Journal of Biotechnology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Engineering biotin prototrophic *Corynebacterium glutamicum* strains for amino acid, diamine and carotenoid production

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
21 December 2013
Accepted 3 January 2014
Available online xxx

Keywords:
Corynebacterium glutamicum
Biotin biosynthesis
Amino acid production
Diamine production
Carotenoid production

ABSTRACT

The Gram-positive *Corynebacterium glutamicum* is auxotrophic for biotin. Besides the biotin uptake system BioYMN and the transcriptional regulator BioQ, this bacterium possesses functional enzymes for the last three reactions of biotin synthesis starting from pimeloyl-CoA. Heterologous expression of *bioF* from the Gram-negative *Escherichia coli* enabled biotin synthesis from pimelic acid added to the medium, but expression of *bioF* together with *bioC* and *bioH* from *E. coli* did not entail biotin prototrophy. Heterologous expression of *bioWAFDBI* from *Bacillus subtilis* encoding another biotin synthesis pathway in *C. glutamicum* allowed for growth in biotin-depleted media. Stable growth of the recombinant was observed without biotin addition for eight transfers to biotin-depleted medium while the empty vector control stopped growth after the first transfer. Expression of *bioWAFDBI* from *B. subtilis* in *C. glutamicum* strains overproducing the amino acids L-lysine and L-arginine, the diamine putrescine, and the carotenoid lycopene, respectively, enabled formation of these products under biotin-depleted conditions. Thus, biotin-prototrophic growth and production by recombinant *C. glutamicum* were achieved.

1. Introduction

Corynebacterium glutamicum is a Gram-positive bacterium originally isolated due to its ability to secrete L-glutamate (Kinoshita et al., 1957). This bacterium became the workhorse of amino acid production and is known for safe production in the food and feed industries since more than fifty years. Currently, 2,160,000 tons of L-glutamate and 1,480,000 tons of L-lysine are produced annually (Ajinomoto, 2013). *C. glutamicum* is not only used for biotechnological production of L-glutamate and L-lysine, but has also been engineered for the production of other amino acids such as L-serine (Stolz et al., 2007) and L-proline (Jensen and Wendisch, 2013), organic acids such as pyruvate (Wieschalka et al., 2012), lactate (Okino et al., 2008a,b), succinate (Litsanov et al., 2012a,b), α-ketoglutarate (Jo et al., 2012), 2-ketoisovalerate (Krause et al., 2010) and 2-ketoisocaproate (Buckle-Vallant et al., 2013). Moreover, the diamines cadaverine and putrescine (Mimitsuka et al., 2007; Schneider and Wendisch, 2010), carotenoids (Heider et al., 2012, 2014), the vitamin pantothenate (Chassagnole et al., 2003; Hüser et al., 2005) and the alcohols ethanol and isobutanol (Blombach et al., 2011; Sakai et al., 2007; Smith et al., 2010) can be produced by recombinant *C. glutamicum* strains.

C. glutamicum grows aerobically on a variety of carbon sources such as the sugars glucose, fructose and sucrose, the sugar alcohols mannitol and inositol, the organic acids gluconate, citrate, acetate, propionate, pyruvate, D-lactate and L-lactate or aromatic compounds like vanillate or protocatechuate (Blombach and Seibold, 2010; Chaudhry et al., 2007; Frunzke et al., 2008; Ikeda, 2012; Krings et al., 2006; Laslo et al., 2012; Merkens et al., 2005; Netzer et al., 2004). Industrially, mostly starch hydrolysates and molasses are used as carbon sources. Direct access to starch and glucans (Seibold et al., 2006; Tsuchidate et al., 2011) as well as to crude glycerol (Meiswinkel et al., 2013b; Rittmann et al., 2008), amino sugars (Uhde et al., 2013) or cellubiose (Adachi et al., 2013) and pentoses in cellulosic hydrolysates (Gopinath et al., 2011; Meiswinkel et al., 2013a) has been realized.

Simple mineral salts media can be used for growth of *C. glutamicum* and growth can be stimulated by iron chelators such as

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http://dx.doi.org/10.1016/j.jbiotec.2014.01.023
0168-1656/

protocatechuic acid. Since *C. glutamicum* is auxotrophic for biotin (vitamin H, B7 or coenzyme R), a source of biotin has to be present in growth media. Biotin is imported into the *C. glutamicum* cell by BioYMN (Schneider et al., 2012b). The biotin requirement of *C. glutamicum* is due to only two proteins requiring this cofactor, namely pyruvate carboxylase and acetyl-CoA carboxylase (Gande et al., 2004, 2007; Jager et al., 1996; Peters-Wendisch et al., 1996, 1997, 1998, 2001; Sato et al., 2008). Both pyruvate carboxylase as the major anaplerotic carboxylase of *C. glutamicum* and acetyl-CoA carboxylase as essential for fatty acid synthesis are biotinylated at a particular lysyl residue by biotin–protein ligase BirA (Peters-Wendisch et al., 2012). Biotin starvation of *C. glutamicum* elicits L-glutamate production (Udaka, 1960). Therefore, production of other amino acids such as L-lysine by *C. glutamicum* requires provision of sufficient biotin (Ko and Chipley, 1984), in particular since biotin-dependent pyruvate carboxylase is a bottleneck of lysine production (Peters-Wendisch et al., 2001).

Biotin-auxotrophic *C. glutamicum* surprisingly possesses not only BioYMN for biotin uptake and BirA for protein biotinylation (Fig. 1) (Peters-Wendisch et al., 2012; Schneider et al., 2012b), but also biotin transcriptional regulator BioQ (Brune et al., 2012) and the enzymes for the three final reactions of biotin synthesis, 7,8-diaminononanoate synthase BioA, dethiobiotin synthase BioD and biotin synthase BioB (Hatakeyama et al., 1993a,b; Hatakeyama et al., 1997). Biotin synthesis can be divided into two parts: synthesis of pimelate thioester (pimeloyl-CoA or pimeloyl-acyl carrier protein (ACP)) followed by the biotin ring assembly (Lin and Cronan, 2011). Of the four highly conserved reactions of biotin ring assembly (Streit and Entcheva, 2003) only 8-amino-7-oxononanoate synthase BioF is not encoded in the *C. glutamicum* genome (Kalinowski et al., 2003). Since desthiobiotin and aminopelargonic acid derivatives, but not pimelic acid, supported growth when added in low concentrations (Okumura et al., 1962), biotin auxotrophy of *C. glutamicum* was considered to be due to the lack of BioF (Hatakeyama et al., 1993a,b; 1997). Alternatively, *C. glutamicum* might lack BioF and in addition a biosynthetic pathway leading to its substrate pimeloyl-CoA/ACP (Fig. 1).

