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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 fold to $2.41 \pm 0.26\ mg\ l^{-1}$ (+)-valencene, which is equivalent to $251 \pm 26\ \mu g\ g^{-1}$ 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 C_5-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 $1\ \mu g\ l^{-1}$ 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-lysin) 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

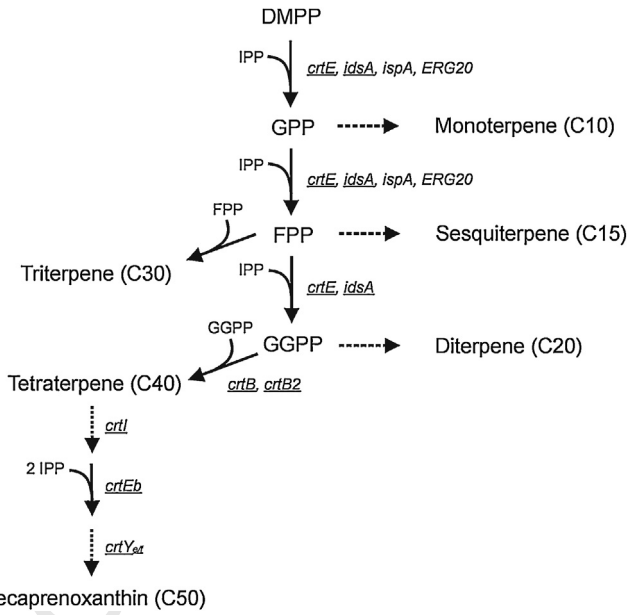

Fig. 1. *Schematic representation of isprenoid biosynthesis.* Gene names of enzymes are depicted next to the arrow representing the reaction. Reactions of prenyltransferases (CrtB, CrtB2, CrtE, IdsA, IspA and ERG20) are given as solid lines, reactions of terpene synthases as dashed lines and other reactions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

of a *n*-dodecane phase to facilitate extraction of hydrophobic compounds such as (+)-valencene.

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). *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 4% 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 genes and plasmids

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

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Table 1
Strains, plasmids and oligonucleotides used in this study.

Strain, plasmid or oligonucleotide	Relevant characteristics or sequence	Source or reference
E. coli strains		
E. coli DH5α	F⁻ *thi*-1 *endA1 hsdR17*(r⁻ m⁻) *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Hanahan (1983)
C. glutamicum strains		
WT[a]	ATCC13032	Abe et al. (1967)
Δ*crtB2*Δ*crtI2-1*/*2*Δ*crtB*	*crtB*Δ*crt2I2-1I2-2* and *crtB* deletion mutant of *C. glutamicum* WT	Heider et al. (2012)
Δ*crtE*Δ*idsA*	*crtE* (cg0723) and *idsA* (cg2384) deletion mutant of *C. glutamicum* WT	This work
VLC1	Δ*crtE*Δ*idsA*(pVWEx1-*ERG20*)(pEKEx3-*CsTPS1*)	This work
VLC2	Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CsTPS1*)	This work
VLC3	Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CnVS*)	This work
Plasmids		
pK19*mobsacB*	Km^R; *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 $oriV_{Ec}$ *sacB lacZ*α)	Schäfer et al. (1993)
pK19*mobsacB-crtE*	pK19*mobsacB* with a *crtE* deletion construct	This work
pK19*mobsacB-idsA*	pK19*mobsacB* with a *idsA* deletion construct	This work
pVWEx1	Km^R; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{q}, pCG1 $oriV_{Cg}$)	Peters-Wendisch et al. (2001)
pVWEx1-*CsTPS1*	pVWEx1 derivative for IPTG-inducible expression of (+)-valencene synthase *CsTPS1* of *Citrus sinensis* containing an artificial ribosome binding site	This work
pVWEx1-*ERG20*	pVWEx1 derivative for IPTG-inducible expression of FPP synthase *ERG20* from *S. cerevisiae* containing an artificial ribosome binding	This work
pVWEx1-*idsA*	pVWEx1 derivative for IPTG-inducible expression of *idsA* from *C. glutamicum* containing an artificial ribosome binding	This work
pVWEx1-*CnVS*	pVWEx1 derivative for IPTG-inducible expression of (+)-valencene synthase *CnVS* of *Callitropsis nootkatensis* containing an artificial ribosome binding site	This work
pEKEx3	$Spec^R$; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{q}, pBL1 $oriV_{Cg}$)	Stansen et al. (2005)
pEKEx3-*CsTPS1*	pEKEx1 derivative for IPTG-inducible expression of (+)-valencene synthase *CsTPS1* of *Citrus sinensis* containing an artificial ribosome binding site	This work
pEKEx3-*ispA*	pEKEx3 derivative for IPTG-inducible expression of FPP synthase *ispA* from *E. coli* containing an artificial ribosome binding	This work
pEKEx3-*crtE*	pEKEx3 derivative for IPTG-inducible expression of *crtE* from *C. glutamicum* containing an artificial ribosome binding	This work
Oligonucleotides sequence (5′ → 3′)		
CsVal_fwd[b]	*GAATTCCTGCAG*GTCGACTCTAGAGGAAAGG	
CsVal_rev[b]	GCGTCGACCCCGGGTTAGAATGGAACGTG	
CnVS_fwd[c]	GCGC**GGATCC**GAAAGGAGGCCCTTCAGATGGCTGAAATGTTTAATGG	
CnVS_rev[c]	CGCC**GAATTCGGTACC**GTTATGGAATGATTGGTTCC	
Erg20_fwd[b,c]	*CTGCAG*GAA**AGGAGG**CCCTTCAGATGGCTTCAGAAAAAGA	
Erg20_rev[b]	GAGGCCC*TTCAGGCTGCAG*TTATTTGCTTCTCTTGTAAAC	
ispA_fwd	GCGCGGATCCGAAAGGAGGCCCTTCAGATGGACTTTCCGCAGCAACTCG	
ispA_rev	CGCCGAATTCGGTACCGTTATTTATTACGCTGGATGATGTAGTCC	
crtE_A[b]	AAAACCCGGG*TAGCTCCATATAACGTGCCG	
crtE_B[d]	***CCCATCCACTAAACTTAAACA***GATTGTCATGCCATTGTCCAT	
crtE_C[d]	***TGTTTAAGTTTAGTGGATGGG***GCCAGCCGCAAATCTTAG	
crtE_D[b]	AAAACCCGGG*ACTTCATCGGAAACTATGTCTA	
crtE_E	GTGACCATGAGGGCGAAAGC	
crtE_F	TCACATAGTCCGGCGTTTGC	
*crtE_Pst*I-fw[b,c]	AAAA*CTGCAG***GAAAGGAGGCCCTTCAG**ATGGACAATGGCATGACAATC	
*crtE_Bam*HI-fw[b,c]	AAAA*GGATCC***GAAAGGAGGCCCTTCAG**ATGGACAATGGCATGACAATC	
*crtE_Pst*I-rv[b]	AAAA*CTGCAG*CTAAGATTTGCGGCTGGC	
idsA_A[b]	AAAACCCGGG*CTCTTCGCGAGTGTGGTTAAC	
idsA_B[d]	***CCCATCCACTAAACTTAAACA***GGCATCGAAACTGCTCAA	
idsA_C[d]	***TGTTTAAGTTTAGTGGATGGG***TCAACCGAACGTCGGATGTAG	
idsA_D[b]	AAAACCCGGG*AGAGACCTCAAGCAACATGG	
idsA_E	GCAGCTTCGCCAGAGTGTAT	
idsA_F	CAATGCGGACAATGCTCCAG	
idsA_SalI-fw[b,c]	AAAA*GTCGAC***GAAAGGAGGCCCTTCAG**TTGAGCAGTTTCGATGCC	
*idsA_Sal*I-rv[b]	AAAA*GTCGAC*CTACATCCGACGTTCGGTTG	
pVWEx-fw	CATCATAACGGTTCTGGC	
pVWEx-rv	ATCTTCTCTCATCCGCCA	
M13 fw	CACAGCGGGAGTGCCTATTGTTTTG	
M13 rv	CAGCGATGATCACTTCTGGCTC	

