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Engineering *Corynebacterium glutamicum* for fast production of L-lysine and L-pipecolic acid

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Abstract The Gram-positive *Corynebacterium glutamicum* is widely used for fermentative production of amino acids. The world production of L-lysine has surpassed 2 million tons per year. Glucose uptake and phosphorylation by *C. glutamicum* mainly occur by the phosphotransferase system (PTS) and to lesser extent by inositol permeases and glucokinases. Heterologous expression of the genes for the high-affinity glucose permease from *Streptomyces coelicolor* and *Bacillus subtilis* glucokinase fully compensated for the absence of the PTS in Δ*hpr* strains. Growth of PTS-positive strains with glucose was accelerated when the endogenous inositol permease IolT2 and glucokinase from *B. subtilis* were overproduced with balanced translation initiation rates using plasmid pEKEx3-IolTBest. When the genome-reduced *C. glutamicum* strain GRLys1 carrying additional *in-frame* deletions of *sugR* and *ldhA* to derepress glycolytic and PTS genes and to circumvent formation of L-lactate as by-product was transformed with this plasmid or with pVWEx1-IolTBest, 18 to 20 % higher volumetric productivities and 70 to 72 % higher specific productivities as compared to the parental strain resulted. The non-proteinogenic amino acid L-pipecolic acid (L-PA), a precursor of immunosuppressants, peptide antibiotics, or piperidine alkaloids, can be derived from L-lysine. To enable production of L-PA by the constructed L-lysine-producing strain, the L-lysine 6-dehydrogenase gene *lysDH* from *Silicibacter pomeroyi* and the endogenous pyrroline 5-carboxylate reductase gene *proC* were overexpressed as synthetic operon. This enabled *C. glutamicum* to produce L-PA with a yield of 0.09 ± 0.01 g g^{-1} and a volumetric productivity of 0.04 ± 0.01 g L^{-1} h^{-1}.To the best of our knowledge, this is the first fermentative process for the production of L-PA from glucose.

Keywords *Corynebacterium glutamicum* · L-lysine · L-pipecolic acid · SugR · Permease · Lysine dehydrogenase

Introduction

L-lysine is one of the essential amino acids in mammals, which have to intake L-lysine through their diet as they lack L-lysine biosynthesis. L-lysine is produced biotechnologically to meet the annual world market of about 2 million tons (Hermann 2003; Wendisch 2007; Yukawa and Inui 2013). Glucose-based fermentations with *C. glutamicum* are in place for efficient L-lysine production (Wendisch et al. 2006a, b; Hirasawa et al. 2012; Blombach et al. 2009b, Heider and Wendisch 2015). This microorganism was initially isolated as a natural L-glutamate producer (Abe et al. 1967). *C. glutamicum* is a non-pathogenic, facultative anaerobic Gram-positive bacterium found in soil, animal feces, fruits, and vegetables with an irregular rod shape (Eggeling and Bott 2005). The physiology, biochemistry, and genetics of L-lysine biosynthesis are well understood and have been employed in rational development of L-lysine producing strains. For example, the L-lysine production strain GRLys1 (Unthan et al. 2015) possesses two gene copies of the key enzyme of L-lysine biosynthesis, aspartokinase (Schrumpf et al. 1992; Cremer et al. 1991; Kalinowski et al. 1991), devoid of feedback inhibition by L-lysine (encoded by $lysC^{T311I}$),

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two copies of the L-lysine biosynthesis genes *asd*, *dapA*, *dapB*, *ddh*, and *lysA*, and two copies of L-lysine export gene *lysE* (Vrljic et al. 1996). Side product formation is reduced due to a restricted homoserine dehydrogenase (encoded by hom^{V59A}) (Eikmanns et al. 1991), anaplerosis is improved due to deletion of the PEP carboxykinase gene (Riedel et al. 2001), and a variant of the pyruvate carboxylase gene (Peters-Wendisch et al. 2001) and the strain was cured of prophages CGP1, CGP2, and CGP3 (Baumgart et al. 2013). A wealth of knowledge on L-lysine biosynthesis and production has accumulated (Wendisch et al. 2006a, b; Hirasawa et al. 2012; Blombach et al. 2009a; Heider and Wendisch 2015; Becker and Wittmann 2012; Wendisch 2014; Mitsuhashi 2014; Park et al. 2010).

C. glutamicum takes up and phosphorylates glucose primarily via the phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS). In addition, glucose can be imported via the non-phosphorylating inositol permeases IolT1 and IolT2 (Lindner et al. 2011; Ikeda et al. 2011; Bäumchen et al. 2009). Intracellular glucose may be phosphorylated by ATP-dependent or polyphosphate-dependent glucokinases PpgK and Glk (Park et al. 2000; Lindner et al. 2010). While the PTS constitutes a high-affinity uptake system (Km of 14 μM), the permeases IolT1 and IolT2 have about hundred fold lower affinities for glucose (Km values of 2.8 mM and 1.9 mM, respectively). As the ATP- or polyphosphate-dependent glucokinases Glk and PpgK do not consume PEP, the availability of PEP for anabolism e.g., for biosynthesis of aromatic amino acids may increase (Tang et al. 2013; Zhang et al. 2015). Since high initial glucose concentrations are typical for biotechnological processes, use of the glucose permease and glucokinase pathway offers an interesting opportunity to enhance glucose utilization while maintaining high PEP availability. Commensurate with this notion, PpgK was shown to be important only at high glucose concentrations (Lindner et al. 2010). Glucose utilization by *C. glutamicum* is subject to transcriptional control (Toyoda et al. 2008, 2009a; Teramoto et al. 2011; Park et al. 2010; Gerstmeir et al. 2004). For example, SugR is a general repressor of the PTS, glycolysis and fermentative L-lactate dehydrogenase genes in *C. glutamicum* (Engels and Wendisch 2007; Engels et al. 2008; Teramoto et al. 2011; Gaigalat et al. 2007). Deletion of *sugR* accelerated glucose utilization (Blombach et al. 2009b; Bartek et al. 2010; Teramoto et al. 2011), while overexpression of *sugR* slowed glucose utilization (Engels et al. 2008). Due to co-utilization of protocatechuic acid, single cells of *C. glutamicum* grow faster with glucose in the highly diluted environments of a Lab Chip than cells in suspension (Unthan et al. 2014). Taken together, glucose uptake and utilization by *C. glutamicum* wild type as well as by L-lysine producing strains may be relevant targets for strain development.