Two distinct major pathways for biosynthesis of pimeloyl-CoA/ACP are known (Fig. 1), the BioC-BioH pathway in *E. coli* and the BioI-BioW pathway in *Bacillus subtilis* (Lin and Cronan, 2011). In *E. coli*, S-adenosyl methionine-dependent methyltransferase BioC methylates malonyl-CoA and the resulting methyl ester enters fatty acid biosynthesis (Lin and Cronan, 2012). After two elongation cycles carboxylesterase BioH demethylates the generated methyl-pimeloyl-ACP to pimeloyl-ACP, the substrate of biotin ring assembly (Agarwal et al., 2012). In *B. subtilis*, the P450 protein BioI oxidatively cleaves a carbon-carbon bond of an acyl-ACP intermediate of fatty acid biosynthesis to generate pimeloyl-ACP (Cryle and Schlichting, 2008; Green et al., 2001; Lin and Cronan, 2011). Alternatively, pimeloyl-CoA synthetase BioW from *B. subtilis* activates externally added pimelic acid (Bower et al., 1996) to yield pimeloyl-CoA as precursor for biotin ring assembly.

In order to render *C. glutamicum* biotin prototroph we initiated experiments regarding heterologous expression of genes either of the *E. coli* BioC-BioH pathway as well as of the *B. subtilis* BioI-BioW pathway. Here we show that expression of *bioF* from *E. coli* allows for biotin synthesis from pimelic acid added to the medium, but expression of *bioF* alone or together with *bioC* and *bioH* from *E. coli* did not entail biotin prototrophy. By contrast, expression of the complete biotin synthesis operon *bioWAFDBI* from *B. subtilis* converted *C. glutamicum* to a biotin prototroph. Very recently, a similar approach was used to construct biotin hyper-auxotrophic and prototrophic *C. glutamicum* strains (Ikeda et al., 2013).

2. Materials and methods

2.1. Bacterial strains, plasmids, oligonucleotides, and culture conditions

Bacterial strains and plasmids used are listed in Table 1. *Escherichia coli*, *Bacillus subtilis* and *C. glutamicum* were grown in lysogeny broth complex medium (LB) as the standard medium (Sambrook and Russell, 2001) and kanamycin (25 μg/l) and/or spectinomycin (100 μg/l) were added when appropriate. The minimal medium used for growth and amino acid production by *C. glutamicum* was described previously (Keilhauer et al., 1993) and contained 100 mM or 220 mM glucose and 0–200 μg biotin l^{-1}, respectively. The cultures (50 ml in 500 ml baffled Erlenmeyer flasks) were inoculated to give an optical density at 600 nm (OD_{600}) of about 1 and then incubated aerobically at 30 °C on a rotary shaker at 120 rpm. For induction of the expression either 10 μM, 50 μM or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added to the cultures.

2.2. Construction of plasmids and strains

Standard methods such as PCR, restriction, and ligation were carried out as described previously (Sambrook and Russell, 2001). Plasmids were constructed in *Escherichia coli* DH5α from PCR-generated fragments (KOD, Novagen) and isolated with the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). All cloned DNA fragments were shown to be correct by sequencing.

For the construction of the expression vectors pEKEX3*bioF* and pEKEX3bio*FCH* chromosomal DNA from *E. coli* K-12 MG1655 and for the construction of pEKEX3*bioWAFDBI* chromosomal DNA from *B. subtilis* sub. *subtilis* str. 168 was isolated as described (Eikmanns et al., 1994). The oligonucleotides used for the PCR amplification of *bioF*, *bioC* and *bioH* from *E. coli* and *bioWAFDBI* from *B. subtilis* as well as for the construction of pVWEx1 are listed in Table 1 and were obtained from Metabion (Martinsried, Germany) or Life Technologies (Carlsbad, CA, USA). The *bioWAFDBI* operon was amplified from genomic DNA of *B. subtilis* sub. *subtilis* str. 168 using the primers bioWAFDBI-fw and bioWAFDBI-rv (Table 1). The PCR product was cloned into pEKEx3 (Stansen et al., 2005) using the restriction sites introduced by the primers to yield pEKEx3-*bioWAFDBI*. Some transformants showed irregular plasmids and were not able to grow without biotin which might be due to homologous recombination between *bioA*, *bioB* or *bioD* on the chromosome and on the plasmid. Plasmid pVWEx1-$argB_{A26VM31V}$ was constructed by digesting pEKEx3-$argB_{A26VM31V}$ (Schneider et al., 2011) with KpnI and BamHI and ligation of the resulting fragment $argB_{A26VM31V}$ into KpnI/BamHI digested pVWEx1 vector. The resulting plasmid pVWEx1-$argB_{A26VM31V}$ was verified by sequencing. Transformation of *E. coli* was performed using the calcium chloride method (Sambrook and Russell, 2001), while *C. glutamicum* was transformed by electroporation as described previously (Eggeling and Reyes, 2005). The resulting strains are listed in Table 1.

2.3. Quantification of amino acid, polyamine and lycopene concentrations

For quantification of extracellular amino acids and polyamines, aliquots of the culture were withdrawn, the optical density (OD_{600}) was measured and cells were removed by centrifugation (13,000 × *g*, 10 min). The supernatant was analyzed using a high-pressure liquid chromatography system (HPLC, 1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany).