[a] WT, wild type.
[b] Sequence in italic: restriction sites.
[c] Sequence in bold: artificial ribosome binding site.
[d] Sequence in bold and italic: linker sequence for hybridization.

(+)-valencene synthase *CsTPS1* (*C. sinensis*) was obtained from NCBI GeneEntrez Database (GenBank: AF441124.1). The sequence was codon optimized for *C. glutamicum* by the Java Codon Adaptation Tool (JCat) (Grote et al., 2005) and the optimized gene was synthesized by GeneArt® life technologies™ (Darmstadt, Germany). An artificial ribosomal binding site (AGGAGG) was added 8 bp before the translational start codon. Upstream of the RBS and downstream of the stop codon of the gene restriction sites for cloning were added. The gene was amplified using primer CsVal_fwd and CsVal_rev (Table 1) and cloned in the expression vectors pEKEx3 (Peters-Wendisch et al., 2001) and pVWEx1 (Stansen et al., 2005) (Table 1), respectively, by blunt end cloning. The (+)-valencene

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synthase gene *CnVS* (*C. nootkatensis*) was amplified from the plasmid pAC-65-3 with primers CnVS_fwd and CnVS_rev (Table 1) and cloned in expression vector pVWEx1 using BamHI and KpnI restriction sites. The FPP synthase genes *ERG20* from *S. cerevisiae* and *ispA* from *E. coli* were amplified from the respective genomic DNA with primers Erg20_fwd/Erg20_rev and ispA_fwd/ispA_rev (Table 1), respectively, and cloned in pVWEx1 or pEKEx3 by blunt end cloning, resulting in pVWEx1-*ERG20* and pEKEx3-*ispA*. The correct sequences of all inserts were confirmed by sequencing.

2.3. Strain optimization and (+)-valencene production in C. glutamicum

For the deletion of prenyltransferases *crtE* and *idsA* of *C. glutamicum* the suicide vector pK19*mobsacB* was used as described previously (Heider et al., 2012). The double knockout strain Δ*crtE*Δ*idsA* was chosen as host for production of (+)-valencene and transformed with pVWEx1-*ERG20* and pEKEx3-*ispA*, respectively, resulting in the strains Δ*crtE*Δ*idsA*(pVWEx1-*ERG20*) and Δ*crtE*Δ*idsA*(pEKEx3-*ispA*). Subsequently, both strains were transformed either with pVWEx1-*CsTPS1*, pEKEx3-*CsTPS1* or pEKEx3-*CnVS* giving rise to Δ*crtE*Δ*idsA*(pVWEx1-*ERG20*)(pEKEx3-*CsTPS1*), named VLC1, Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CsTPS1*), named VLC2, and Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CnVS*), named VLC3. Production of (+)-valencene was realized as described in 2.1.

2.4. GC/MS measurements

The harvested *n*-dodecane phases of the production cultures were analyzed using a Thermo Scientific TRACE GC ULTRA connected to a Thermo Scientific ISQ Single Quadrupole Mass Spectrometer using a TG-5MS column (length: 30 m, I.D.: 0.25 mm, film thickness: 0.25 μm) (Thermo Scientific, Waltham, Massachusetts, USA). The *n*-dodecane phase was diluted in hexane prior to measurement in the GC/MS. After splitless injection of 1 μl, the initial temperature of 40 °C was increased for 10 °C min^{-1} to 160 °C then for 15 °C min^{-1} to 250 °C with a 2 min ramp at 250 °C at the end of the measurement with a constant helium gas flow of 1 ml min^{-1}. The MS operating parameters were ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature were 230 °C. (+)-valencene was identified by the comparison of retention time and mass spectrum to technical (+)-valencene (Sigma Aldrich, Steinheim, Germany). For quantitative analysis of (+)-valencene a calibration curve with technical (+)-valencene was used. β-Elemene and germacrene A were putatively identified by comparison of the mass spectrum to the NIST library. Concentrations of β-elemene and germacrene A were estimated using (+)-valencene as reference.

2.5. Tolerance experiments with n-dodecane and (+)-valencene and determination of the partitioning coefficient

To determine whether overlaying with *n*-dodecane is harmful to the cells and is interfering with their growth, growth with different volumes of *n*-dodecane as overlay was analyzed. *n*-Dodecane was added aseptically as overlay to cultures of *C. glutamicum* WT in the early exponential growth phase and volumes from one tenth to an equivalent volume as the culture volume were used. Growth was monitored until the cultures reached the stationary phase. To test for toxicity of (+)-valencene, 2–64 mM of (+)-valencene were added aseptically to cultures of *C. glutamicum* WT in the early exponential growth phase and growth was followed. The partitioning coefficient K_d was determined as described previously (Brennan et al., 2012) by quantitation of (+)-valencene in the aqueous CgXII medium, to which 100 mM (+)-valencene was added initially, and the organic *n*-dodecane phase after stable concentrations were reached.

2.6. DNA microarray analysis of n-dodecane- and (+)-valencene specific gene expression changes

To determine gene expression changes due to the addition of (+)-valencene or due to overlay with *n*-dodecane, *C. glutamicum* WT 13032 was cultivated in CGXII minimal media as described earlier. Two hours after inoculation a tenth of the culture volume (1 ml) *n*-dodecane or 4 mM (+)-valencene, respectively, were added aseptically to the cultures. Cells were harvested 3 h afterwards. Preparation of total RNA, cDNA synthesis and DNA microarray hybridization were performed as described before (Ishige et al., 2003; Lange et al., 2003; Wendisch, 2003). After microarray hybridization the slides were analyzed with ImaGene software (BioDiscovery, Hawthorne, CA, USA), for data processing the EMMA platform was utilized (Dondrup et al., 2003).