L-lysine can be converted to other value-added compounds such as the diamine cadaverine (Mimitsuka et al. 2007; Tateno et al. 2009; Kind et al. 2014; Oh et al. 2015; Shimizu 2013; Leßmeier et al. 2015), 5-aminovalerate (5AVA) (Park et al. 2013) and L-PA (Fujii et al. 2002; Gatto et al. 2006; Neshich et al. 2013). *C. glutamicum* has been engineered for cadaverine production but not for production of L-PA. The non-proteinogenic amino acid L-PA is a precursor of immunosuppressants (Maddess et al. 2008), peptide antibiotics (Takayama et al. 1984), or piperidine alkaloids (Clevenstine et al. 1979). In plants, L-PA is a critical regulator of inducible plant immunity, mediating defense amplification and priming (Návarová et al. 2012). Lysine degradation yields L-PA as intermediate. DL-lysine and L-lysine are oxidatively deaminated by stereospecific L-lysine α-oxidases from *Pseudomonas putida* (Miller and Rodwell 1971) and *Trichoderma viride* (Kusakabe et al. 1980), respectively, to α-keto-ε-aminocaproate, which spontaneously dehydrates to Δ^1-piperideine-2-carboxylate (P2C). P2C reductase yields L-PA from P2C (Payton and Chang 1982). As shown in Fig. 1, L-PA may also be derived from Δ^1-piperideine-6-carboxylate (P6C) in an NADPH-dependent reduction reaction catalyzed by pyrroline 5-carboxylate reductase ProC (Fujii et al. 2002), an enzyme of L-proline biosynthesis in many bacteria including *C. glutamicum* (Ankri et al. 1996). P6C arises from α-aminoadipic semialdehyde (AASA) by spontaneous cyclization (Fig. 1). Four reactions give rise to AASA from L-lysine: (1) L-lysine cyclodeaminase (LCD) from streptomycetes such as *S. hygroscopicus* that produce polyketide immunosuppressants such as rapamycin or meridiamycin (Molnár et al. 1996); (2) L-lysine 6-aminotransferase (LAT) from *Flavobacterium lutencens* (Soda et al. 1968); and (3) L-lysine 6-dehydrogenase e.g., from *Agrobacterium tumefaciens* (Misono et al. 1989) or from *Silicibacter pomeroyi* (Neshich et al. 2013) (Fig. 1). Furthermore, L-lysine can be converted to AASA via saccharopine by L-lysine α-ketoglutarate reductase and saccharopine dehydrogenase from *Rizoctonia leguminicola* (Wickwire et al. 1990). L-PA accumulated intracellularly when *Escherichia coli* overproducing L-lysine 6-dehydrogenase from *S. pomeroyi* was grown in L-lysine-containing media (Neshich et al. 2013). L-PA was produced from L-lysine in biotransformations with *E. coli* overproducing either L-lysine α-oxidase from *Scomber japonicus* (Tani et al. 2015) or LAT (Fujii et al. 2002).

In this study, L-lysine production from glucose was accelerated by overexpression of endogenous *iolT2* encoding inositol permease and by heterologous expression of the glucokinase gene from *B. subtilis*. In addition, deletion of *sugR* led to derepression of glycolytic and PTS genes and deletion of *ldhA* precluded formation of L-lactate as by-product. This strain was used as basis for strain development for fermentative production of L-PA from glucose.

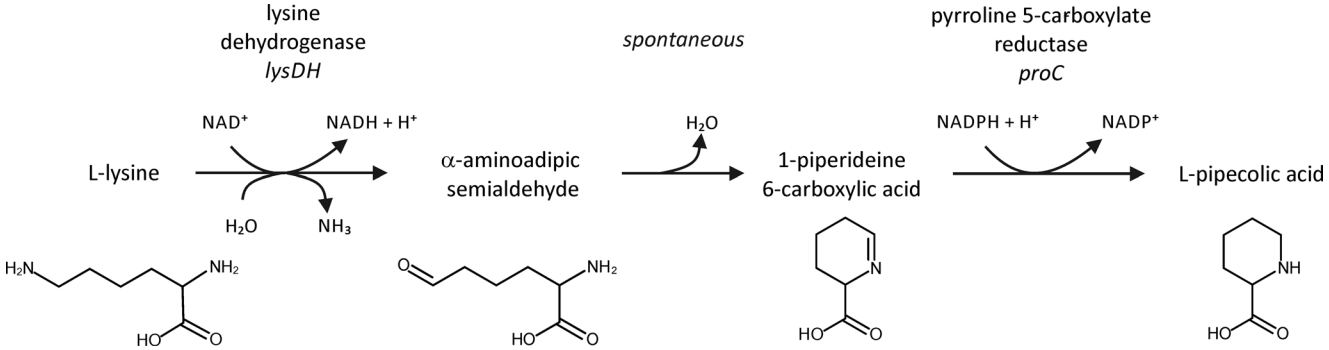

Fig. 1 Metabolic pathway for conversion of L-lysine to L-PA in recombinant *C. glutamicum*. L-lysine 6-dehydrogenase (deaminating) (EC 1.4.1.18) from *S. pomeroyi* encoded by *lysDH* and pyrroline 5-carboxylate reductase (EC 1.2.1.41) from *C. glutamicum* encoded by *proC* were used. The combined reactions of L-lysine 6-dehydrogenase (EC 1.4.1.18) and the spontaneous cyclization of α-aminoadipic semialdehyde are sometimes referred to as L-lysine:NAD^+ oxidoreductase (deaminating, cyclizing) (EC 1.4.1.15)