Amino acids were determined by automatic precolumn derivatization with *ortho*-phthaldialdehyde as described previously (Georgi et al., 2005) and separated on a reversed phase

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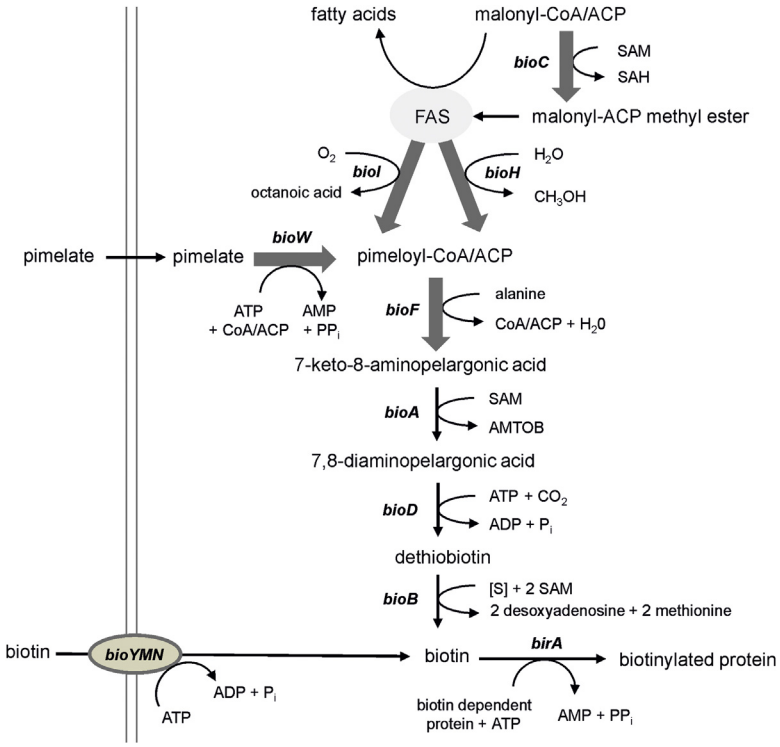

Fig. 1. Scheme of biotin synthesis in *C. glutamicum*, *E. coli* and *B. subtilis*. Reactions not present in *C. glutamicum* are accentuated by broad arrows. Gene names of enzymes are given: biotin protein ligase (*birA*), biotin uptake system (*bioYMN*), biotin synthetase (*bioB*), dethiobiotin synthetase (*bioD*), 7,8-diaminopelargonic acid aminotransferase (*bioA*), 7-keto-8-aminopelargonic acid synthase (*bioF*), 6-carboxyhexanoate-CoA ligase (*bioW*), pimeloyl-ACP-methylester esterase (*bioH*), malonyl-CoA O-methyltransferase (*bioC*), cytochrome P450 hydroxylase (*bioI*). *Abbreviations*: AMTOB, S-adenosyl-4-methylthio-2-oxobutanoate; FAS, type-I or type-II fatty acid synthase complex; SAH, S-adenosyl-homocysteine; SAM-S-adenosyl-L-methionine.

column (LiCrosphere 125 mm × 4 mm, 5-μm particle size, end-capped; Chromatographie Service GmbH, Langerwehe, Germany). The respective fluorescent isoindole derivatives were detected using a fluorescent detector and excitation at 230 nm and emission at 450 nm. For elution a buffer gradient consisted of 0.1 M sodium acetate, pH 7.2 (with 0.03% sodium azide), as the polar phase and methanol as the nonpolar phase was used. L-Asparagine served as an internal standard.

Polyamines were determined by precolumn derivatization with *ortho*-phthaldialdehyde as described above for amino acids. Hexamethylenediamine served as an internal standard.

Carotenoid extraction from the *C. glutamicum* cells was performed by a modified protocol as described earlier (Heider et al., 2012). 1 ml aliquots of the cell cultures were centrifuged at 13,000 × *g* for 1 min. The pigments were extracted with 1 ml methanol:acetone mixture (7:3) at 60 °C for 30 min with thorough vortexing every 10 min. Two extraction cycles were performed to remove all visible colors from the cell pellet. The extraction mixture was centrifuged 10,000 × *g* for 15 min and the supernatant was transferred to a new tube. The lycopene content in the extracts was quantified through absorbance at 470 nm by HPLC analysis (see below) and the concentrations were calculated using a standard curve and appropriate dilutions. High performance liquid chromatography (HPLC) analyses of the *C. glutamicum* extracts were performed on an Agilent 1200 series HPLC system (Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn), including a diode array detector (DAD) for UV/visible (vis) spectrum recording. For separation a column system consisting of a precolumn (10 mm × 4 mm MultoHigh 100 RP18-5, CS Chromatographie Service GmbH, Langerwehe, Germany) and a main column (ProntoSIL 200-5 C30, 250 mm × 4 mm, CS Chromatographie Service GmbH, Langerwehe, Germany) was used. Quantification of carotenoids was performed using the extracted wavelength chromatogram at 470 nm. Lycopene from tomato (Sigma, Steinheim, Germany) was used as standard. It was dissolved in chloroform according to its solubility and diluted in methanol:acetone (7:3). The HPLC protocol comprised a gradient elution for 10 min and a mobile phase composition of (A) methanol and (B) methanol/methyl tert-butyl ether/ethyl acetate (5:4:1) starting from 10% to 100% eluent B followed by 20 min of isocratic elution with 100% B. After that the eluent composition is set back to 10% B for 3 min. The injection volume was 50 μl and the flow rate was kept constant at 1.4 ml/min.