3. Results

3.1. (+)-Valencene is inhibiting growth of C. glutamicum

Sesquiterpenes such as (+)-valencene are volatile and known to be growth inhibitory to many microorganisms. To evaluate the toxicity of (+)-valencene to *C. glutamicum*, growth experiments with rising concentrations of (+)-valencene were carried out in CgXII minimal media in 100 ml non-baffled shake flasks. (+)-valencene was added aseptically to exponentially growing cultures of *C. glutamicum* WT 4 h after inoculation. Addition of 4 mM (+)-valencene led to a reduction of the growth rate ($0.14 \pm 0.01\ h^{-1}$) by nearly 40% compared to the control without (+)-valencene ($0.22 \pm 0.01\ h^{-1}$) (Fig. 2A). However, growth of *C. glutamicum* with 8 mM ($0.12 \pm 0.01\ h^{-1}$) and 64 mM ($0.12 \pm 0.01\ h^{-1}$) was not much slower than with 4 mM (+)-valencene (Fig. 2A).

To determine gene expression changes due to the addition of (+)-valencene, DNA microarray experiments were performed. A total of 53 genes showed significant ($p \leq 0.05$) RNA level changes by at least a factor of four (Table 2). Genes of ethanol, acetate and propionate catabolism, namely those encoding alcohol dehydrogenase and aldehyde dehydrogenase (*adhA* and *aldA*) (Arndt et al., 2008; Arndt and Eikmanns, 2007), the glyoxylate cycle enzymes malate synthase and isocitrate lyase (*aceB* and *aceA*) (Auchter et al., 2011; Gerstmeir et al., 2003), and methylcitrate synthase and methylcitrate dehydratase (*prpC2* and *prpD2*) (Claes et al., 2002) showed about 10 fold decreased RNA levels in the presence of (+)-valencene. Most notably, a gene encoding for a putative multidrug efflux protein (*mmpL2*, cg1054) and the adjacent putative transcriptional regulator gene (cg1053) showed 100 fold and 30 fold higher expression levels, respectively. Thus, (+)-valencene addition elicits a gene expression response which may include (+)-valencene extrusion via MmpL2. Taken together, to achieve high (+)-valencene titers it is desirable to avoid accumulation of (+)-valencene in the cultivation broth e.g. by in situ extraction into an organic phase.

3.2. n-Dodecane is suitable for extraction of (+)-valencene from C. glutamicum cultures and compatible with its growth

Given the relatively high toxicity of the hydrophobic (+)-valencene to *C. glutamicum*, a two-phase extractive cultivation strategy with *n*-dodecane overlay was followed. First, it was tested whether *n*-dodecane is suitable as an extractant for cultures of *C. glutamicum*. *n*-Dodecane was added to the cultures in the exponential growth phase to avoid confounding by lag phase phenomena. Growth rates of *C. glutamicum* in glucose minimal medium were $0.22 \pm 0.01\ h^{-1}$ without, $0.21 \pm 0.01\ h^{-1}$ with one tenth, and

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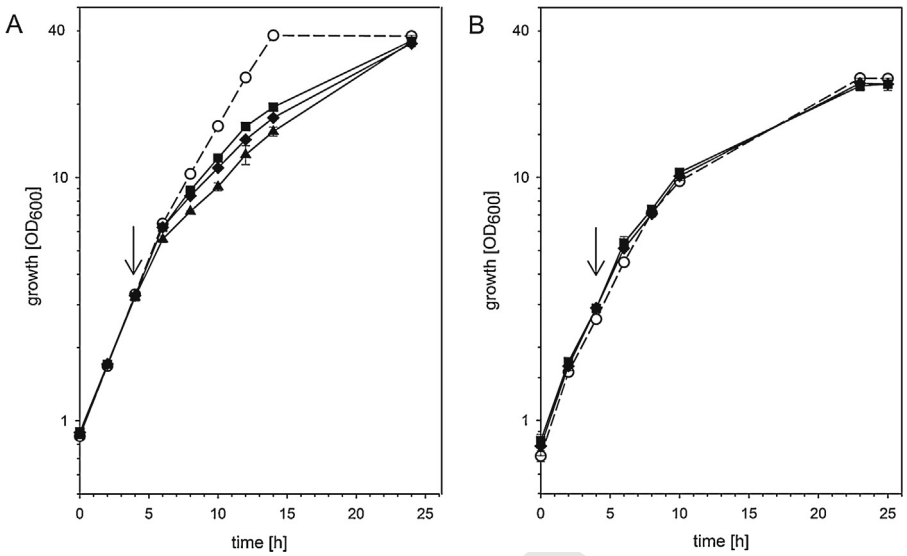

Fig. 2. *Growth of C. glutamicum WT in the presence of (+)-valencene or n-dodecane.* (A) Growth of *C. glutamicum* WT without (white circles), with 4 mM (black squares), 8 mM (black diamonds) or 64 mM (+)-valencene (black triangles) in 100 ml flasks with 10 ml CgXII minimal media with 4% glucose as carbon source. Means and standard deviations of duplicate cultivations are given. (B) Growth of *C. glutamicum* WT without *n*-dodecane overlay (white circles), with one tenth of the culture volume (black squares) or an equal volume of the culture volume of *n*-dodecane (black diamonds) in 100 ml flasks with 10 ml CgXII minimal medium with 2% glucose as carbon source. Means and standard deviations of triplicate cultivations are given.

$0.20 \pm 0.01\ h^{-1}$ with an equal volume of *n*-dodecane as overlay, respectively (Fig. 2B). The cultures grew to OD_{600} of 25.5 ± 0.7, 24.2 ± 0.4, and 24.2 ± 1.4, respectively (Fig. 2B). To determine if overlying with *n*-dodecane changes global gene expression, DNA microarray experiments were performed. Only four genes showed altered RNA levels ($p \leq 0.05$) by at least a factor of four (Table 3). Taken together, overlaying the cultures with *n*-dodecane as organic phase hardly affected growth of *C. glutamicum* WT.

To assay if *n*-dodecane is suitable for extraction of (+)-valencene from the *C. glutamicum* culture medium, the partition coefficient (K_d) of (+)-valencene between the aqueous CgXII medium and *n*-dodecane was determined. It could be shown that (+)-valencene preferentially partitioned to the *n*-dodecane phase with a $\log K_d$ 2.29 ± 0.25 (data not shown). Since *n*-dodecane was also shown to be compatible with fast growth of *C. glutamicum*, *n*-dodecane can be considered suitable for (+)-valencene extraction from *C. glutamicum* cultivation broth.

