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Production of the sesquiterpene (+)-valencene by metabolically engineered *Corynebacterium glutamicum*

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ABSTRACT

The sesquiterpene (+)-valencene is an aroma compound of citrus fruits and is used to flavor foods and drinks. Biosynthesis of (+)-valencene starts from farnesyl pyrophosphate, an intermediate of carotenoid biosynthesis. *Corynebacterium glutamicum*, the workhorse of the million-ton scale amino acid industry, is naturally pigmented as it synthesizes the rare fifty carbon atoms (C50) containing carotenoid decaprenoxanthin. Since the carotenoid pathway of this Gram-positive bacterium has previously been engineered for efficient production of several C50 and C40 carotenoids, its potential to produce a sesquiterpene was assessed. Growth of *C. glutamicum* was negatively affected by (+)-valencene, but overlaying *n*-dodecane as organic phase for extraction of (+)-valencene was shown to be biocompatible. Heterologous expression of the (+)-valencene synthase gene from the sweet orange *Citrus sinensis* was not sufficient to enable (+)-valencene production, likely because provision of farnesyl pyrophosphate (FPP) by endogenous prenyltransferases was too low. However, upon deletion of two endogenous prenyltransferase genes and heterologous expression of either FPP synthase gene *ispA* from *Escherichia coli* or *ERG20* from *Saccharomyces cerevisiae* (+)-valence production by *C. sinensis* valencene synthase was observed. Employing the valencene synthase from Nootka cypress improved (+)-valencene titers 10 fold to 2.41 ± 0.26 mg l^{-1} (+)-valencene, which is equivalent to 0.25 ± 0.03 mg g^{-1} cell dry weight (CDW). This is the first report on sesquiterpene overproduction by recombinant *C. glutamicum*.

1. Introduction

Terpenoids, also referred to as isoprenoids, are a large and highly diverse group of natural products that are derived from the five carbon (C5) precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMPP). The classification of terpenoids is based on the number of C5-subunits they consist of, e.g. monoterpenes (C10) and sequiterpenes (C15) (Kirby and Keasling, 2009). In most bacteria, algae and in the plastids of plants, IPP and DMPP are synthesized in the 2-C-methyl-D-erythritol 4-phosphate (MEP) or 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway (Eisenreich et al., 2001). In this pathway, glyceraldehyde 3-phosphate and pyruvate are condensed and converted to IPP in seven enzymatic steps. IPP and DMPP are subsequently converted by enzymes of the prenyltransferase family to the precursor metabolites of monoterpenes (geranyl pyrophosphate, GPP), sesquiterpenes (farnesyl pyrophosphate, FPP) and diterpenes and carotenoids (geranylgeranyl pyrophosphate, GGPP) by head-to-tail or head-to-head condensation (Takahashi and Koyama, 2006).

The bicyclic sesquiterpene (+)-valencene is a constituent of the essential oils of different members of the *Citrus* genus, including the sweet orange (*Citrus sinensis*). Due to its fruity, woody flavor it is commercially used as an additive in drinks and food with a market volume of approximately 10,000 kg per year (Beekwilder et al., 2014). Besides the use as a food additive, (+)-valencene can be oxidized to (+)-nootkatone (Girhard et al., 2009). (+)-Nootkatone is a high value product used to add a grapefruit flavor to drinks, since it exhibits a slightly bitter taste and has a low odor threshold of about 1 μg l^{-1} water (Fraatz et al., 2009). (+)-Valencene is produced by (+)-valencene synthases belonging to the family of sesquiterpene synthases, which catalyze the conversion of FPP to linear and cyclic sesquiterpenes (Chappell, 2004). To the present day there are only a few functional (+)-valencene synthases described in literature, e.g. CnTPS1 from *C. sinensis*, VvVal from *Vitis vinifera* and CnVS from *Callitropsis nootkatensis* (Beekwilder et al., 2014; Lucker et al., 2004; Sharon-Asa et al., 2003). Currently, (+)-valencene is routinely

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extracted from citrus oil glands by steam distillation, but giving the fact that only a minor part of the fruits contain (+)-valencene, it is desirable to genetically modify microorganisms for the production of (+)-valencene.