3. Results

3.1. Heterologous expression of bioF, bioC and bioH from E. coli is not sufficient for biotin prototrophy of C. glutamicum

C. glutamicum possesses the genes encoding for the enzymes BioA, BioD and BioB functional for the biotin ring assembly, but a *bioF* gene is not found in its genome (Kalinowski et al., 2003). Therefore, the question if heterologous expression of *bioF* from *E. coli* alone can establish biotin prototrophy in *C. glutamicum* was addressed experimentally. For IPTG-inducible expression *bioF* from *E. coli* was cloned into vector pEKEx3 (Stansen et al., 2005). When strain WT(pEKEx3-*bioF*) was grown on minimal medium with IPTG but without biotin it showed the same growth behavior as the respective empty vector control strain (Fig. 2). However, when pimelic acid, which when activated by CoA serves as substrate of the *bioF* encoded 7-keto-8-aminopelargonic acid synthetase, was added to the culture medium, growth without biotin was observed for strain WT(pEKEx3-*bioF*) but not for the empty vector control (Fig. 3). Thus, *bioF* is not sufficient to render *C. glutamicum* a biotin prototrophic bacterium. On the other hand pimelic acid can be

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

Strain, plasmid or oligonucleotide	Relevant characteristics or sequence	Reference or source
E. coli		
DH5α	F⁻ *thi*-1 *endA1 hsdR17*(r⁻ m⁻) *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Hanahan (1983)
MG1655	K-12, wild-type, CGSC6300	CGSC
B. subtilis		
168	Wild-type, *trpC2*, auxotrophic for tryptophan	BGSC
C. glutamicum		
WT	Wild-type, ATCC 13032, auxotrophic for biotin	Udaka (1960)
WT(pEKEx3-*bioF*)	ATCC 13032 carrying the expression vector pEKEX3-*bioF* encoding *bioF* from *E. coli*, spec^r	This work
WT(pEKEx3-*bioFCH*)	ATCC 13032 carrying the expression vector pEKEX3-*bioFCH* encoding *bioF, bioC* and *bioH* from *E. coli*, spec^r	This work
WT(pEKEx3-*bioWAFDBI*)	ATCC 13032 carrying the expression vector pEKEX3-*bioWAFDBI* encoding *bioW*, *bioA*, *bioF*, *bioD*, *bioB* and *bioI* from *B. subtilis* 168, spec^r	This work
DM1933	Δ*pck pyc*$_{P458S}$ *hom*$_{V59A}$, 2 copies of *lysC*$_{T311I}$, 2 copies of *asd*, 2 copies of *dapA*, 2 copies of *dapB*, 2 copies of *ddh*, 2 copies of *lysA*, 2 copies of *lysE* derived from WT *C. glutamicum*	Blombach et al. (2009b)
DM1933(pEKEx3-*bioWAFDBI*)	DM1933 carrying the expression vector pEKEX3-*bioWAFDBI*, spec^r	This work
LYC1	Δ*crtYEb* (pVWEx1-*crtEBI*); in-frame deletion of *crtY* and *crtEb* carrying the expression vector pVWEx1-*crtEBI*, kan^r	Heider et al. (2014)
LYC1(pEKEx3-*bioWAFDBI*)	LYC1 carrying the expression vector pEKEX3-*bioWAFDBI*, kan^r, spec^r	This work
ARG2	Δ*argR* (pVWEx1-*argB*$_{A26VM31V}$); in-frame deletion of *argR* including pVWEx1-*argB*$_{A26VM31V}$, kan^r	This work
ARG2(pEKEx3-*bioWAFDBI*)	ARG2 carrying the expression vector pEKEX3-*bioWAFDBI*, kan^r, spec^r	This work
ORN1	Δ*argF*Δ*argR*; auxotrophic for L-arginine	Schneider et al. (2011)
PUT21	ORN1 carrying pVWEx1-*speC*-5′ 21-*argF*, kan^r	Schneider et al. (2012b)
PUT21(pEKEx3-*bioWAFDBI*)	PUT21 carrying the expression vector pEKEX3-*bioWAFDBI*, kan^r, spec^r	This work
Plasmids		
pEKEx3	Spec^R; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{q}, pBL1 *ori*V_{Cg})	Stansen et al. (2005)
pEKEx3-*bioF*	pEKEx3 containing *bioF* from *E. coli* MG1655	This work
pEKEx3-*bioFCH*	pEKEx3 containing *bioF*, *bioC* and *bioH* from *E. coli* MG1655	This work
pEKEx3-*bioWAFDBI*	pEKEx3 containing *bioW*, *bioA*, *bioF*, *bioD*, *bioB* and *bioI* from *B. subtilis* 168	This work
pVWEx1	Km^R; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, *lacI*^{q}, pCG1 *ori*V_{Cg})	Peters-Wendisch et al. (2001)
pVWEx1-*crtEBI*	pVWEx1 derivative for IPTG-inducible expression of *crtE*, *crtB* and *crtI* from *C. glutamicum* containing artificial ribosome binding sites in front of *crtE* and *crtBI*	Heider et al. (2014) and Schneider and Wendisch (2010)
pVWEx1-*speC*	Kan^R, pVWEx1 with *speC* from *E. coli* MG1655	
pVWEx1-speC-5′ 21-argF	pVWEx1-speC carrying argF from C. glutamicum ATCC 13032 with synthetic 5′-region	Schneider et al. (2012a)
Oligonucleotides[a]		
bioWAFDBI-fw	ca*gtcgac***agaaaggaggccctcag**ATGCAAGAAGAAACTTTTTATAG	This work
bioWAFDBI-rv	ca*gaattc*TTATTCAAAAGTCACCGGCAGC	This work
bioF-fw	*ggatcc***aaggagatatagat**ATGATCTGGCAGGAGAAAATCGAC	This work
bioF-rv	ggatccTTAACCGTTGCCATGCAGCA	This work
bioFC-fw	ca*gtcgac***agaaaggaggccctcag**ATGAGCTGGCAGGAGAAAATC	This work
bioFC-rv	ca*ctcgag*TTACTCACGAGCAATCACTCC	This work
bioH-fw	ca*ctcgag*ATGAATAACATCTGGTGGCAG	This work
bioH-rv	caggatccCTACACCCTCTGCTTCAACGC	This work

[a] The restriction sites are given in italics, the region containing the ribosomal binding site in bold.

activated to pimeloyl-CoA although the genome of *C. glutamicum* lacks a *bioW* homolog encoding for 6-carboxyhexanoate-CoA ligase.