3.3. Expression of the (+)-valencene synthase gene CsTPS1 from Citrus sinensis is not sufficient for (+)-valencene production by C. glutamicum

The C15 precursor FPP, an intermediate of carotenoid biosynthesis in *C. glutamicum*, is the immediate precursor of (+)-valencene biosynthesis. To block conversion of FPP to geranylgeranyl pyrophosphate, phytoene and further to the native carotenoid decaprenoxanthin and, thus, to provide sufficient farnesyl pyrophosphate, the double deletion strain *C. glutamicum* Δ*crtB2*Δ*crtI2-1/2*Δ*crtB* was used (Table 1). This mutant lacked both phytoene synthases and was not pigmented (Heider et al., 2012). Plasmid-borne expression of either *crtB1* or *crtB2* complemented the mutant since the recombinant strains showed yellow pigmentation and synthesized decaprenoxanthin (data not shown).

Since the genome of *C. glutamicum* possesses two genes encoding prenyltransferases (*crtE* and *idsA*) putatively involved in condensation of IPP with DMPP to geranyl pyrophosphate and/or farnesyl pyrophosphate and/or geranylgeranyl pyrophosphate, which might differ with respect to the chain length of their products (C10, C15 or C20), both genes were used for strain construction. IPTG-inducible expression plasmids were constructed for heterologous expression of either *crtE* or *idsA* and of the gene for *C. sinensis* (+)-valencene synthase *CsTPS1* and used for transformation of *C. glutamicum* Δ*crtB2*Δ*crtI2-1/2*Δ*crtB*. However, in cultivation experiments with these strains accumulation of (+)-valencene was not observed (Table 4).

3.4. Expression of heterologous FPP synthase genes in combination with (+)-valencene synthase genes CsTPS1 or CnVS led to (+)-valencene production

Since overexpression of endogenous *crtE* and *idsA*, both of which are annotated as GGPP synthases, did not entail (+)-valencene production when combined with heterologous (+)-valencene synthase, it was hypothesized that primarily GGPP is available and that the pool of FPP was too low to support (+)-valencene production. Consequently, heterologous expression of a FPP synthase gene in a *C. glutamicum* strain lacking both CrtE and IdsA was tested in order to provide sufficient FPP for (+)-valencene synthesis. Therefore, the double deletion mutant Δ*crtE*Δ*idsA* was constructed by sequential in-frame deletion of *crtE* and *idsA*. The resulting strain was white due to a lack of pigmentation as expected for a strain devoid of GGPP synthase activity (data not shown).

To provide *C. glutamicum* Δ*crtE*Δ*idsA* with FPP synthase activity, plasmids were constructed for heterologous expression of either *ispA* from *E. coli* or *ERG20* from *S. cerevisiae*, respectively. Transformation of *C. glutamicum* Δ*crtE*Δ*idsA* with the plasmid for expression of *ispA* from *E. coli* led to pigmentation, while expression of *ERG20* did not (data not shown). The (+)-valencene synthase genes *CsTPS1* or *CnVS*, respectively, were expressed from a compatible expression plasmid. The resulting strain *C. glutamicum* Δ*crtE*Δ*idsA*(pVWEx1-*ERG20*)(pEKEx1-*CsTPS1*) was named VLC1, Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CsTPS1*) was named VLC2. Since production titer of (+)-valencene were higher with heterologous expression of *ispA* compared to *ERG20* (Table 4), pVWEx1-*CnVS* was expressed in Δ*crtE*Δ*idsA*(pEKEx3-*ispA*) and the resulting strain was named VLC3 (Table 1).

Cultivations with *C. glutamicum* VLC1, VLC2 and VLC3 were conducted in 9 ml glucose minimal medium overlaid with 1 ml of *n*-dodecane. After 48 h *C. glutamicum* VLC1

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Table 2
Gene expression differences in response to 4 mM valencene stress. The mRNA values are averages of two Arrays from two independent cultivations, only mRNA levels ≤0.25 or ≥4 are shown.

Gene ID[a]	Gene name, description	mRNA level[b] with/without 4 mM (+)-valencene
cg0018	Putative membrane protein	6.4
cg0508	Putative ABC transporter spermidine/putrescine/iron-binding protein	0.2
cg0566	*gapT*, putative 4-aminobutyrate aminotransferase	0.2
cg0706	Conserved putative membrane protein	17.2
cg0753	Putative secreted protein	6.1
cg0759	*prpD2*, 2-methylcitrate dehydratase	0.1
cg0762	*prpC2*, 2-methylcitrate synthase	0.1
cg0833	Conserved hypothetical protein	0.2
cg0952	Putative integral membrane protein	0.2
cg0953	Putative solute: sodium symporter (SSS)	0.2
cg1053	Putative transcriptional regulator, TetR-family	29.7
cg1054	*mmpL2*, putative multidrug efflux protein	100.0
cg1068	Putative oxidoreductase	4.2
cg1291	Putative membrane protein	5.1
cg1300	*cydB*, cytochrome *d* ubiquinol oxidase subunit II	4.3
cg1301	*cydA*, cytochrome *d* ubiquinol oxidase subunit I	5.2
cg1325	Conserved hypothetical protein	19.7
cg1373	Putative glyoxalase	0.2
cg1537	*ptsG*, glucose-specific enzyme phosphotransferase system protein	0.3
cg1612	Putative acetyltransferase	0.1
cg1626	Conserved hypothetical protein	5.9
cg1628	Putative hydrolase, alpha/beta superfamily	9.2
cg1761	*nifS2*, Cysteine desulfhydrase, AT class IV/selenocysteine lyase	4.8
cg1908	Hypothetical protein	0.2
cg1930	Putative secreted hydrolase	0.2
cg2069	Putative secreted protein	0.2
cg2117	*ptsI*, phosphotransferase system (PTS), Enzyme I	0.2
cg2136	*gluA*, ABC-type glutamate transporter, ATPase subunit	0.2
cg2137	*gluB*, ABC-type glutamate transporter, substrate-binding protein	0.2
cg2138	*gluC*, ABC-type glutamate transporter, permease subunit	0.2
cg2181	ABC-type transporter, putative dipeptide-binding protein	0.2
cg2182	ABC-type transporter, permease subunit	0.2
cg2183	ABC-type transporter, permease subunit	0.2
cg2184	ABC-type transporter, ATPase subunit	0.1
cg2444	Hypothetical protein	5.1
cg2559	*aceB*, malate synthase	0.1
cg2560	*aceA*, isocitrate lyase	0.1
cg2630	*pcaG*, protocatechuate 3,4-dioxygenase, alpha subunit	0.2
cg2636	*catA*, catechol 1,2-dioxygenase	0.1
cg2707	Conserved hypothetical protein	0.2
cg2796	Conserved hypothetical protein	4.1
cg2811	ABC-type lipoprotein release transporter, permease subunit	4.5
cg2812	ABC-type lipoprotein release transporter, ATPase subunit	5.1
cg3048	*pta*, phosphate acetyltransferase	0.2
cg3078	Hypothetical protein	5.7
cg3079	*clpB*, ATP-dependent protease	6.7
cg3096	*aldA*, aldehyde dehydrogenase	0.1
cg3107	*adhA*, alcohol dehydrogenase	0.1
cg3195	Putative flavin-containing monooxygenase	0.1
cg3226	Putative MFS-type L-lactate permease	0.2
cg3320	ABC-type transporter, ATPase and permease subunit	7.7
cg3321	ABC-type transporter, ATPase and permease subunit	15.4
cg3322	Putative secreted membrane-fusion protein	5.8

[a] Gene ID, name and annotation is according to BX927147.
[b] Differential gene expression as calculated for two technical replicates. Values listed were selected for $p < 0.05$.