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids are listed in Table 1. *E. coli* DH5α (Hanahan 1983) was used for vector construction. The precultivation of *C. glutamicum* and cultivation of *E. coli* were carried out in Luria-Bertani (LB) medium (Sambrook et al. 1989) inoculated from a fresh LB agar plate. For growth of *C. glutamicum* strains, the cells from the preculture were washed in CGXII minimal medium (Eggeling and Bott 2005) without glucose, before 50 ml of main CGXII culture with glucose was inoculated to an optical density of 0.5 (OD_{600}). *C. glutamicum* precultures and cultures were incubated at 30 °C and 120 rpm in 500 ml baffled flasks. *E. coli* cultures were incubated at 37 °C and 200 rpm in 500 ml baffled flasks. The final glucose concentration varied between 0.5 % (*w*/*v*) and 4 % (*w*/*v*). For selection of pVWEx1 and pSH1, 25 μg ml^{-1} of kanamycin was added to cultures (Peters-Wendisch et al. 2001). For selection of pEKEx3, 100 μg ml^{-1} of spectinomycin was added to cultures (Stansen et al. 2005).

Construction of expression vectors

Genes were amplified via PCR from genomic DNA of *C. glutamicum* ATCC 13032 (Kalinowski et al. 2003), *Mycobacterium smegmatis* mc^2 155 (Pimentel-Schmitt et al. 2009), *S. coelicolor* A3(2) (van Wezel et al. 2005; Imriskova et al. 2005), *E. coli* K-12 MG 1655 (Meyer et al. 1997), *B. subtilis* subsp. *subtilis* str. 168 (Mesak et al. 2004), or *S. pomeroyi* DSS-3 (DSMZ-15171) (González et al. 2003). Genomic DNA was prepared following the same protocol described previously (Eikmanns et al. 1995). The primers for the vector constructions are listed in Table 2.

Balanced expression of glucose permease and kinase genes

For the balanced expression of glucose permease and kinase genes, a combinatorial approach was performed by combining four ribosomal binding site (AAAGGAGG, AAAGGTGG, AAAGGCGG, and AAAGGGGG) and two transcriptional start site (ATG and GTG) options for the permease genes cloned together with the glucokinase gene, which was not varied. Primers pgk13, pgk14, pgk15, pgk16, pk35, and pk36 (s. Table 2) were used for PCR, and genes were joined via Gibson assembly to yield pEKEx3-$glcK^{Bs}$-$glcP^{Sco}$ and pEKEx3-$glcK^{Bs}$-$iolT2^{Cg}$ vectors (Gibson 2011). These vectors were used to transform *C. glutamicum* Δ*hpr*, and the best combinations were chosen according to fastest growth in minimal medium with 2 % (*w*/*v*) of glucose and different IPTG concentrations. IPTG concentrations of 100 μM were optimal for (pEKEx3-$glcK^{Bs}$-$glcP^{Sco}$) and 50 μM for (pEKEx3-$glcK^{Bs}$-$iolT2^{Cg}$).

Deletion of *sugR and ldhA*

The two-step homologous recombination procedure was used to generate *in-frame* deletions of Δ*sugR* and Δ*ldhA* in the strain GRLys1 (Rittmann et al. 2003). For that purpose, the vectors pK19*mobsacB*-Δ*sugR* (Engels et al. 2008) and pK19*mobsacB*-Δ*ldhA* (Blombach et al. 2011) were used as described previously (Engels and Wendisch 2007). The deletions were verified by PCR using the primer pairs Δ*sugR*-Ver-fw/Δ*sugR*-Ver-rv and Δ*ldhA*-Ver-fw/Δ*ldhAR*-Ver-rv (Table 2).

Analytical procedures

Extracellular amino acids and carbohydrates were quantified using a high-pressure liquid chromatography system (1200 series, Agilent Technologies Deutschland GmbH,