In *E. coli*, the precursor pimeloyl-ACP of the BioF reaction is provided by a modified fatty acid biosynthesis pathway elongating methylmalonyl-thioester, which is generated from malonyl-thioester by BioC (Lin et al., 2010). After two iterations of fatty acid biosynthesis, pimeloyl-ACP methyl ester is hydrolyzed by BioH to methanol and pimeloyl-ACP (Fig. 1). *C. glutamicum* possesses two FAS-I type fatty acid synthases FasIA and FasIB that are active with CoA derivatives (Radmacher et al., 2005a,b) that are different from FAS-II type fatty acid synthase of *E. coli*. As expected, when the *E. coli* genes *bioF*, *bioC* and *bioH* were cloned as an artificial operon into the expression vector pEKEx3 and *C. glutamicum* was transformed with the resulting vector pEKEx3-*bioFCH*, the constructed strain WT(pEKEx3-*bioFCH*) did not grow in minimal medium without biotin (Fig. 2).

3.2. Heterologous expression of the bioWAFDBI operon from B. subtilis entails biotin prototrophy of C. glutamicum

Unlike *E. coli*, the Gram-positive *B. subtilis* synthesizes pimeloyl-CoA either from externally added pimelic acid by pimeloyl-CoA synthetase BioW or by interception of fatty acid biosynthesis by P450-dependent BioI (Bower et al., 1996; Lin and Cronan, 2011). Although *C. glutamicum* possesses functional *bioA*, *bioB*, and *bioD* genes, the whole *bioWAFDBI* operon of *B. subtilis* including *bioW,*

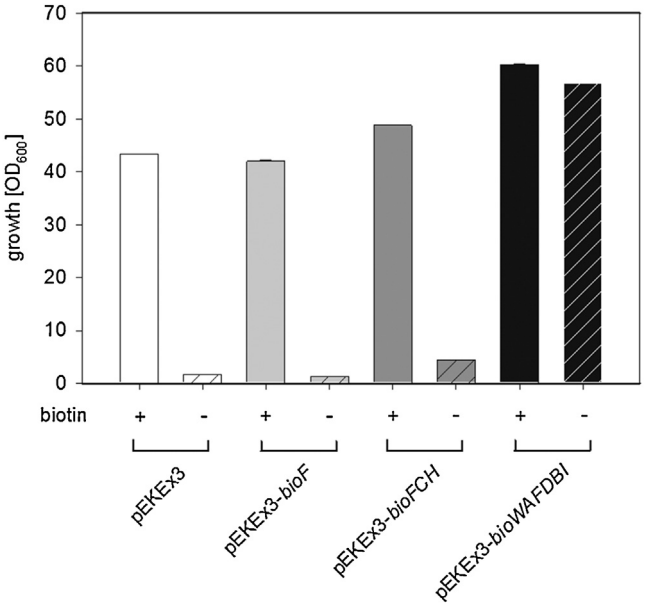

Fig. 2. Biomass formation of various *C. glutamicum* strains in glucose minimal medium with or without biotin. Means and standard deviations optical densities at 600 nm of three independent cultivations inoculated from biotin-limited glucose minimal medium pre-cultures after 24 h incubation are shown. IPTG (1 mM) and spectinomycin were added.

bioF and *bioI* was cloned into the expression vector pEKEx3 and *C. glutamicum* was transformed with the resulting vector pEKEx3-*bioWAFDBI*.

Comparative growth experiments with *C. glutamicum* WT(pEKEx3) and WT(pEKEx3-*bioWAFDBI*) were performed. Upon transfer from biotin-rich medium to biotin-depleted glucose minimal medium, both strains grew comparably, but did not reach

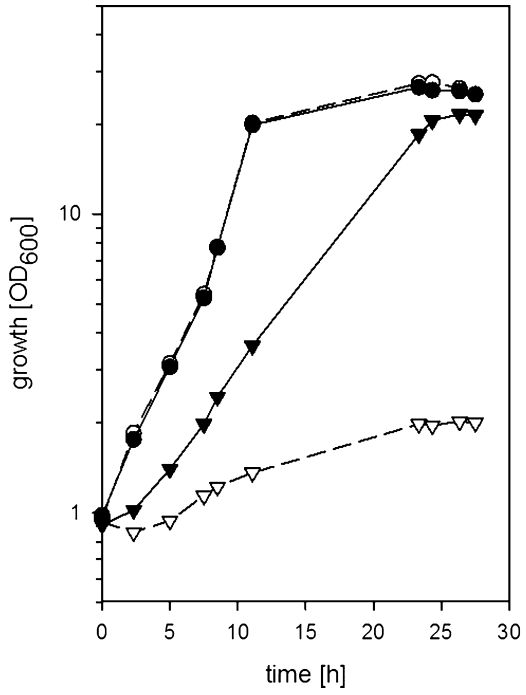

Fig. 3. Growth of *C. glutamicum* WT(pEKEX3) (closed symbols) and WT(pEKEX3-*bioF*) (open symbols) with 200 μg/l biotin (circles) or with 20 mg/l pimelic acid (triangles). Cultivations were inoculated from biotin-limited glucose minimal medium pre-cultures after 24 h incubation. IPTG (1 mM) and spectinomycin were added.

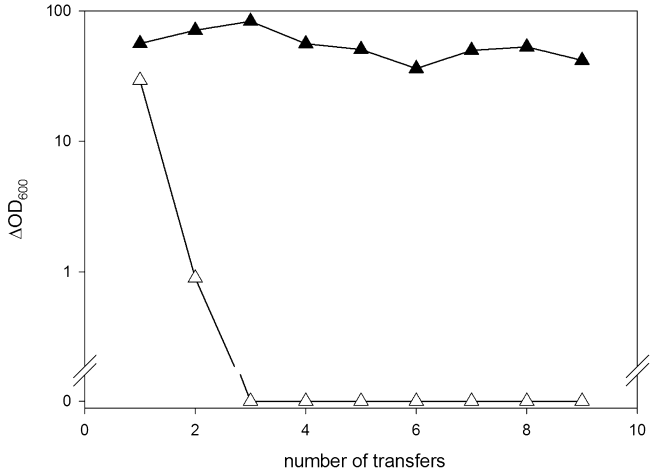

Fig. 4. Biomass formation of *C. glutamicum* WT(pEKEX3-*bioWAFDBI*) and WT(pEKEX3) in glucose minimal medium without added biotin. Repeated transfers from biotin-limited glucose minimal medium pre-cultures of *C. glutamicum* WT(pEKEX3) and WT(pEKEX3-*bioWAFDBI*) to biotin-free glucose minimal medium is shown. IPTG (1 mM) and spectinomycin were added. A representive data set is shown. In total, *C. glutamicum* WT(pEKEX3-*bioWAFDBI*) grew in biotin-free medium for about 52 generations in 315 h.