Table 3
Gene expression changes in the absence or presence of 1 ml *n*-dodecane overlay. The mRNA values are averages of three DNA microarrays from three independent cultivations. Only genes differentially expressed by a factor of four or more are shown.

Gene ID[a]	Gene name, description	mRNA level[b] with/without 1 ml *n*-dodecane
cg1650	*pctC*, ABC transporter, permease subunit	0.22
cg1869	*ruvB*, holliday junction resolvasome helicase subunit	0.23
cg2207	Hypothetical zinc metalloprotease	0.24
cg3397	Hypothetical protein	0.21

[a] Gene ID, name and annotation is according to BX927147.
[b] Differential gene expression as calculated for three technical replicates. Values listed were selected for $p < 0.05$.

accumulated 0.15 ± 0.01 mg l^{-1} (+)-valencene (equivalent to 20 ± 1 μg g^{-1} CDW), *C. glutamicum* VLC2 0.22 ± 0.04 mg l^{-1} or 31 ± 6 μg g^{-1} CDW (+)-valencene and *C. glutamicum* VLC3 produced 2.41 ± 0.26 mg l^{-1} or 251 ± 26 μg g^{-1} CDW (+)-valencene (Fig. 3). Strains harboring the empty vector did not produce any detectable (+)-valencene (Fig. 3). While (+)-valencene accumulation by the CsTPS1 producing strain *C. glutamicum* VLC1 increased during cultivation about seven fold from 24 h (0.02 ± 0.01 mg l^{-1}) to 48 h (0.15 ± 0.01 mg l^{-1}), the CnVS producing strain *C. glutamicum* VLC3 accumulated almost as much (+)-valencene after 24 h (2.21 ± 0.09 mg l^{-1}) as after 48 h (2.41 ± 0.26 mg l^{-1}) (data not shown and Table 4) although the *n*-dodecane phase was not yet saturated (see Section 2). GC–MS analysis revealed that, besides (+)-valencene, *C. glutamicum* strains VLC1, VLC2 and VLC3 accumulated β-elemene (Fig. 3). Concentrations of β-elemene were estimated based on referencing to (+)-valencene and were about 0.38, 0.55 and 0.98 mg l^{-1}, respectively, for *C. glutamicum* VLC1, VLC2, and VLC3, respectively, after 48 h of cultivation (data not shown). While the CsTPS1 expressing strains VLC1 and VLC2 accumulated about 2.5 fold more β-elemene than (+)-valencene, the CnVS expressing strain VLC3 more than two fold more (+)-valencene than β-elemene. Only *C. glutamicum* VLC3 accumulated germacrene A at 0.25 mg l^{-1} based on referencing to (+)-valencene after 48 h (data not shown). Taken together, by combining FPP synthase and (+)-valencene synthase activities in a *C. glutamicum* strain led to the first demonstration of production of a sesquiterpene in this bacterium. Moreover, production based on (+)-valencene synthase CnVS was superior to production by CsTPS1.

4. Discussion

The sesquiterpene (+)-valencene is a frequently used food additive with a considerable economic value. To our knowledge, this paper is the first report of production of a sesquiterpene by *C. glutamicum*. (+)-Valencene production has previously been shown for

Table 4
(+)-Valencene production by various *C. glutamicum* strains in glucose minimal medium.

Strain	(+)-Valencene concentration (mg l^{-1})[a]
Δ*crtB*Δ*crt2I2-1*/*2*Δ*crtB*(pEKEx3-*idsA*)(pVWEx1-*CsTPS1*)	<0.02
Δ*crtB*Δ*crt2I2-1*/*2*Δ*crtB*(pEKEx3-*crtE*)(pVWEx1-*CsTPS1*)	<0.02
VLC1 = Δ*crtE*Δ*idsA*(pVWEx1-*ERG20*)(pEKEx3-*CsTPS1*)	0.15 ± 0.01
VLC2 = Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CsTPS1*)	0.22 ± 0.04
VLC3 = Δ*crtE*Δ*idsA*(pEKEx3-*ispA*)(pVWEx1-*CnVS*)	2.41 ± 0.26

[a] Values represent means ± standard deviation from at least triplicate cultures.

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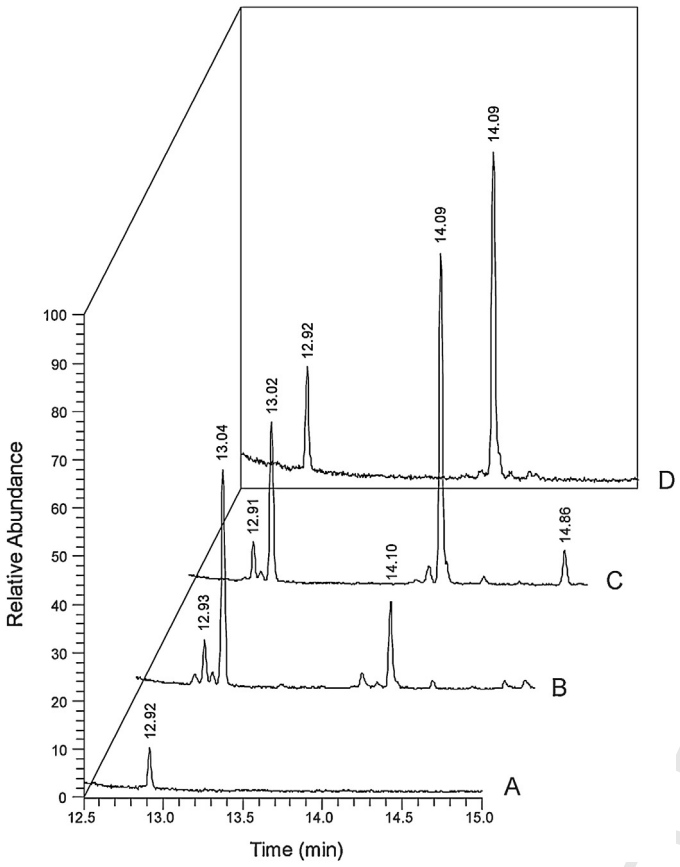