Table 1 Strains and plasmids used in this work

Strains and plasmids	Description	Source
Strains		
WT	*C. glutamicum* wild type, ATCC 13032	ATCC
GRLys1	*C. glutamicum* ATCC13032 with the following modifications: *Δpck*, 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*, in-frame deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752) and CGP3 (cg1890-cg2071); also named DM1933ΔCGP123	Unthan et al. 2015
GRLys1*ΔsugR*	In-frame deletion of *sugR* (cg2115) in GRLys1	This work
GRLys1*ΔsugRΔldhA*	In-frame deletion of *sugR* (cg2115) and *ldhA* (cg3219) in GRLys1	This work
Plasmids		
pVWEx1	Kan^R, *C. glutamicum*/*E. coli* shuttle vector (P_{tac}, *lacI*, pHM1519 $oriV_{C.g.}$)	Peters-Wendisch et al. 2001
pEKEx3	$Spec^R$,*C. glutamicum*/*E. coli* shuttle vector (P_{tac}, *lacI*, pBL1 $OriV_{C.g.}$)	Stansen et al. 2005
pSH1	Kan^R, *C. glutamicum*/*E. coli* shuttle vector (P_{tuf}, pHM1519 $OriV_{C.g.}$)	Heider & Wendisch unpublished
pVWEx1-$glcP^{Sco}$	Kan^R, pVWEx1 overexpressing *glcP* from *S. coelicolor* A3(2)	This work
pVWEx1-$glcP^{Smeg}$	Kan^R, pVWEx1 overexpressing *glcP* from *M. smegmatis* MC^2 155	This work
pVWEx1-$galP^{Ec}$	Kan^R, pVWEx1 overexpressing *galP* from *E. coli* MG 1655	This work
pVWEx1-$iolT2^{Cg}$	Kan^R, pVWEx1 overexpressing *iolT2* from *C. glutamicum* ATCC 13032	Lindner et al. 2011
pEKEx3-$ppgK^{Cg}$	$Spec^R$, pEKEx3 overexpressing *ppgK* from *C. glutamicum* ATCC 13032	Lindner et al. 2010
pEKEx3-glk^{Cg}	$Spec^R$, pEKEx3 overexpressing *glk* from *C. glutamicum* ATCC 13032	Lindner et al. 2011
pEKEx3-glk^{Ec}	$Spec^R$, pEKEx3 overexpressing *glk* from *E. coli* MG 1655	Lindner et al. 2010
pEKEx3-glk^{Sco}	$Spec^R$, pEKEx3 overexpressing *glk* from *S. coelicolor* A3(2)	This work
pEKEx3-$glcK^{Bs}$	$Spec^R$, pEKEx3 overexpressing *glcK* from *B. subtilis* subsp. *Subtilis* str. 168	This work
pEKEx3-ScoBest	$Spec^R$, pEKEx3 overexpressing *glcP* from *S. coelicolor* A3(2) with RBS sequence AAAGGTGG and translational start codon and *glcK* from *B. subtilis* subsp. *Subtilis* str. 168	This work
pVWEx1-ScoBest	Kan^R, pVWEx1 overexpressing *glcP* from *S. coelicolor* A3(2) with RBS sequence AAAGGTGG and translational start codon GTG and *glcK* from *B. subtilis* subsp. *Subtilis* str. 168	This work
pEKEx3-IolTBest	$Spec^R$, pEKEx3 overexpressing *iolT2* from *C. glutamicum* ATCC 13032 with RBS sequence AAAGGGGG and translational start codon GTG and *glcK* from *B. subtilis* subsp. *Subtilis* str. 168	This work
pVWEx1-IolTBest	Kan^R, pVWEx1 overexpressing *iolT2* from *C. glutamicum* ATCC 13032 with RBS sequence AAAGGGGG and translational start codon GTG and *glcK* from *B. subtilis* subsp. *Subtilis* str. 168	This work
pSH1-*lysDH-proC*	Kan^R, pSH1 overexpressing *lysDH* from *S. pomeroyi* DSS-3 and *proC* from *C. glutamicum* ATCC 13032	This work
pK19*mobsacB*-*ΔsugR*	Kan^R, mobilizable *E. coli* vector for the construction of insertion and deletion mutants of *C. glutamicum* (oriV, sacB, lacZ) with the deletion construct for *sugR* (cg2115)	Engels and Wendisch 2007
pK19*mobsacB*-*ΔldhA*	Kan^R, mobilizable *E. coli* vector for the construction of insertion and deletion mutants of *C. glutamicum* (oriV, sacB, lacZ) with the deletion construct for *ldhA* (cg3219)	Blombach et al. 2011

Böblingen, Germany). Cell cultures were centrifuged (13,000×g, 10 min), and the supernatant was used for analysis. For the detection of L-lysine, L-glutamate, L-valine and L-alanine, samples were derivatized with *ortho*-phthaldialdehyde and for detection of L-PA with fluorenylmethyl chloroformate (FMOC) as described (Schneider and Wendisch 2010). All amino acids were separated on a system consisting of a pre-column (LiChrospher 100 RP18 EC-5μ (40 × 4 mm), CS Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5 μ (125 × 4 mm), CS Chromatographie Service GmbH), and detected with a fluorescence detector (FLD G1321A, 1200 series, Agilent Technologies). L-Asparagine was used as internal standard. For quantification of carbohydrates, a column for organic acids

