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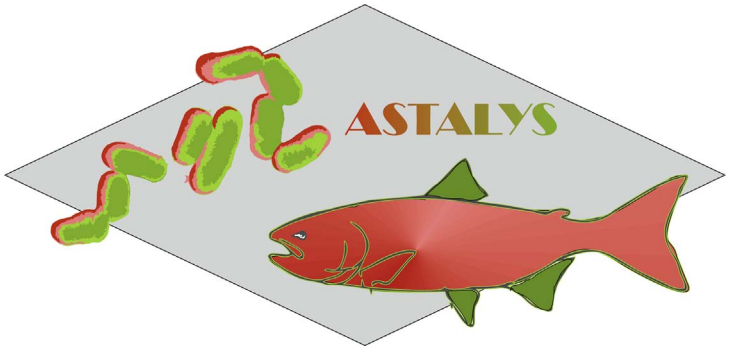
Coproduction of cell-bound and secreted value-added compounds: Simultaneous production of carotenoids and amino acids by *Corynebacterium glutamicum*

Nadja A. Henke, Daniela Wiebe, Fernando Pérez-García, Petra Peters-Wendisch, Volker F. Wendisch*

Genetics of Prokaryotes, Faculty of Biology & Center for Biotechnology (CeBiTec), Bielefeld University, Universitaetsstraße 25, Bielefeld, Germany

GRAPHICAL ABSTRACT



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ABSTRACT

Corynebacterium glutamicum is used for production of the food and feed amino acids L-glutamate and L-lysine at the million-ton-scale. One feed formulation of L-lysine simply involves spray-drying of the fermentation broth, thus, including secreted L-lysine and *C. glutamicum* cells which are pigmented by the C50 carotenoid decaprenoxanthin. *C. glutamicum* has been engineered for overproduction of various compounds including carotenoids. In this study, *C. glutamicum* was engineered for coproduction of a secreted amino acid with a cell-bound carotenoid. As a proof of principle, coproduction of L-glutamate with the industrially relevant astaxanthin was shown. This strategy was applied to engineer L-lysine overproducing strains for combined overproduction of secreted L-lysine with the cell-bound carotenoids decaprenoxanthin, lycopene, β-carotene, zeaxanthin, canthaxanthin and astaxanthin. By fed-batch fermentation 48 g/L L-lysine and 10 mg/L astaxanthin were coproduced. Moreover, *C. glutamicum* was engineered for coproduction of L-lysine and β-carotene from xylose and arabinose as alternative feedstocks.

1. Introduction

Corynebacterium glutamicum was isolated in the 1950s as a natural glutamate producer (Kinoshita et al., 1957). L-Glutamate is used in the food industry as a flavor enhancer in large scale (Hashimoto, 2016). This bacterium is also used for production of L-lysine (Wendisch et al., 2016b) which finds application as feed additive for the breeding of swine, poultry and in aquaculture (Eggeling and Bott, 2015;

* Corresponding author.
E-mail address: volker.wendisch@uni-bielefeld.de (V.F. Wendisch).

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Mitsuhashi, 2014) and its annual market volume is expected to reach 2.8 million tons by 2020 (Lee and Wendisch, 2017). Rational metabolic engineering of *C. glutamicum* wild type enabled overproduction of various value-added compounds (Heider and Wendisch, 2015) and led to strains producing L-lysine with relevant titers, yields and productivities (Becker and Wittmann, 2015; Ikeda, 2016; Pérez-García et al., 2016).

C. glutamicum is yellow-colored due to biosynthesis of the pigment decaprenoxanthin and its glycosides. Decaprenoxanthin belongs to the rare C50 carotenoids and is produced from the precursors IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate) which are formed form the glycolytic intermediates pyruvate and GAP (glyceraldehyde 3-phosphate) in the MEP (methylerythritolphosphate) pathway (Heider et al., 2012; Henke et al., 2017; Krubasik et al., 2001). Whereas the genes of the MEP pathway and for the GGPP (geranylgeranyl pyrophosphate) synthase IdsA are scattered, seven carotenogenic genes (*crtE*, cg0722, *crtB*, *crtI*, $crtY_e$, $crtY_f$, *crtEb*) are co-transcribed as *crt* operon (Heider et al., 2012). The MarR-type regulator CrtR represses the *crt* operon by binding to a target DNA sequence overlapping with the promoter of this operon (Henke et al., 2017). Notably, isoprenoid pyrophosphates serve as inducers of CrtR, thus, the *crt* operon is derepressed upon sufficient supply of the precursors of carotenogenesis (Henke et al., 2017). Thus, this CrtR-mediated metabolite-dependent regulatory mechanism of carotenogenesis differs fundamentally from the various light-induced regulatory mechanisms known from other non-phototrophic and phototrophic bacteria (Henke et al., 2017). Regulatory engineering involving deletion of *crtR* (Henke et al., 2017) and overexpression of sigma factor genes (Taniguchi et al., 2017) were combined with metabolic engineering to overproduce the native carotenoid decaprenoxanthin and its precursors and to enable production of non-native C50 and C40 carotenoids such as β-carotene and astaxanthin with volumetric productivities that are competitive to commercially used production hosts (Heider et al., 2014; Henke et al., 2016). These carotenoids are the colorful representatives of the large and versatile group of terpenoids that are mainly used as additives in the food, feed and cosmetics industries (Dembitsky, 2005; Downham and Collins, 2000; Winterhalter and Rouseff, 2001), but due to their excellent antioxidant properties they are also receiving increasing attention as a high-performance ingredient with beneficial effects on human health (Sandmann, 2001; Umeno et al., 2005). The market volume of carotenoids is increasing and expected to reach $1.8 billion in 2019.