Fig. 3. *GC/MS measurements of (+)-valencene production.* 3-D plot of representative GC/MS chromatograms of the organic phase of cultures of (A) the negative control strain *ΔcrtEΔidsA*(pEKEx3-*ispA*)(pVWEx1), (B) *C. glutamicum* VLC2, (C) *C. glutamicum* VLC3 and (D) (+)-valencene standard. The intensity range of ((A)–(C)) was normalized to the *n*-decane peak and intensity of (D) was chosen to match the (+)-valencene peak in **C**. Retention times are indicated above each peak. Retention times were about 12.9 for *n*-decane, about 13.0 for β-elemene, about 14.1 for (+)-valencene, and about 14.9 for germacrene A.

several other eukaryotic (e.g. yeast) and prokaryotic (e.g. *Rhodobacter sphaeroides*) hosts (Beekwilder et al., 2014; Farhi et al., 2011; Scholtmeijer et al., 2014). *C. glutamicum*, the workhorse of the million-ton-scale amino acid production industry, has not only been engineered for utilization of a broad range of carbon sources e.g. pentoses, starch, glycerol, and cellubiose (Adachi et al., 2013; Meiswinkel et al., 2013a,b; Song et al., 2013), but has also been shown to produce a variety of carotenoids with titers in the mg g^{-1} CDW range (Heider et al., 2012, 2014a). The demonstration of (+)-valencene production by recombinant *C. glutamicum* reported here indicates that *C. glutamicum* not only is able to overproduce various carotenoids, but also a terpene.

C. glutamicum was shown to be compatible to two-phase extractive cultivation with *n*-dodecane overlay since neither growth nor global gene expression were changed much (Table 3). By contrast, addition of (+)-valencene exerted stress on *C. glutamicum* since the growth rate decreased (Fig. 2A) and more than fifty genes showed up to 100 fold altered RNA levels (Table 2). The highest change affected the operon *mmpl2*-cg1053. RNA levels were increased 30 fold (cg1053) and 100 fold (*mmpl2*), respectively. While cg1053 encodes a putative TetR-family transcriptional regulator, *mmpl2* codes for a drug exporter of the RND superfamily. Members of this family share an extremely broad substrate specificity for the export of e.g. various drugs, heavy metals, aliphatic and aromatic solvents or fatty acids (Domenech et al., 2005; Paulsen et al., 1996). Thus, it is conceivable, that (+)-valencene, which can be considered an aromatic solvent, may be exported by Mmpl2.

Interference of terpenes with cell growth and evaporation of the terpenes was counter-acted by overlaying *n*-dodecane at one-tenth of the culture volume as shown for other microbial systems (Brennan et al., 2012; Jang et al., 2011). The determined partition coefficient ($\log K_d$) of (+)-valencene of 2.29 ± 0.25 in CgXII medium was in the range of values described previously (Girhard et al., 2009). Thus, more generally two-phase extractive cultivation with *n*-dodecane overlay may be helpful for production of various volatile and hydrophobic compounds by *C. glutamicum*.

Overproduction of the endogenous prenyltransferases CrtE and IdsA in a strain lacking phytoene synthase activity and expressing a heterologous codon-optimized version of the (+)-valencene synthase CsTPS1 gene of *C. sinensis* did result in detectable (+)-valencene accumulation. This may indicate that CrtE and IdsA act as geranylgeranyl synthases without producing significant amounts of farnesyl pyrophosphate, the precursor for sesquiterpenoids. In support of this notion, Ohnuma and coworkers described elongation of DMPP with IPP by prenyltransferases to be a consecutive process without release of the intermediates (Ohnuma et al., 1998). However, it remains to be shown whether CrtE and IdsA of *C. glutamicum* indeed do not release significant amounts of GPP or FPP during catalysis and if the intracellular FPP pool in *crtE* and *idsA* overexpressing strains is too low for the chosen sesquiterpene synthases.

Expression of heterologous genes for prenyltransferases with FPP synthase activity in a *C. glutamicum* strain devoid of endogenous GGPP synthases allowed for (+)-valencene production when combined with expression of a valencene synthase gene indicating that FPP supply was sufficient for sesquiterpene biosynthesis. Both, a FPP synthases of eukaryotic origin (encoded by *ERG20* from *S. cerevisiae*) and one of prokaryotic origin (encoded by *ispA* from *E. coli*) that previously had been shown to improve isoprenoid production in different hosts (Ignea et al., 2011; Ro et al., 2006; Wang et al., 2010) could be used in the double deletion *C. glutamicum* mutant *ΔcrtEΔidsA* for (+)-valencene production. However, subtle differences between ERG20 and IspA were observed. The white phenotype of *C. glutamicum ΔcrtEΔidsA* due to its lack of decaprenoxanthin synthesis was overcome when *ispA* from *E. coli* was expressed, while expression of *ERG20* did not lead to significant pigmentation (data not shown). This may indicate that while catalysis by IspA does not only yield GPP and FPP, but also GGPP, catalysis by ERG20 stops at the level of FPP. Nevertheless, FPP provision by IspA in recombinant *C. glutamicum* appeared more efficient than by ERG20 since more (+)-valencene accumulated. However, this might also reflect different gene expression levels and use of different vectors.

Simultaneous overexpression of *CsTPS1* with either *ERG20* or *ispA* in *ΔcrtEΔidsA* led to titers of 0.15 and 0.22 mg l^{-1} (+)-valencene, respectively, for strains VLC1 and VLC2. The equivalent product concentrations of about 20 and 31 μg g^{-1} CDW, respectively, are considerably lower than those obtained for different carotenoids produced by *C. glutamicum* which are in the range of 2–4 mg g^{-1} CDW (Heider et al., 2012, 2014a). Currently, it is unknown why (+)-valencene production is only at about 1% of its potential, i.e. the level of carotenoid production in this bacterium. This might be due to a rather low activity and/or low robustness of the (+)-valencene synthase as described before in *S. cerevisiae* and *R. sphaeroides* (Beekwilder et al., 2014; Farhi et al., 2011).

To enhance (+)-valencene production a second (+)-valencene synthase, namely CnVS from *C. nootkatensis* which was just recently discovered and showed a significantly higher yield of (+)-valencene in *S. cerevisiae* and *R. sphaeroides* compared to *CsTPS1* (Beekwilder et al., 2014) was used. Comparing *C. glutamicum* strain VLC3, in which *CsTPS1* of

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C. sinensis was replaced with *CnVS* of *C. nootkatensis*, with VLC2, a tenfold increase of (+)-valencene production was observed. VLC3 produced 2.41 mg l^{-1} (+)-valencene which is equivalent to 251 μg g^{-1} CDW. While *CsTPS1* was codon-optimized for expression in *C. glutamicum*, *CnVs* was not. Since the *CnVS* gene contains several codons not preferred by *C. glutamicum* including the rare codons ATA for isoleucine and AGA for arginine (Liu et al., 2010), employing *CnVS* codon-optimized for *C. glutamicum* might further increase of (+)-valencene production. Moreover, targeted mutagenesis of the coding sequence might allow to select a more active variant of CnVs (Nov et al., 2013). Expression of codon-optimized CnVs in *S. commune* yielded 16 mg l^{-1} (+)-valenence (Scholtmeijer et al., 2014). Recently, it could be shown that 52 mg l^{-1} (+)-valencene were produced in a non-optimized *R. sphaeroides* strain using a codon optimized CnVS and that 352 mg l^{-1} were reached after additional heterologous expression of the MVA pathway of *Paracoccus zeaxanthinifaciens* (Beekwilder et al., 2014). It remains to be shown if similar improvements are also possible in *C. glutamicum*.