Corynebacterium glutamicum, a gram-positive, rod-shaped soil bacterium, is a known producer of amino acids (e.g. L-glutamate and L-lysine) at a million ton scale per year (Ajinomoto, Inc., available http://www.ajinomoto.com/en/ir/pdf/FY13Q1_data_E.pdf, Cited 27 February 2014) and is considered as a *generally-regarded-as-safe* (GRAS) organism. *C. glutamicum* has a history of more than five decades of safe use in food and feed biotechnology. *C. glutamicum* grows naturally on various carbon sources, e.g. sugars, alcohols, organic and amino acids (Blombach and Seibold, 2010). Access to alternative carbon sources was gained by metabolic engineering enabling *C. glutamicum* to grow and produce e.g. with xylose (Meiswinkel et al., 2013a), crude glycerol (Meiswinkel et al., 2013b), cellobiose (Adachi et al., 2013), or starch (Song et al., 2013). Its versatility as biotechnological production platform was further extended by metabolic engineering to include overproduction of other amino acids such as L-proline (Jensen and Wendisch, 2013). Moreover, besides amino acids, also production of a number of other compounds with economical interest such as glycolate (Zahoor et al., 2014), 1,4-diaminobutane (putrescine) (Schneider and Wendisch, 2010), 1,5-diaminopentane (cadavarine) (Mimitsuka et al., 2007), pyruvate and other organic acids (Wieschalka et al., 2012, 2013) as well as succinate (Litsanov et al., 2012a,b; Okino et al., 2008) by engineered *C. glutamicum* became possible. Recently, its potential as a producer of a variety of carotenoids was revealed (Heider et al., 2012, 2014a).

C. glutamicum WT possesses all genes for the MEP pathway of IPP synthesis as well as carotenogenic genes for synthesis of its pigment, the rare C50 carotenoid decaprenoxanthin (Heider et al., 2014b; Sandmann and Yukawa, 2005). The carotenogenic genes of *C. glutamicum* WT are organized in two gene clusters, the first one, consisting of the genes *crtE-cg0722-crtBIY$_e$Y$_f$Eb*, coding for a geranylgeranyl-pyrophosphate synthase, a putative drug exporter of the RND superfamily, a phytoene synthase, a phytoene desaturase, a carotenoid C45/C50 ε-cyclase and a lycopene elongase, respectively. The second gene cluster contains the three genes *crtB2*, *crtI2-1*, and *crtI2-2*. While no evidence for activity of CrtI2-1 and CrtI2-2 as phytoene desaturase was obtained although they show high similarity to parts of CrtI, *crtB2* was shown to encode a second functional phytoene synthase (Heider et al., 2012). In *C. glutamicum*, carotenoid biosynthesis starts with the conversion of IPP and DMPP to GGPP catalyzed by prenyltransferases CrtE and/or IdsA (Fig. 1). Subsequently, two molecules of GGPP are condensed by CrtB and/or CrtB2 to yield phytoene. After four desaturation reactions catalyzed by phytoene desaturase CrtI the resulting lycopene undergoes two prenylation reactions catalyzed by the gene product of *crtEb* to yield the acyclic C50 carotenoid flavuxanthin (Fig. 1). The heterodimeric ε-cyclase CrtY$_e$ and CrtY$_f$ converts favuxanthin to decaprenoxanthin, the natural pigment of *C. glutamicum* (Heider et al., 2014a).

