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Pushing product formation to its limit: Metabolic engineering of *Corynebacterium glutamicum* for L-leucine overproduction

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ABSTRACT

Using metabolic engineering, an efficient L-leucine production strain of *Corynebacterium glutamicum* was developed. In the wild type of *C. glutamicum*, the *leuA*-encoded 2-isopropylmalate synthase (IPMS) is inhibited by low L-leucine concentrations with a K_i of 0.4 mM. We identified a feedback-resistant IMPS variant, which carries two amino acid exchanges (R529H, G532D). The corresponding *leuA*^{fbr} gene devoid of the attenuator region and under control of a strong promoter was integrated in one, two or three copies into the genome and combined with additional genomic modifications aimed at increasing L-leucine production. These modifications involved (i) deletion of the gene encoding the repressor LtbR to increase expression of *leuBCD*, (ii) deletion of the gene encoding the transcriptional regulator IolR to increase glucose uptake, (iii) reduction of citrate synthase activity to increase precursor supply, and (iv) introduction of a gene encoding a feedback-resistant acetohydroxyacid synthase. The production performance of the resulting strains was characterized in bioreactor cultivations. Under fed-batch conditions, the best producer strain accumulated L-leucine to levels exceeding the solubility limit of about 24 g/l. The molar product yield was 0.30 mol L-leucine per mol glucose and the volumetric productivity was 4.3 mmol l^{-1} h^{-1}. These values were obtained in a defined minimal medium with a prototrophic and plasmid-free strain, making this process highly interesting for industrial application.

1. Introduction

Corynebacterium glutamicum is used in industrial biotechnology to produce several million tons of amino acids annually, in particular the flavor enhancer L-glutamate (2,200,000 t/year) and the feed additive L-lysine (1,500,000 t/year). Based on the increasing knowledge on this organism, which is documented in three monographs (Eggeling and Bott, 2005; Burkovski, 2008; Yukawa and Inui, 2013), strains have been developed for a variety of other commercially interesting compounds (Becker and Wittmann, 2012), such as organic acids (Okino et al., 2008; Litsanov et al., 2012a, 2012b; Wieschalka et al., 2013), diamines (Schneider and Wendisch, 2011; Mimitsuka et al., 2007; Kind and Wittmann, 2011), or alcohols (Inui et al., 2004; Smith et al., 2010; Blombach et al., 2011). Moreover, *C. glutamicum* is also an efficient host for the production of heterologous proteins (Scheele et al., 2013, and references therein). Thus, *C. glutamicum* has become a platform organism in industrial biotechnology.

Besides L-glutamate and L-lysine, *C. glutamicum* can also be used for the production of a variety of other amino acids, including the branched-chain amino acids (BCAAs) L-valine, L-isoleucine, and L-leucine, which are made in much smaller quantities of 1,000–3,000 t per year (Becker and Wittmann, 2012). However, the market is substantially growing, requiring efficient production processes. BCAAs are essential amino acids and used as components of pharmaceuticals, in animal feed industry, and as additives of infusion solutions and dietary products (Park and Lee, 2010). The role of L-leucine in stimulation of muscle protein synthesis and glucose homeostasis is discussed in the literature (Garlick, 2005; Layman, 2003). It is used in combination with the other BCAAs for patients with hepatic diseases to improve the nutritional status (Freund et al., 1982). L-Leucine is also applied as a flavoring substance and as a lubricant for tablet production (Leuchtenberger, 1996; Rotthäuser et al., 1998). Recently, the production of the biofuel 3-methyl-1-butanol from the L-leucine precursor 2-ketoisocaproate has been reported (Cann and Liao, 2010). The overlapping biosynthesis pathways of the three BCAAS, partly sharing the same precursors and enzymes, are depicted in Fig. 1.

Only a few studies deal with the microbial production of L-leucine using either *Brevibacterium lactofermentum* (later renamed to *C. glutamicum*) or *Escherichia coli* as hosts, which were reviewed recently by Park and Lee (2010). One of the first L-leucine producers was a 2-thiazolealanine-resistant, methionine-isoleucine-auxotrophic mutant derived from the glutamic acid-producing *B. lactofermentum* 2256 by nitrosoguanidine treatment (Tsuchida et al., 1974). The