Apart from the commercially used flavor compound (+)-valencene, formation of β-elemene was observed in the GC–MS chromatograms of *C. glutamicum* VLC1, VLC2 and VLC3 (Fig. 3). β-Elemene is known to occur as a high-temperature induced Cope-rearrangement product of germacrene A, which itself is a by-product of both CsTPS1 and CnVS (Beekwilder et al., 2014). β-Elemene might find application as it is known as a compound with anti-proliferative effects toward some cancer cell types (Wang et al., 2005; Zhu et al., 2011). Furthermore, (+)-valencene can be oxidized to (+)-nookatone (Girhard et al., 2009), a high value additive for drinks due to its grapefruit flavor (Fraatz et al., 2009).

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References

Abe, S., Takayarna, K., Kinoshita, S., 1967. Taxonomical studies on glutamic acid producing bacteria. J. Gen. Appl. Microbiol. 13, 279–301.
Adachi, N., Takahashi, C., Ono-Murota, N., Yamaguchi, R., Tanaka, T., Kondo, A., 2013. Direct L-lysine production from cellobiose by *Corynebacterium glutamicum* displaying beta-glucosidase on its cell surface. Appl. Microbiol. Biotechnol. 97, 7165–7172.
Arndt, A., Auchter, M., Ishige, T., Wendisch, V.F., Eikmanns, B.J., 2008. Ethanol catabolism in *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol..
Arndt, A., Eikmanns, B.J., 2007. The alcohol dehydrogenase gene *adhA* in *Corynebacterium glutamicum* is subject to carbon catabolite repression. J. Bacteriol. 189, 7408–7416.
Auchter, M., Cramer, A., Huser, A., Ruckert, C., Emer, D., Schwarz, P., Arndt, A., Lange, C., Kalinowski, J., Wendisch, V.F., Eikmanns, B.J., 2011. RamA and RamB are global transcriptional regulators in *Corynebacterium glutamicum* and control genes for enzymes of the central metabolism. J. Biotechnol. 154, 126–139.
Beekwilder, J., van Houwelingen, A., Cankar, K., van Dijk, A.D., de Jong, R.M., Stoopen, G., Bouwmeester, H., Achkar, J., Sonke, T., Bosch, D., 2014. Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant Biotechnol. J. 12, 174–182.
Blombach, B., Seibold, G.M., 2010. Carbohydrate metabolism in *Corynebacterium glutamicum* and applications for the metabolic engineering of L-lysine production strains. Appl. Microbiol. Biotechnol. 86, 1313–1322.
Brennan, T.C., Turner, C.D., Kromer, J.O., Nielsen, L.K., 2012. Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 109, 2513–2522.
Chappell, J., 2004. Valencene synthase—a biochemical magician and harbinger of transgenic aromas. Trends Plant Sci. 9, 266–269.
Claes, W.A., Puhler, A., Kalinowski, J., 2002. Identification of two *prpDBC* gene clusters in *Corynebacterium glutamicum* and their involvement in propionate degradation via the 2-methylcitrate cycle. J. Bacteriol. 184, 2728–2739.
Dagert, M., Ehrlich, S.D., 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene 6, 23–28.
Domenech, P., Reed, M.B., Barry 3rd, C.E., 2005. Contribution of the *Mycobacterium tuberculosis* MmpL protein family to virulence and drug resistance. Infect. Immun. 73, 3492–3501.
Dondrup, M., Goesmann, A., Bartels, D., Kalinowski, J., Krause, L., Linke, B., Rupp, O., Sczyrba, A., Puhler, A., Meyer, F., 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. J. Biotechnol. 106, 135–146.
Eggeling, L., Reyes, O., 2005. Experiments. In: Eggeling, L., Bott, M. (Eds.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL, pp. 3535–3566.
Eisenreich, W., Rohdich, F., Bacher, A., 2001. Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci. 6, 78–84.
Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., Abeliovich, H., Vainstein, A., 2011. Harnessing yeast subcellular compartments for the production of plant terpenoids. Metab. Eng. 13, 474–481.
Fraatz, M.A., Berger, R.G., Zorn, H., 2009. Nootkatone—a biotechnological challenge. Appl. Microbiol. Biotechnol. 83, 35–41.
Gerstmeir, R., Wendisch, V.F., Schnicke, S., Ruan, H., Farwick, M., Reinscheid, D., Eikmanns, B.J., 2003. Acetate metabolism and its regulation in *Corynebacterium glutamicum*. J. Biotechnol. 104, 99–122.
Girhard, M., Machida, K., Itoh, M., Schmid, R.D., Arisawa, A., Urlacher, V.B., 2009. Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from Bacillus subtilis in a two-liquid-phase system. Microb. Cell Fact. 8 (36).
Grote, A., Hiller, K., Scheer, M., Munch, R., Nortemann, B., Hempel, D.C., Jahn, D., 2005. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 33, W526–W531.
Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557–580.
Heider, S.A., Peters-Wendisch, P., Netzer, R., Stafnes, M., Brautaset, T., Wendisch, V.F., 2014a. Production and glucosylation of C50 and C40 carotenoids by metabolically engineered *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 98, 1223–1235.
Heider, S.A., Peters-Wendisch, P., Wendisch, V.F., 2012. Carotenoid biosynthesis and overproduction in *Corynebacterium glutamicum*. BMC Microbiol. 12, 198.
Heider, S.A., Peters-Wendisch, P., Wendisch, V.F., Beekwilder, J., Brautaset, T., 2014b. Metabolic engineering for the microbial production of carotenoids and related products with a focus on the rare C50 carotenoids. Appl. Microbiol. Biotechnol. 98, 4355–4368.
Ignea, C., Cvetkovic, I., Loupassaki, S., Kefalas, P., Johnson, C.B., Kampranis, S.C., Makris, A.M., 2011. Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. Microb. Cell Fact. 10, 4.
Ishige, T., Krause, M., Bott, M., Wendisch, V.F., Sahm, H., 2003. The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. J. Bacteriol. 185, 4519–4529.
Jang, H.J., Yoon, S.H., Ryu, H.K., Kim, J.H., Wang, C.L., Kim, J.Y., Oh, D.K., Kim, S.W., 2011. Retinoid production using metabolically engineered *Escherichia coli* with a two-phase culture system. Microb. Cell Fact. 10, 59.
Jensen, J.V., Wendisch, V.F., 2013. Ornithine cyclodeaminase-based proline production by *Corynebacterium glutamicum*. Microb. Cell Fact. 12, 63.
Kirby, J., Keasling, J.D., 2009. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu. Rev. Plant Biol. 60, 335–355.
Lange, C., Rittmann, D., Wendisch, V.F., Bott, M., Sahm, H., 2003. Global expression profiling and physiological characterization of *Corynebacterium glutamicum* grown in the presence of L-valine. Appl. Environ. Microbiol. 69, 2521–2532.
Litsanov, B., Brocker, M., Bott, M., 2012a. Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. Appl. Environ. Microbiol. 78, 3325–3337.
Litsanov, B., Kabus, A., Brocker, M., Bott, M., 2012b. Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. Microb. Biotechnol. 5, 116–128.
Liu, G., Wu, J., Yang, H., Bao, Q., 2010. Codon usage patterns in *Corynebacterium glutamicum*: mutational bias, natural selection and amino acid conservation. Comp. Funct. Genomics 2010, 343569.
Lucker, J., Bowen, P., Bohlmann, J., 2004. Vitis vinifera terpenoid cyclases: functional identification of two sesquiterpene synthase cDNAs encoding (+)-valencene synthase and (−)-germacrene D synthase and expression of mono- and sesquiterpene synthases in grapevine flowers and berries. Phytochemistry 65, 2649–2659.
Meiswinkel, T.M., Gopinath, V., Lindner, S.N., Nampoothiri, K.M., Wendisch, V.F., 2013a. Accelerated pentose utilization by *Corynebacterium glutamicum* for accelerated production of lysine, glutamate, ornithine and putrescine. Microb. Biotechnol. 6, 131–140.
Meiswinkel, T.M., Rittmann, D., Lindner, S.N., Wendisch, V.F., 2013b. Crude glycerol-based production of amino acids and putrescine by *Corynebacterium glutamicum*. Bioresour. Technol. 145, 254–258.
Mimitsuka, T., Sawai, H., Hatsu, M., Yamada, K., 2007. Metabolic engineering of *Corynebacterium glutamicum* for cadaverine fermentation. Biosci. Biotechnol. Biochem. 71, 2130–2135.
Nov, Y., Fulton, A., Jaeger, K.E., 2013. Optimal scanning of all single-point mutants of a protein. J. Comput. Biol. 20, 990–997.
Ohnuma, S., Hirooka, K., Tsuruoka, N., Yano, M., Ohto, C., Nakane, H., Nishino, T., 1998. A pathway where polyprenyl diphosphate elongates in prenyltransferase. Insight into a common mechanism of chain length determination of prenyltransferases. J. Biol. Chem. 273, 26705–26713.
Okino, S., Noburyu, R., Suda, M., Jojima, T., Inui, M., Yukawa, H., 2008. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl. Microbiol. Biotechnol. 81, 459–464.
Paulsen, I.T., Brown, M.H., Skurray, R.A., 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. 60, 575–608.
Peters-Wendisch, P.G., Schiel, B., Wendisch, V.F., Katsoulidis, E., Mockel, B., Sahm, H., Eikmanns, B.J., 2001. Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol. 3, 295–300.

