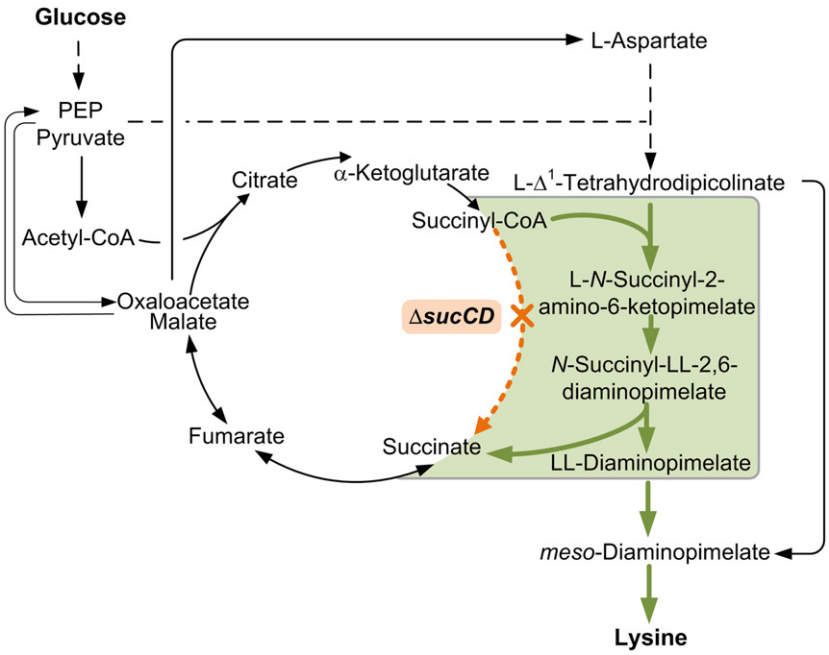

Fig. 1. Novel metabolic engineering strategy to increase the availability of succinyl-CoA for enhanced lysine production in *Corynebacterium glutamicum*: Coupling of the TCA cycle to the succinylase branch of lysine biosynthesis by deleting the TCA cycle enzyme succinyl-CoA synthetase, which competes with lysine biosynthesis for the pathway driver succinyl-CoA.

engineering of *C. glutamicum* was subsequently extended to increase the supply of NADPH. Successful strategies have provided strains capable of an increased lysine production by the metabolic flux engineering of the NADPH supply, which is brought about by the amplification of the pentose phosphate pathway (PPP) at the levels of either glucose 6-phosphate dehydrogenase (Becker et al., 2007), 6-phosphogluconate dehydrogenase (Ohnishi et al., 2005), glucose 6-phosphate isomerase (Marx et al., 2003), or fructose-1,6-bisphosphatase (Becker et al., 2005). In addition to carbon building blocks and redox power, lysine biosynthesis via the succinylase branch further involves succinyl-CoA as the activator of the pathway intermediates for introducing nitrogen. The major role of succinyl-CoA in the aerobic bacterium *C. glutamicum* is the operation of the TCA cycle for obtaining energy. In the TCA cycle, succinyl-CoA is formed by the enzyme α-ketoglutarate dehydrogenase and is further converted to succinate by the enzyme succinyl-CoA synthetase, releasing ATP (Eikmanns, 2005). The succinylase branch of the lysine biosynthesis pathway is responsible for approximately 70% of the lysine produced by the lysine-overproducing strains. This branch might even display the exclusive route under certain conditions, thereby necessitating the recruitment of stoichiometric amounts of succinyl-CoA for the product formed (Sonntag et al., 1993; van Gulik and Heijnen, 1995). However, the availability of succinyl-CoA has thus far not been considered as a metabolic engineering target for lysine overproduction.

In the present study, we propose a novel metabolic engineering strategy for increased lysine production in *C. glutamicum* using pathway coupling as the driving force. This process is mediated by the targeted interruption of the TCA cycle at the level of the enzyme succinyl-CoA synthetase in order to eliminate this reaction consuming succinyl-CoA and by integrating the corresponding part of the lysine biosynthetic pathway by bridging it to the disrupted TCA cycle. For this purpose, the two genes *sucCD* that encode both subunits of the succinyl-CoA synthetase were deleted in a recently obtained lysine overproducer (Becker et al., 2009). Subsequently, the production performance of the novel strain was evaluated and compared to the parent strain. This comparison was complemented by analysis at the systems level, and it involved transcriptome and ^{13}C fluxome analysis to resolve the cellular response to the genetic modification and to unravel targets for further strain engineering. The systems wide insight was of great interest with respect to the function of the enzyme succinyl-CoA synthetase, which remains to be fully characterized in *C. glutamicum* (Eikmanns, 2005), except for its transcriptional regulation (Cho et al., 2010; Han et al., 2008b).

2. Materials and methods

2.1. Microorganisms and plasmids

The lysine-producing strain *C. glutamicum* BS87 (Becker et al., 2009), obtained by metabolic engineering of the lysine pathway and supporting pathways from the wild-type *C. glutamicum* ATCC 13032, was used as the parent strain. The *Escherichia coli* strains DH5α and NM522 (Invitrogen, Karlsruhe, Germany) and the plasmids pClik int *sacB* and pTC were used in genetic engineering, as described previously (Kind et al., 2010a).

2.2. Genetic engineering

The *sucCD* operon with the two genes *cgl2565* and *cgl2566* that encode the two subunits of the enzyme succinyl-CoA synthetase was deleted by replacing the coding region by a shorter DNA fragment that lacked 1050 bp. This approach has been proven successful for other deletions in *C. glutamicum* (Becker et al., 2008; Kind et al., 2010b). Transformation was performed with the integrative vector pClik int *sacB*, using kanamycin resistance and sucrose tolerance as positive selection markers (Jäger et al., 1992). Vector construction was carried out with standard cloning strategies involving PCR, enzymatic digestion and DNA ligation. Strain validation was performed by PCR and by the determination of enzymatic activity. The primers used for construction and verification of the *sucCD* deletion mutant are listed in Table 1. Successful deletion of *sucCD* was confirmed by the 1088 bp-long