the same biomass concentrations as in biotin-sufficient glucose minimal medium. After a second transfer to biotin-depleted glucose minimal medium the empty vector control strain hardly grew, while *C. glutamicum* WT(pEKEx3-*bioWAFDBI*) utilized glucose completely and reached a high biomass concentration (Fig. 2). *C. glutamicum* WT(pEKEx3-*bioWAFDBI*) was repeatedly transferred to biotin-depleted glucose minimal medium and its biotin-independent growth was followed for seven additional transfers (Fig. 4). Thus, since *C. glutamicum* WT(pEKEx3-*bioWAFDBI*) grew in 8 transfers for more than 51 generations in biotin-depleted glucose minimal medium, it can be considered a biotin-prototroph.

3.3. Amino acid production by biotin-prototrophic *C. glutamicum* strains

To test if amino acid production can be achieved in biotin-free media, L-lysine as well as L-arginine production was analyzed by the respective producer strains (Blombach et al., 2009b; Schneider et al., 2011) carrying plasmid pEKEx3-*bioWAFDBI*. The L-lysine producing strain DM1933 (Blombach et al., 2009b) was transformed with the vector pEKEx3-*bioWAFDBI* or the empty vector pEKEx3 and L-lysine production was followed in biotin-depleted glucose medium. While the empty vector control strain grew only in the first culture without biotin, DM1933(pEKEX3-*bioWAFDBI*) grew, utilized glucose completely and produced about 13 mM L-lysine upon repeated transfer to biotin-depleted medium (Fig. 5A). Comparable L-lysine concentrations accumulated when the empty vector control or DM1933(pEKEX3-*bioWAFDBI*) were cultivated with biotin (Fig. 5A).

To assay for L-alanine production by *C. glutamicum* in biotin-free media, a variant of the L-alanine producing strain ARG1 was constructed since $argB_{A26VM31V}$ is expressed in *C. glutamicum* Δ*argR* (Schneider et al., 2011) using the same vector as used for *bioWAFDBI* overexpression. Thus, *C. glutamicum* Δ*argR* was transformed with the newly constructed pVWEx1-$argB_{A26VM31V}$ to yield ARG2 and additional introduction of pEKEX3-*bioWAFDBI* yielded ARG2(pEKEX3-*bioWAFDBI*). *C. glutamicum* ARG2(pEKEX3-*bioWAFDBI*) utilized glucose, grew and produced about 25 mM arginine in biotin-depleted glucose minimal media, while ARG2(pEKEx3) stopped growth and arginine

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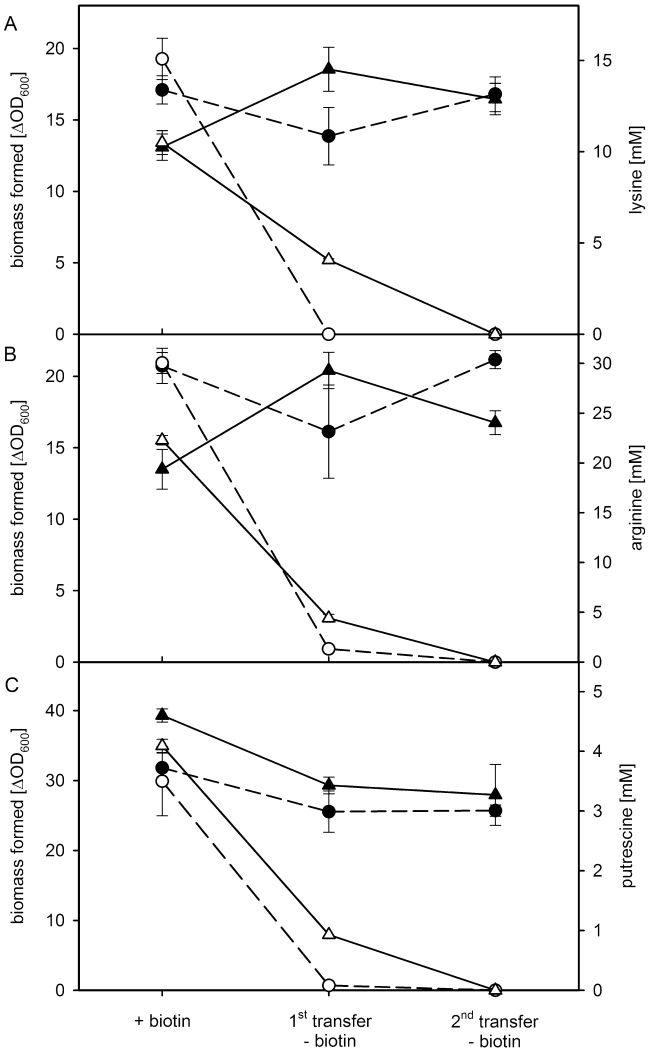

Fig. 5. Growth, L-lysine, L-arginine and putrescine production by (A) *C. glutamicum* DM1933(pEKEX3-*bioWAFDBI*), (B) ARG2(pEKEX3-*bioWAFDBI*) and (C) PUT21(pEKEX3-*bioWAFDBI*), respectively, and the respective empty vector carrying control strains in glucose minimal medium without biotin. Means and standard deviations of biomass (triangles) formed (ΔOD_{600}) and of product titers (circles) of three independent cultivations inoculated from biotin-limited glucose minimal medium pre-cultures at the indicated time points are shown. Data obtained for strains carrying the empty vector are represented by open symbols and for strains carrying by pEKEX3-*bioWAFDBI* closed symbols. IPTG (1 mM) and spectinomycin were added.

production after the first transfer to biotin-free medium (Fig. 5B). Thus, plasmid pEKEX3-*bioWAFDBI* can be transferred to amino acid producing strains to sustain efficient production of the amino acids arginine and L-lysine in biotin-free media.