Table 2 List of primers used in this work

Primer name	Oligonucleotide sequence (5′ - 3′)	Description
X1fw	CATCATAACGGTTCTGGC	Forward primer for colony PCR with the vectors pEKEx3 and pVWEx1
X1rv	ATCTTCTCTCATCCGCCA	Reverse primer for colony PCR with the vectors pEKEx3 and pVWEx1
SHfw	GAAAGTCGTAGCCACCAC	Forward primer for colony PCR with the vector pSH1
SHrv	GACCGCTTCTGCGTTCTG	Reverse primer for colony PCR with the vector pSH1
Scfw	GATGGTACC*GAAAGGAGGCCCTTCAG*GTGGCCAGCACATCGCAG	Forward primer for overexpression of $glcP^{Sco}$. Restriction site for *Kpn*I
Scrv	GATGGTACCGTCAGCCCATTTCCTCCAG	Reverse primer for overexpression of $glcP^{Sco}$. Restriction site for *Kpn*I
Smfw	GATCCTGCAGG*GAAAGGAGGCCCTTCAG*ATGCGCCAGACGGGCAGTC	Forward primer for overexpression of $glcP^{Smeg}$. Restriction site for *Sbf*I
Smrv	GATTCTAGACTAGATCTTCTCGGGCTTGGG	Reverse primer for overexpression of $glcP^{Smeg}$. Restriction site for *Xba*I
EcGf	GATCCTGCAGG *GAAAGGAGGCCCTTCAG*ATGCCTGACGCTAAAAAACAGGG	Forward primer for overexpression of $galP^{Eco}$. Restriction site for *Sbf*I
EcGr	GATTCTAGATTAATCGTGAGCGCCTATTTCG	Reverse primer for overexpression of $galP^{Eco}$. Restriction site for *Xba*I
BsFw	GATGGATCC*GAAAGGAGGCCCTTCAG*ATGGACGAGATATGGTTTG	Forward primer for overexpression of $glcK^{Bs}$. Restriction site for *Bam*HI
BsRv	GATGGATCCTTAACAATTTTGATGTTTCAGCC	Reverse primer for overexpression of $glcK^{Bs}$. Restriction site for *Bam*HI
C2Fw	GATCCTGCAGG *GAAAGGAGGCCCTTCAG*ATGGGACTCACCATCGGCGTCGAC	Forward primer for overexpression of glK^{Sco}. Restriction site for *Sbf*I
C2Rv	GATGAATTCTCACATGATCGGGTCGGGTTCTCTC	Forward primer for overexpression of glK^{Sco}. Restriction site for *Eco*RI
gpk13	**CTGCAGGTCGACTCTAGAG***GAAAGGAGGCCCTTCA* *G*ATGGACGAGATATGGTTTGC	Forward primer for overexpression of $glcK^{Bs}$. Vector overlapping region (pEKEx3 and pVWEx1)
gpk14	**GGGTCTTTCGAA**TTAACAATTTTGATGTTTCAG	Reverse primer for overexpression of $glcK^{Bs}$. Genes overlapping region (ScoBest)
gpk15	**CATCAAAATTGTTAATTCGAAAGACCC***GA* *AAGGnGGCCCTTCAG*rTGGCCAGCACATCGCAGGC	Forward primer for overexpression of $glcP^{Sco}$. Genes overlapping region (ScoBest)
gpk16	**CGGTACCCGGGGATC**TCAGCCCATTTCCTCCAGGG	Reverse primer for overexpression of $glcP^{Sco}$. Vector overlapping region (pEKEx3 and pVWEx1)
ScSe	AGGCCGCGATCGTCATCGGCATC	Sequencing primer for intergene region of ScoBest vector
pk35	**CATCAAAATTGTTAATTCGAAAGACCC***GAAAGGnG* *GCCCTTCAG*rTGACGGACATCAAGGCCAC	Forward primer for overexpression of $iolT2^{Cg}$. Genes overlapping region (IolTBest)
pk36	**CGGTACCCGGGGATC**TTAAGCCTTCTTGAAGATCTG	Reverse primer for overexpression of $iolT2^{Cg}$. Vector overlapping region (pEKEx3 and pVWEx1)
IoSe	CTCGCTGAACTCGCACCACTAG	Sequencing primer for intergene region of IolTBest vector
ΔsugR-Ver-fw	GTTCGTCGCGGCAATGATTGACG	Forward primer for verification of the deletion of *sugR*
ΔsugR-Ver-rv	CTCACCACATCCACAAACCACGC	Reverse primer for verification of the deletion of *sugR*
ΔldhA-Ver-fw	TGATGGCACCAGTTGCGATGT	Forward primer for verification of the deletion of *ldhA*
ΔldhA-Ver-rv	CCATGATGCAGGATGGAGTA	Reverse primer for verification of the deletion of *ldhA*
P001	**CCTGCAGGTCGACTCTAGAG***G* *AAAGGAGGCCCTTCAG*ATGCGCTGGAACATTTGTGT	Forward primer for overexpression of *lysDH*. Vector overlapping region (pSH1)
P002	**GATTACAGCAATTGTTGTCAT***CTGAAGGG* *CCTCCTTTC*TCAAGCGGCCTTGTTTTGGG	Reverse primer for overexpression of *lysDH*. Genes overlapping region (*lysDH-proC*)
P003	**CCCAAAACAAGGCCGCTTGA***GAAAG* *GAGGCCCTTCAG*ATGACAACAATTGCTGTAATC	Forward primer for overexpression of *proC*. Genes overlapping region (*lysDH-proC*)
P004	**GAGCTCGGTACCCGGGGATC**CTAGCGCTTTCCGAGTTCTTC	Reverse primer for overexpression of *proC*. Vector overlapping region (pSH1)

Underlined letters restriction site, *italic letters* ribosomal binding sites + spacer, *bold letters* overlapping region

(300 × 8 mm, 10 μm particle size, 25 Å pore diameter, CS Chromatographie Service GmbH) and a refractive index detector (RID G1362A, 1200 series, Agilent Technologies) was used (Peters-Wendisch et al. 2014).

Transcriptome analysis using DNA microarrays

To identify gene expression changes due to the addition of L-PA to the growth medium, *C. glutamicum* WT was cultivated

in CGXII minimal medium with 4 % (*w*/*v*) glucose supplemented with either 200 mM L-PA or 200 mM sodium chloride. Exponentially growing cells were harvested by centrifugation (4000 × g, 10 min, 4 °C) and kept at −80 °C. RNA isolation was performed as described (Wendisch 2003). DNA microarray analysis, synthesis of fluorescently labeled cDNA from total RNA, DNA microarray hybridization, and gene expression analysis were performed as described previously (Ishige et al. 2003; Lange et al. 2003). The data are available as Gene Expression Omnibus GSE79690 data set at http://www.ncbi.nlm.nih.gov/geo/.