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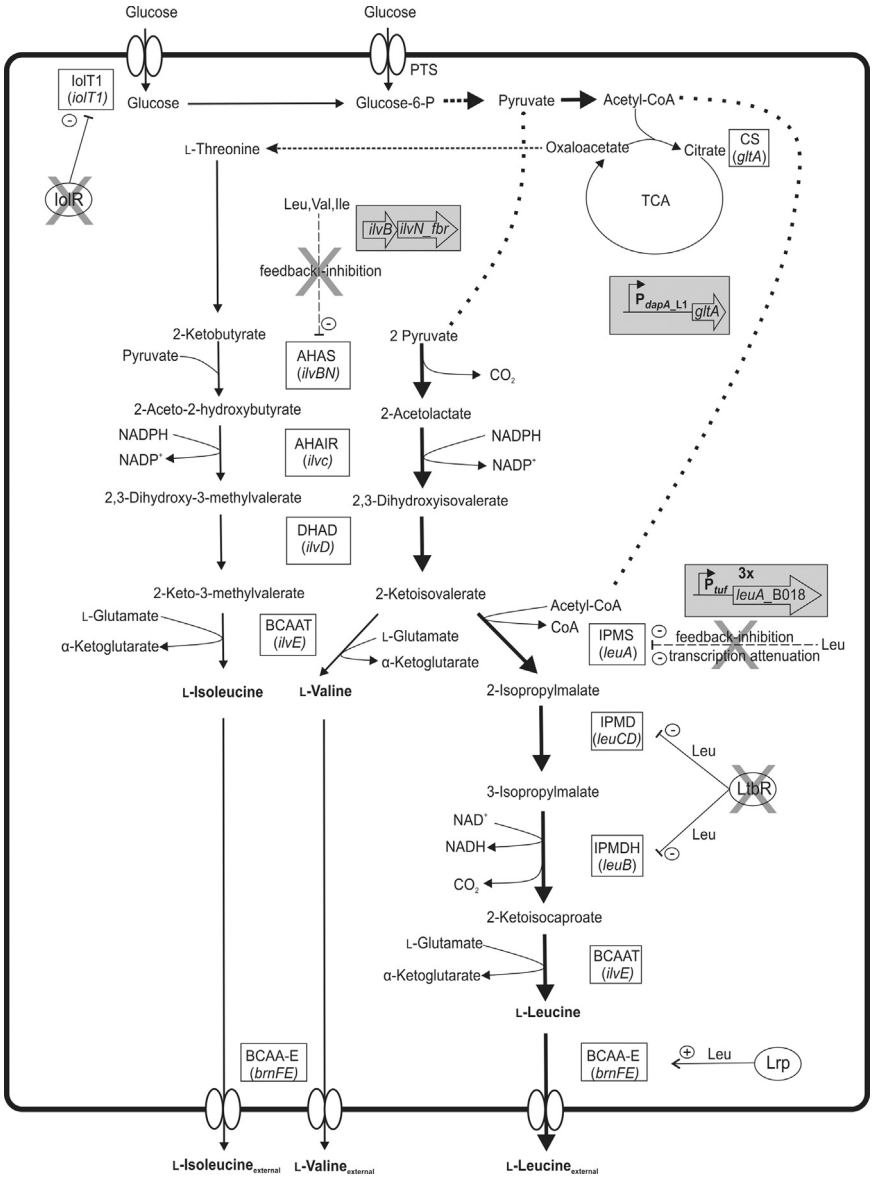

Fig. 1. Schematic representation of the L-leucine biosynthesis pathway of *C. glutamicum* and of the metabolic engineering steps performed in this study. Enzymes and their corresponding genes relevant for this study are shown in boxes. Regulatory proteins are shown in cycles. Lines with "+" indicate activation of gene expression, "-" indicates repression of gene expression (solid lines) or feedback-inhibition and transcription attenuation (dashed lines). "Leu", "Val", and Ile" indicate the presence of L-leucine, L-valine, and L-isoleucine, respectively. Deletion of genes and the respective proteins as well as abolishment of feedback-inhibition and transcription attenuation are indicated by "X". Metabolic engineering steps, like promoter exchanges and integration of genes encoding feedback-resistant enzyme variants, are highlighted in grey. Thick arrows indicate increased metabolic fluxes. Not shown is the *avtA* gene encoding the branched-chain amino acid transaminase AvtA, which transaminates 2-ketoisovalerate to L-valine in an L-alanine dependent manner. Abbreviations: AHAS, acetohydroxyacid synthetase; AHAIR, acetohydroxyacid isomeroreductase; BCAA-E, branched-chain amino acid exporter (BrnFE); BCAAT, branched-chain amino acid transaminase IlvE; CS, citrate synthase; DHAD, dihydroxyacid dehydratase; fbr, feedback-resistant; IPMD, 3-isopropylmalate dehydratase; IPMDH, 3-isopropylmalate dehydrogenase; IPMS, 2-isopropylmalate synthase; Lrp, leucine-responsive protein; LtbR, leucine and tryptophan biosynthesis regulator; P, promoter; PTS, phosphotransferase system; TCA, tricarboxylic acid cycle.

productivity of this strain was further optimized by additional mutagenesis steps (Ambe-Ono et al., 1996; Tsuchida and Momose, 1986). Another *S*-(2-aminoethyl)-L-cysteine resistant mutant was derived from *C. glutamicum* ATCC 13032 (Azuma et al., 1987). All *C. glutamicum*-based production strains were created by random mutagenesis, which is known to cause adverse effects, such as growth retardation and by-product formation (Park et al., 2007). Especially the accumulation of large amounts of by-products is a problem in the production of the three BCAAs due to their overlapping biosynthesis pathways, which negatively affects yield and downstream processing. Since the genome of *C. glutamicum* is known (Kalinowski et al., 2003; Ikeda and Nakagawa, 2003) and efficient techniques for its genetic engineering exist (Kirchner and Tauch, 2003), the creation of tailor-made strains of this organism with desired properties is feasible. In this study, we report on the rational design of a *C. glutamicum* L-leucine producer. Starting from wild type ATCC 13032, we constructed a potent, genetically defined and plasmid-free producer strain that is suitable for industrial fermentation processes.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

All bacterial strains and plasmids used in this study as well as their relevant characteristics are listed in Table 1. The *C. glutamicum*