3.4. Diamine production by recombinant C. glutamicum under biotin-depleted conditions

The diamine 1,4-diaminobutane (also called putrescine) can be efficiently produced by recombinant *C. glutamicum* strains employing a plasmid addiction system (Schneider et al., 2012a; Schneider and Wendisch, 2010). To achieve production of 1,4-diaminobutane on biotin-depleted medium *C. glutamicum* PUT21 was transformed with pEKEX3-*bioWAFDBI* and the empty vector pEKEx3 and growth and putrescine formation was followed. Whereas the empty vector control strain grew only in the first culture without biotin, PUT21(pEKEX3-*bioWAFDBI*) grew, utilized glucose completely and produced 2–5 mM putrescine upon repeated transfer to biotin-depleted medium (Fig. 5C).

3.5. Carotenoid production by recombinant C. glutamicum without added biotin

Disruption of the synthesis of the natural yellow pigment decaprenoxanthin by deletion of genes *crtY* and *crtEb* lycopene cyclase and elongase resulted in lycopene accumulation which could be enhanced by overexpressing the genes for lycopene synthesis *crtE*, *crtB* and *crtI* from vector pVWEx1 (Heider et al., 2014). After transformation of this lycopene producing strain which is named LYC1 with pEKEx3-*bioWAFDBI* or the empty vector, growth and lycopene accumulation was determined in biotin-rich medium and in medium without added biotin. *C. glutamicum* LYC1(pEKEx3) could not grow after one passage in medium without added biotin whereas LYC1(pEKEx3-*bioWAFDBI*) grew upon repeated transfer to biotin-depleted medium and accumulated about 3–6 μM lycopene (Table 2). Thus, transformation with pEKEx3-*bioWAFDBI* entailed biotin-prototrophic lycopene production by *C. glutamicum*.

4. Discussion

Production of amino acids, diamines and carotenoids by biotin-prototrophic producing strains of *C. glutamicum* as described here is a desired feature for corynebacterial fermentation processes since no source of biotin has to be provided by the medium. L-glutamate is formed as unwanted by-product whenever biotin is limiting e.g. by polyhydroxyalkanoate producing recombinant *C. glutamicum* (Jo et al., 2009). Besides avoidance of L-glutamate formation, provision of sufficient biotin was required for L-lysine production by the hitherto available biotin-auxotrophic *C. glutamicum* strains for high levels of anaplerotic pyruvate carboxylase and acetyl-CoA carboxylase to enable fast growth at the low carbon dioxide concentrations encountered during the initial fermentation phase and for high anaplerotic flux to supply oxaloacetate as precursor for L-lysine biosynthesis. Accordingly, overproduction of biotin protein ligase BirA led to higher levels of the biotin-containing protein pyruvate carboxylase and improved growth and L-lysine production in glucose medium (Peters-Wendisch et al., 2012). Potentially, the combined overexpression of the genes for complete biotin synthesis as well as for biotin protein ligase and pyruvate carboxylase could further improve L-lysine production. As shown here, plasmid pEKEx3-*bioWAFDBI* enabled production in biotin-free medium not only of L-lysine, but also of L-arginine, putrescine and the carotenoid lycopene. More generally, growth-associated production of further amino acids such as L-serine (Stolz et al., 2007), L-proline (Jensen and Wendisch, 2013), branched-chain amino acids (Blombach et al., 2009a), L-alanine (Okino et al., 2008b), the diamine cadaverine (Mimitsuka et al., 2007), organic acids such as pyruvate (Wieschalka et al., 2012) and succinate (Litsanov et al., 2012a) polyhydroxyalkanoate (Jo et al., 2009) or proteins (Scheele et al., 2013) in biotin-free medium appears very likely possible.

Heterologous expression of *bioWAFDBI* from *B. subtilis* as shown here and of *bioBF* from *E. coli* combined with *bioI* from *B. subtilis* as shown in parallel work (Ikeda et al., 2013) renders *C. glutamicum* biotin prototrophic. As *C. glutamicum* possesses functional *bioB* (Hatakeyama et al., 1993a,b, 1997) it is likely that heterologous expression of *bioI* combined with *bioF* would be sufficient to endow *C. glutamicum* with biotin prototrophy. However, it remains to be shown whether expression of *bioI* and *bioF* are indeed the minimum requirement and whether additional *bio* genes possibly from other microorganims are needed to match biotin biosynthesis capacity with high level production requirements. Although *bioW* from *B. subtilis* functions to activate externally added pimelic acid (Ploux et al., 1992), growth of the biotin-prototrophic *C. glutamicum* WT(pEKEx3-*bioWAFDBI*) was not accelerated when 5 or 20 mg/l pimelic acid were added to biotin-free medium (data not shown). This finding suggests that biosynthesis of biotin de novo via BioI

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Table 2
Growth and lycopene production of *C. glutamicum* LYC1(pEKEx3) and LYC1(pEKEx3-*bioWAFDBI*) in biotin-free media.

	LYC1(pEKEx3)				LYC1(pEKEx3-*bioWAFDBI*)			
		– Biotin				– Biotin		
	+ Biotin	1st passage	2nd passage	3rd passage	+ Biotin	1st passage	2nd passage	3rd passage
c_{Lyc} [μM]	6.3 ± 0.2	5.2 ± 0.4	n.d.	n.d.	5.7 ± 0.8	5.0 ± 0.5	3.7 ± 0.4	4.5 ± 0.3
ΔOD_{600}	21 ± 1	19 ± 1	n.g.	n.g.	22 ± 1	19 ± 1	20 ± 1	23 ± 1
$Y_{P/S}$ [$mg_{Lyc}\ g_{Glu}^{-1}$]	0.19 ± 0.01	0.16 ± 0.01	n.d.	n.d.	0.17 ± 0.02	0.15 ± 0.02	0.11 ± 0.01	0.14 ± 0.01
q_P [$mg_{Lyc}\ g_{DW}^{-1}\ h^{-1}$]	0.03 ± 0.00	0.02 ± 0.00	n.d.	n.d.	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00

n.g., no growth; n.d. not determined.

coupled to ring assembly by BioF, BioA, BioD and BioB and from external pimelic acid via BioW coupled to ring assembly by BioF, BioA, BioD and BioB are not synergistic.