Results

Potential of alternative glucose permeases to substitute for the glucose PTS

C. glutamicum not only takes up and phosphorylates glucose mainly via the high-affinity (Km of 14 μM) phosphotransferase system (PTS) but also can use inositol permeases IolT1 and IolT2, which have about hundred fold lower affinities for glucose and require subsequent phosphorylation by glucokinases (Lindner et al. 2011; Ikeda et al. 2015). The latter systems may be important at the high glucose concentrations employed in industrial fermentations. In order to test if uptake and phosphorylation via glucose permeases and glucokinase can fully substitute for the glucose PTS at medium to high glucose concentrations (1 % (*w*/*v*) to 4 % (*w*/*v*)), glucose permease and glucokinase genes from various organisms were expressed in the PTS-deficient *C. glutamicum* strain *Δhpr* (Lindner et al. 2011). The heat-stable protein HPr is an essential component of the PTS system, which is phosphorylated by PTS component EI and, subsequently, phospho-HPr transfers the phosphoryl group to the PTS permease component. The genes for the glucose permease from *S. coelicolor* A3(2) ($GlcP^{Sco}$) with a Km of 41 μM (van Wezel et al. 2005), the glucose permease from *M. smegmatis* mc^2 155 ($GlcP^{Smeg}$) with a Km of 19.4 μM (Pimentel-Schmitt et al. 2009), the galactose permease from *E. coli* MG1655 ($GalP^{Eco}$) with a Km of 10.2 μM (McDonald et al. 1997), and the inositol transporter 2 from *C. glutamicum* ATCC 13032 ($IolT2^{Cg}$) with a Km of 1.9 mM (Lindner et al. 2011) were cloned into the IPTG-inducible expression vector pVWEx1 (Peters-Wendisch et al. 2001) and used to transform *C. glutamicum Δhpr*. In minimal medium with 4 % (*w*/*v*) of glucose, *C. glutamicum* WT (pVWEx1) grew with a growth rate of 0.42 ± 0.01 h^{-1} (Fig. 2), while *C. glutamicum Δhpr*(pVWEx1) hardly grew (0.03 ± 0.01 h^{-1}), which is due to low expression of *iolT1* and *iolT2* in the absence of inositol (Krings et al. 2006). Of the glucose permease genes tested, overexpression of $iolT2^{Cg}$ supported fastest growth (μ = 0.27 ± 0.01 h^{-1}); however, even after variation of the IPTG concentration used for induction, the wild-type growth rate could not be restored. Thus, overexpression of glucose permease alone was not sufficient to substitute for the PTS in *C. glutamicum Δhpr*.

Combined overexpression of glucokinase and glucose permease genes

C. glutamicum possesses ATP-dependent glucokinase Glk (Park et al. 2000) and polyphosphate/ATP-dependent glucokinase PpgK with a Km for glucose of 1 mM (Lindner et al. 2010). Combined overexpression of *ppgK* with either *iolT1* or *iolT2* in a PTS-negative strain led to slightly slower glucose utilization rates than in the parent strain (Lindner et al. 2011). Thus, combinations of alternative glucokinases with glucose permeases were tested for their potential to fully compensate for the lack of the PTS. Genes encoding glucokinase from *E. coli* K-12 MG1655 (Glk^{Eco}) with Km of 0.78 mM (Meyer et al. 1997), the glucokinase from *S. coelicolor* A3(2) (Glk^{Sco}) with Km of 1.4 mM (Imriskova et al. 2005), the glucokinase from *B. subtilis* subsp. *Subtilis* str. 168 ($GlcK^{Bs}$) with Km of 0.24 mM (Mesak et al. 2004), the glucokinase (Glk^{Cg}) and the polyphosphate (PolyP)/ATP-dependent glucokinase from *C. glutamicum* ($PpgK^{Cg}$) with Km of 1 mM (Lindner et al. 2010) were cloned into the IPTG-inducible expression vector pEKEx3 (Stansen et al. 2005). The resulting vectors were used to transform *C. glutamicum Δhpr*(pVWEx1- $iolT2^{Cg}$), *Δhpr*(pVWEx1-$galP^{Eco}$), and *Δhpr*(pVWEx1-$glcP^{Sco}$), respectively. All strains

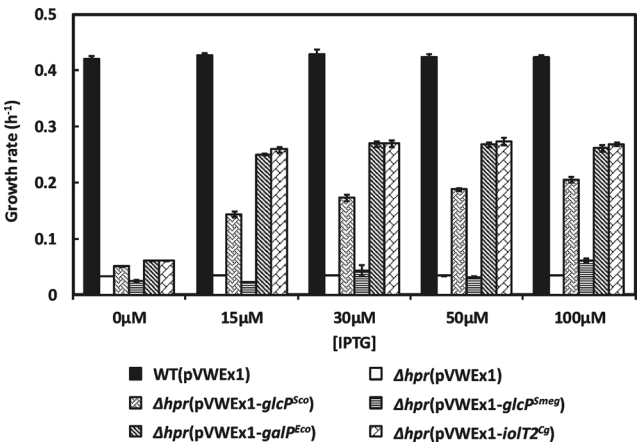

Fig. 2 Influence of sugar permease gene overexpression on growth of *C. glutamicum Δhpr* in glucose minimal medium. Growth rates in minimal medium with 4 % (*w*/*v*) glucose at the indicated IPTG concentrations are given for strains WT(pVWEx1), *Δhpr*(pVWEx1), *Δhpr*(pVWEx1-$glcP^{Sco}$), *Δhpr*(pVWEx1-$glcP^{Smeg}$), *Δhpr*(pVWEx1-$galP^{Eco}$), and *Δhpr*(pVWEx1-$iolT^{Cg}$) as means and standard deviations of three replicates

were grown in CGXII minimal medium with 4 % (*w*/*v*) glucose (Fig. 3). The fastest growing strains were also cultivated at lower glucose concentrations (Fig. 3). At 1 % (*w*/*v*) and 0.5 % (*w*/*v*) glucose, none of the combinations allowed growth as fast as the wild-type control. With 4 % (*w*/*v*) glucose, the combination of $IolT2^{Cg}$ and $GlcK^{Bs}$ allowed faster growth ($\mu = 0.35\ h^{-1}$) than the wild-type control ($\mu = 0.32\ h^{-1}$). With 2 % (*w*/*v*) glucose, the combination of $GlcP^{Sco}$/$GlcK^{Bs}$ grew faster ($\mu = 0.37\ h^{-1}$) than the wild-type control ($\mu = 0.32\ h^{-1}$).