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Ro, D.K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.

Sandmann, G., Yukawa, H., 2005. Vitamin synthesis: carotenoids, biotin, and pantothenate. In: Eggeling, L., Bott, M. (Eds.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL, pp. 397–415.

Schäfer, T., et al., 1993. Acetyl-CoA synthetase (ADP forming) in archaea, a novle enzyme involved in acetate formation and ATP synthesis. Arch. Microbiol. 159, 72–83.

Schneider, J., Wendisch, V.F., 2010. Putrescine production by engineered *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 88, 859–868.

Scholtmeijer, K., Cankar, K., Beekwilder, J., Wosten, H.A., Lugones, L.G., Bosch, D., 2014. Production of (+)-valencene in the mushroom-forming fungus *S. commune*. Appl. Microbiol. Biotechnol..

Sharon-Asa, L., Shalit, M., Frydman, A., Bar, E., Holland, D., Or, E., Lavi, U., Lewinsohn, E., Eyal, Y., 2003. Citrus fruit flavor and aroma biosynthesis: isolation, functional characterization, and developmental regulation of Cstps1, a key gene in the production of the sesquiterpene aroma compound valencene. Plant J. 36, 664–674.

Song, Y., Matsumoto, K., Tanaka, T., Kondo, A., Taguchi, S., 2013. Single-step production of polyhydroxybutyrate from starch by using alpha-amylase cell-surface displaying system of *Corynebacterium glutamicum*. J. Biosci. Bioeng. 115, 12–14.

Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J.L., Wendisch, V.F., 2005. Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. Appl. Environ. Microbiol. 71, 5920–5928.

Takahashi, S., Koyama, T., 2006. Structure and function of *cis*-prenyl chain elongating enzymes. Chem. Rec. 6, 194–205.

van der Rest, M.E., Lange, C., Molenaar, D., 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. 52, 541–545.

Wang, C., Yoon, S.H., Shah, A.A., Chung, Y.R., Kim, J.Y., Choi, E.S., Keasling, J.D., Kim, S.W., 2010. Farnesol production from *Escherichia coli* by harnessing the exogenous mevalonate pathway. Biotechnol. Bioeng. 107, 421–429.

Wang, G., Li, X., Huang, F., Zhao, J., Ding, H., Cunningham, C., Coad, J.E., Flynn, D.C., Reed, E., Li, Q.Q., 2005. Antitumor effect of beta-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death. Cell. Mol. Life Sci. 62, 881–893.

Wendisch, V.F., 2003. Genome-wide expression analysis in *Corynebacterium glutamicum* using DNA microarrays. J. Biotechnol. 104, 273–285.

Wieschalka, S., Blombach, B., Bott, M., Eikmanns, B.J., 2013. Bio-based production of organic acids with *Corynebacterium glutamicum*. Microb. Biotechnol. 6, 87–102.

Wieschalka, S., Blombach, B., Eikmanns, B.J., 2012. Engineering *Corynebacterium glutamicum* for the production of pyruvate. Appl. Microbiol. Biotechnol. 94, 449–459.

Zahoor, A., Otten, A., Wendisch, V.F., 2014. Metabolic engineering of *Corynebacterium glutamicum* for glycolate production. J. Biotechnol. (in press). Q4

Zhu, T., Xu, Y., Dong, B., Zhang, J., Wei, Z., Xu, Y., Yao, Y., 2011. Beta-elemene inhibits proliferation of human glioblastoma cells through the activation of glia maturation factor beta and induces sensitization to cisplatin. Oncol. Rep. 26, 405–413.

Please cite this article in press as: Frohwitter, J., et al., Production of the sesquiterpene (+)-valencene by metabolically engineered *Corynebacterium glutamicum*. J. Biotechnol. (2014), http://dx.doi.org/10.1016/j.jbiotec.2014.05.032