Pimelic acid could substitute for biotin if *E. coli bioF* (as shown here) or *bioBF* (Ikeda et al., 2013) were expressed in *C. glutamicum* (Fig. 2). Whereas the Km value for pimeloyl-CoA of BioF from *E. coli* is low (25 μM) and might be even lower for pimeloyl-ACP (Turbeville et al., 2011), high concentrations of pimelic acid (20 and 100 mg/l) were required, possibly because import and/or activation of pimelic acid by *C. glutamicum* occurs with low efficiency. Similarly, a high pimelic acid concentration (30 mg/l) was required for *E. coli* strains expressing *bioW* from *B. subtilis* or *bioW* and *bioF* from *B. sphaericus* (Bower et al., 1996; Ploux et al., 1992), however, the Km values for pimelic acid of BioW from *B. subtilis* (145 μM or 23 mg/l) and PauA from *Peudomonas mendocina* (490 μM or 78 mg/l) are comparable to the concentrations used to supplement pimelic acid (Binieda et al., 1999; Ploux et al., 1992). Currently, it is unknown whether pimelic acid uptake into the *C. glutamicum* cell is active or occurs by passive diffusion and which enzyme(s) activate pimelic acid. Acetate kinase and phosphotransacetylase activate acetic acid and propionic acid but do not activate longer fatty acids to the respective CoA esters (Reinscheid et al., 1999; Veit et al., 2009). Alternatively, these acids and succinate may be activated by CoA transferase CAT in *C. glutamicum* (Veit et al., 2009; Yasuda et al., 2007). Since pimeloyl-CoA synthetases have limited similarity to succinyl-CoA synthetases (Binieda et al., 1999) this TCA cycle enzyme may activate pimelic acid as a side reaction. Various enzymes presumably involved in activation of fatty acids including FadD32 exist. FadD32 of *C. glutamicum* activates long-chain fatty acids, however, it does not generate acyl-CoA but acyl-AMP which are substrates of mycolic acid condensase PKS13 (Laneelle et al., 2013; Portevin et al., 2005). In *Vibrio harveyi*, acyl-ACP synthetase AasS is indeed involved in activating pimelic acid to pimeloyl-ACP (Jiang et al., 2010; Lin et al., 2010). Other candidates are FadD1 (cg0341), FadD2 (cg3179), FadD4 (cg1353), FadD5 (cg0480) or FadD15 (cg2521).

Expression of *bioC* and *bioH* of *E. coli* did not lead to biotin prototrophy in *C. glutamicum*, which may be due to BioH generating toxic methanol during hydrolysis of the methyl-pimeloyl-ACP ester (Fig. 1). This possibility, however, appears unlikely as *C. glutamicum* possesses enzymes to degrade toxic methanol and formaldehyde (Lessmeier et al., 2013; Witthoff et al., 2013). The nonfunctionality of the BioC-BioH pathway in *C. glutamicum* could be due to the fact that this bacterium possesses type-I fatty acid synthase complex (FAS-I) whereas both *E. coli* and *B. subtilis* possess type-II fatty acid synthase (FAS-II) (Radmacher et al., 2005a) and mycobacteria possess both and elongate FAS-I products by FAS-II (Laneelle et al., 2013). Thus, it is tempting to speculate that either methyl-malonyl-CoA is not recognized by FAS-I from *C. glutamicum* and/or that BioH cannot gain access to methyl-pimeloyl-ACP in FAS-I from *C. glutamicum* to liberate pimeloyl-ACP after two rounds of fatty acid elongation. Generation of pimeloyl-CoA/ACP by BioI from *B. subtilis* which is known to complement mutations in either *bioC* or *bioH* of *E. coli* (Bower et al., 1996) appeared also to be possible from FAS-I from *C. glutamicum*. It is conceivable that the BioI pathway which differs from the BioC-BioH pathway by its oxygen requirement is compatible with both types of fatty acid synthase complexes FAS-I and FAS-II in other microorganisms.

In its ecological niche the biotin requirement of *C. glutamicum* might pose a competitive disadvantage against other biotin-prototrophic bacteria. In fact, biotin is the target of defense proteins such as the broad antimicrobial and insecticidal biotin-binding proteins avidin in chicken egg-white, streptavidin in streptomycetes, bradavidin in bradyrhizobia and tamavidins in fungi including edible mushrooms (Bleuler-Martinez et al., 2012; Chaiet and Wolf, 1964) with biotin–streptavidin being the strongest noncovalent biological interaction known.

Based on the production of L-lysine, L-arginine, putrescine and lycopene by biotin-prototrophic *C. glutamicum* strains described here transfer of this concept to other *C. glutamicum* production strains using the described plasmid appears straight forward. It might be of further advantage to define a minimal set of heterologous *bio* genes required for biotin prototrophy, possibly just *bioI* and *bioF*, and to integrate these into the corynebacterial genome. L-lysine and L-glutamate are produced in the million-ton-scale and reducing operating costs such as for media components immediately increases competitiveness. Biotin prototrophy provides an excellent means to improve a wide range of biotechnological processes with *C. glutamicum*.

Acknowledgements

AQN is a fellow of the CLIB2021 graduate cluster at Bielefeld University. VFW, PPW and SAEH acknowledge support by the FP7 EU project PROMYSE (289540). VFW and GKR acknowledge support by the BMBF project SysEnCor (0315598E).

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Please cite this article in press as: Peters-Wendisch, P., et al., Engineering biotin prototrophic *Corynebacterium glutamicum* strains for amino acid, diamine and carotenoid production. J. Biotechnol. (2014), http://dx.doi.org/10.1016/j.jbiotec.2014.01.023

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