Since sesquiterpenes such as (+)-valencene are derived from the same precursors IPP and DMAPP as carotenoids, *C. glutamicum* might be a potential production host for a variety of terpenoids other than carotenoids. In this study, the potential of *C. glutamicum* as a production host for (+)-valencene was evaluated. It was shown that simply by heterologous expression of a (+)-valencene synthase gene *C. glutamicum* was not able to produce the sesquiterpene (+)-valencene. However, when endogenous prenyltransferases were replaced by FPP synthase from *Saccharomyces cerevisiae* or from *Escherichia coli*, the heterologous expression of (+)-valencene synthase genes resulted in production of (+)-valencene. Furthermore, *C. glutamicum* growth was shown to be compatible with overlay of a *n*-dodecane phase to facilitate extraction of hydrophobic compounds such as (+)-valencene.

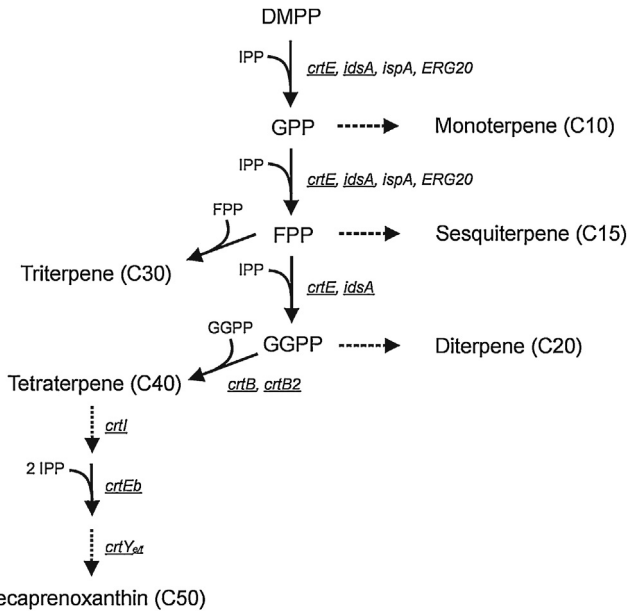

Fig. 1. *Schematic representation of isprenoid biosynthesis.* Gene names of enzymes are depicted next to the arrow representing the reaction. Reactions of prenyltransferases (CrtB, CrtB2, CrtE, IdsA, IspA and ERG20) are given as solid lines, reactions of terpene synthases as dashed lines and other reactions as dotted lines. Abbreviations: IPP, isopentenyl pyrophosphate; DMPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

All strains of *C. glutamicum* used in this work were derived from the wild-type strain ATCC 13032 (Table 1). coli *Escherichia* strain *DH5α* was used for cloning and plasmid maintenance. For transformation of *E. coli*, the $CaCl_2$ method was used (Dagert and Ehrlich, 1979) and *C. glutamicum* was transformed via electroporation (van der Rest et al., 1999) at 2.5 kV, 200 Ω and 25 μF. Growth of bacteria was conducted in 10 ml media in 100 ml non-baffled flasks at 30 °C (*C. glutamicum*) and 37 °C (*E. coli*). Precultures of *C. glutamicum* were grown in BHI or LB supplemented with 50 mM of glucose, respectively. For production experiments the cells were transferred to CgXII minimal media (Eggeling and Reyes, 2005) supplemented with 40 g L^{-1} D-glucose monohydrate as carbon source, directly induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultivated at 120 rpm for 28 h and 50 h, respectively. When necessary, kanamycin and/or spectinomycin were added to the media to concentrations of 25 μg ml^{-1} and 100 μg ml^{-1}, respectively. The production cultures were inoculated with an initial OD_{600} of 1 and growth was followed by monitoring OD_{600}. Biomass concentrations are given as g CDW l^{-1} and were calculated using an experimentally determined factor of 0.25 g CDW l^{-1} for OD_{600} of 1. For extraction of volatile products, the cultures were aseptically overlaid with *n*-dodecane (one tenth of the culture volume) (Sigma Aldrich, Steinheim, Germany) 4 h after induction. After cultivation, cells were harvested by centrifugation for 20 min at 4000 × *g* in glass test tubes and the *n*-dodecane phase was harvested.

2.2. Construction of plasmids

All genes were amplified with KOD Hot Start DNA Polymerase (Novagen, Darmstadt, Germany). The coding sequence of