Varying translation initiation rates for balanced glucokinase and glucose permease gene expression

Since overexpression of genes for permeases and other membrane proteins often perturbs growth, various translation initiation sequences for expression of the permease genes $glcP^{Sco}$ and $iolT2^{Cg}$ were tested for combined expression with $glcK^{Bs}$ in one vector. Expression of $glcK^{Bs}$ was kept constant, and translation initiation rates of the permease gene ($glcP^{Sco}$ or $iolT2^{Cg}$) were varied by modifying ribosomal binding sites (RBS) and the translational start codons (TLS). The pool of vectors carrying different RBS and TLS sequences was used to transform *Δhpr*, and 24 clones for each combination were assayed for growth in minimal medium containing 2 % (*w*/*v*) glucose. The fastest-growing strains were selected and the inserts of the vectors were sequenced. Vector pEKEx3-ScoBest carried the RBS sequence of AAAGGTGG and the TLS sequence of GTG (Fig. 4a) for expression of $glcK^{Bs}$ and $glcP^{Sco}$, and vector pEKEx3-IolTBest carried the RBS sequence of AAAGGGGG and the TLS sequence of GTG (Fig. 4b) for expression of $glcK^{Bs}$ and $iolT2^{Cg}$.

To assay the effect of vectors pEKEx3-ScoBest and pEKEx3-IolTBest on glucose utilization by PTS-positive *C. glutamicum*, both vectors were used to transform *C. glutamicum* WT and growth, glucose consumption, and by-products formation in minimal medium with 2 % (*w*/*v*) glucose were compared to the empty vector carrying control strain (Table 3). Under these conditions, both WT(pEKEx3-ScoBest) and WT(pEKEx3-IolTBest) grew faster than the control strain with growth rates of 0.35 and 0.37 h^{-1}, respectively, which were 9 and 15 % higher than that of WT(pEKEx3) ($\mu = 0.32\ h^{-1}$). L-Lactate formation increased by about 30 % for WT(pEKEx3-ScoBest) and by about 50 % for WT(pEKEx3-IolTBest) as compared to WT(pEKEx3) (about 6 mM; Table 3 (A)). Since vectors pEKEx3-ScoBest and pEKEx3-IolTBest accelerated growth in glucose minimal medium by PTS-positive (WT) and PTS-negative (*Δhpr*) strains, both were used in subsequent experiments with the L-lysine-producing *C. glutamicum* strain GRLys1.

However, *C. glutamicum* WT transformed with the empty vector pEKEx3 (replication origin of pBL1, $Spec^R$) grew slower than WT carrying the empty vector pVWEx1 (replication origin of pHM1519, Kan^R) reflecting different burdens by these plasmids. To exclude vector specific effects, vectors pVWEx1-IolTBest and pVWEx1-ScoBest were constructed and used to transform *C. glutamicum* WT. In line with the results obtained with pEKEx3-ScoBest and pEKEx3-IolTBest (Table 3 (A)), strains WT(pVWEx1-IolTBest) and WT(pVWEx1-

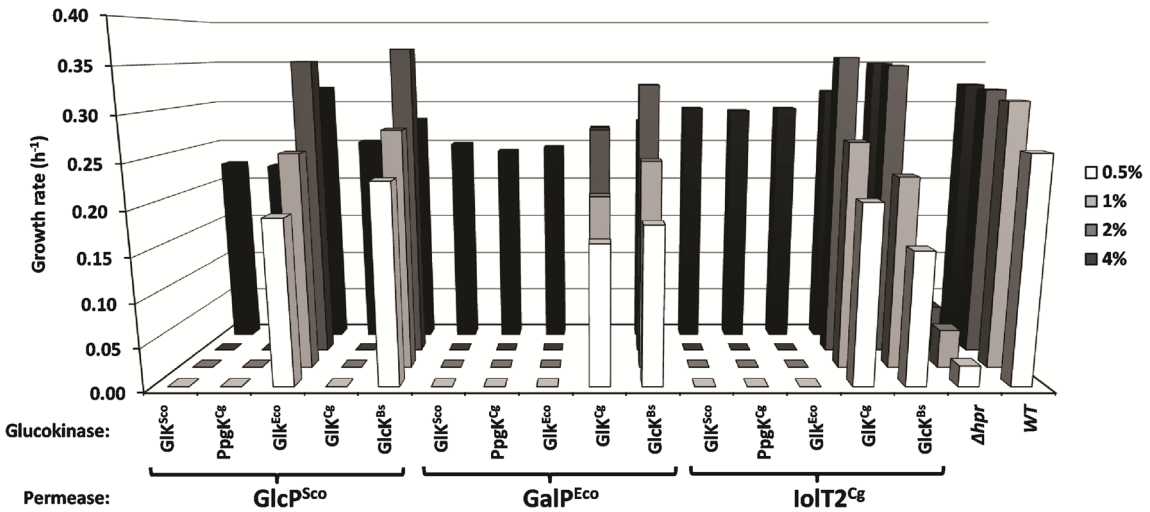

Fig. 3 Influence of sugar permease and glucokinase gene overexpression on growth of *C. glutamicum Δhpr* in glucose minimal medium. Growth rates in minimal medium with the indicated glucose concentration (4 %, 2 %, 1 % or 0.5 % (*w*/*v*)) are given as means of three replicates. The strain *C. glutamicum* WT(pVWEx1)(pEKEx3) was used as positive control (labeled *WT* in the figure). The strain *Δhpr*(pVWEx1)(pEKEx3) was used as negative control (labeled *Δhpr* in the figure). Combinations of the indicated glucokinases and permeases were tested in *Δhpr* with permease genes expressed from plasmid pVWEx1 and glucokinase genes expressed from plasmid pEKEx3. A growth rate of zero is depicted if growth was not tested