This study describes metabolic engineering of *C. glutamicum* for the combined production of cell-bound and secreted value-added compounds. As proof-of-concept, combined production of cell-bound carotenoids such as β-carotene or astaxanthin and amino acids such as L-glutamate or L-lysine is demonstrated. One commercial L-lysine formulation, namely spray-dried L-lysine fermentation broth (Kelle et al., 2005) is compatible to the concept of coproduction of cell-bound and secreted value-added compounds by *C. glutamicum* reported here.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

The strains and plasmids used in this work are listed in Table 1. The prophage-cured *C. glutamicum* MB001 (Baumgart et al., 2013) as well as the L-lysine producing prophage-cured strain GRLys1Δ*sugR*Δ*ldhA* (Pérez-García et al., 2016) were used as the basal strains for genetic engineering. The modifications of the latter strain are focused on increased precursor supply, reduced by-product formation, increased glucose utilization, and enhanced L-lysine export (Baumgart et al., 2013; Kalinowski et al., 1991; Pérez-García et al., 2016; Peters-Wendisch et al., 2001; Unthan et al., 2014; Vrljic et al., 1996). Precultivations of *C. glutamicum* were performed in 50 ml BHIS (brain heart infusion supplemented sorbitol) with 50 mM of glucose and appropriate antibiotics. Main cultivations of *C. glutamicum* were performed in CGXII medium with 100 mM glucose as carbon and energy source (Eggeling and Bott, 2005) with an initial OD_{600} of 1 at 30 °C in a volume of 50 ml in 500 ml flasks with two baffles shaking at 120 rpm. L-Glutamate excretion was trigged as described elsewhere applying biotin limitation or ethambutol addition (50 mg/L) (Eggeling and Bott, 2005). Biotin limitation (1 μg/L) was established after a prelimitation in CGXII with 10 μg/L. When appropriate, kanamycin and/or tetracycline were added to concentrations of 25 μg/ml and 5 μg/ml, respectively. If not stated otherwise, an IPTG concentration of 1 mM for induction of gene expression was added at inoculation of the main culture. The optical density (OD_{600}) of cell cultures was measured using a Shimadzu UV-1202 spectrophotometer (Duisburg, Germany).

2.2. Chromosomal deletions and integrations in GRLys1 derivatives

The L-lysine producing platform strain GRLys1Δ*sugR*Δ*ldhA* was transformed into a coproducing strain of astaxanthin and L-lysine (ASTALYS). During the transformation several coproduction strains were generated. Genomic deletions and integrations were performed using the suicide vector pK19*mobsacB* (Schäfer et al., 1994). The genomic regions flanking the respective gene for homologous recombination were amplified from genomic DNA of *C. glutamicum* WT as described elsewhere (Heider et al., 2014; Henke et al., 2016). The PCR products were purified and assembled and simultaneously cloned into restricted pK19*mobsacB* by Gibson assembly, which resulted in the specific derivatives pK19*mobsacB* (Table 1). Targeted gene deletion/integration proceeds via two-step homologous recombination using the before mentioned deletion/integration vectors as described previously (Eggeling and Bott, 2005). Transfer of the suicide vectors was carried out via transconjugation using *E. coli* S17-1 as donor strain (Eggeling and Bott, 2005). The first recombination, the integration of the vector into one of the gene flanking regions, was selected via kanamycin resistance. Integration of the vector into the genome yields sucrose sensitivity due to the expression of *sacB*, encoding a levansucrase. In the second recombination the suicide vector is excised and clones can be selected upon sucrose-resistance. The deletion/integration of the respective gene could be verified by PCR analysis of the selected mutant, using the primer pairs E/F of the respective genes (Table 1).

2.3. Plasmid-driven heterologous overexpression of genes

Genes encoding for enzymes of functionalization of cyclic C40 carotenoids were expressed with a two-vector system. The expression vectors pSH1 (Henke et al., 2016) and pECXT99A (Kirchner and Tauch, 2003) were used to enable constitutive and inducible overproduction of the β-carotene ketolase and β-carotene hydroxylase from *F. pelagi*, respectively. Utilization of arabinose and xylose was mediated via plasmid-driven overexpression of *araBAD* (*E. coli*) or *xylA* (*X. campestris*) and *xylB* (*C. glutamicum*) under 500 μM IPTG induction (Meiswinkel et al., 2013). Transformation of *C. glutamicum* was performed via electroporation using a Gene Pulser Xcell™ (Bio-Rad Laboratories GmbH, Munich, Germany) at 2.5 kV, 200 Ω and 25 μF (Eggeling and Bott, 2005).

2.4. Fed-batch fermentation

A bioreactor with a total volume of 20 L and a working volume of 15 L was used (MBR Bioreactor AG, Switzerland). It was equipped with three six-bladed Rushton turbines and four baffles. Operating pH and oxygen saturation in the medium (pO_2) were followed by electrodes (Ingold, Germany). By automated addition of KOH (4 M) and phosphoric acid (10%) pH was kept at 7.0. Samples for quantification were taken by an autosampler and cooled down to 4 °C until use. Initial volume of the fermentation was 12 L with additional feeding volume of 3 L. Fermentation was carried out with 0.4 bar overpressure and