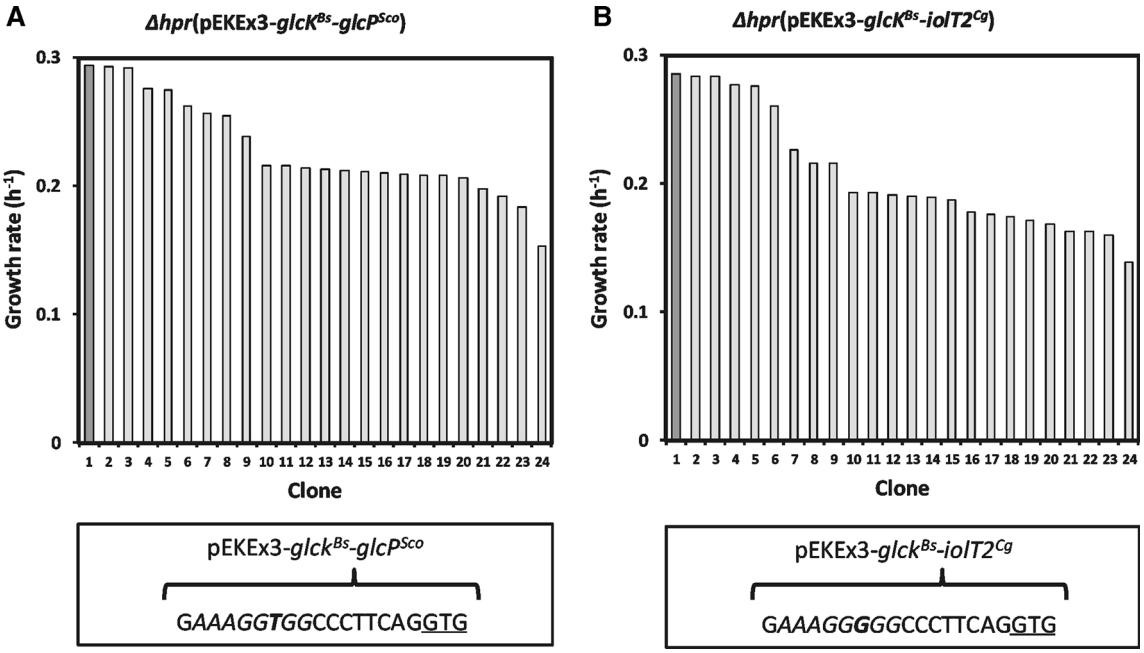

Fig. 4 Influence of varied ribosome binding site and translational start codon sequences of the permease gene $glcP^{Sco}$ (**a**) or $iolT^{Cg}$ (**b**) for expression in combination with glucokinase gene $glcK^{Bs}$ on growth in minimal medium with 2 % (*w*/*v*) glucose. Growth rates of 24 clones with varied $glcP^{Sco}$ (**a**) or $iolT^{Cg}$ (**b**) ribosome binding site and translational start codon sequences expressed in strain *Δhpr* in combination with $glcK^{Bs}$ are indicated. The translational start codon (*underlined*) and ribosome binding site (in *italics*) sequences for the fastest growing clones (highlighted in *dark gray*) are depicted, and differences to the consensus ribosome binding site are given in *bold*. The respective plasmids were named pEKEx3-ScoBest and pEKEx3-IolTBest, respectively

ScoBest) grew faster in glucose minimal medium and showed improved glucose consumption rates as compared to WT(pVWEx1) (Table 3 (B) and Fig. S1).

Engineering *C. glutamicum* strain GRLys1 for increased L-lysine productivity

The L-lysine-producing strain GRLys1 was transformed with vectors pEKEx3, pEKEx3-ScoBest, and pEKEx3-IolTBest, and growth, $Y_{x/s}$, lactate, and L-lysine production were monitored in 2 % (*w*/*v*) glucose minimal medium. GRLys1(pEKEx3) showed a growth rate of 0.30 ± 0.01 h^{-1}, while GRLys1 (pEKEx3-ScoBest) and GRLys1 (pEKEx3-IolTBest) showed growth rates of 0.34 ± 0.01 and 0.35 ± 0.01 h^{-1}, respectively (Fig. 5). However, the L-lysine titer decreased in comparison to GRLys1(pEKEx3) (21 ± 1 mM) as 20 ± 1 mM L-lysine was produced by GRLys1(pEKEx3-ScoBest) and 18 ± 1 mM by GRLys1(pEKEx3-IolTBest) (Fig. 5). Thus, faster growth reduced L-lysine formation. A similar trend is often observed in L-lysine strain development, i.e., an increase of the L-lysine titer is often accompanied by a reduction of the growth rate.

To test if relieving glycolytic genes from transcriptional repression improved growth rates and L-lysine titers, the repressor gene *sugR* was deleted because deletion of *sugR* in *C. glutamicum* has been shown previously to increase expression of glycolysis genes and glucose utilization (Engels et al. 2008; Toyoda et al. 2008). The resulting strain GRLys1*ΔsugR* was transformed with the vectors pEKEx3-ScoBest, pEKEx3-IolTBest, as well as the empty vector pEKEx3. The absence of *sugR* led to higher titer and volumetric productivities of 30 ± 1 mM and 0.19 ± 0.01 g L^{-1} h^{-1}, respectively, as compared to GRLys1(pEKEx3) (Fig. 5). However, deletion of *sugR* reduced the growth rate to 0.20 ± 0.01 h^{-1} (Fig. 5).

As consequence of overexpression of $glcP^{Sco}$ and $glcK^{Bs}$ in GRLys1*ΔsugR*, the resulting strain GRLys1*ΔsugR*(pEKEx3-ScoBest) grew faster (μ = 0.27 ± 0.01 h^{-1}), but produced less L-lysine (25 ± 1 mM), which resulted in a volumetric productivity (0.19 ± 0.01 g L^{-1} h^{-1}) comparable to the empty vector control GRLys1*ΔsugR*(pEKEx3) (Fig. 5). On the other hand, overexpression of $iolT2^{Cg}$ and $glcK^{Bs}$ in GRLys1*ΔsugR* improved L-lysine titer by 57 % to 33 ± 1 mM and volumetric productivity by 22 % to 0.22 ± 0.01 g L^{-1} h^{-1} as compared to GRLys1(pEKEx3) (Fig. 5). Taken together, *C. glutamicum* GRLys1*ΔsugR*(pEKEx3-IolTBest) showed the fastest L-lysine production of the strains constructed here.

C. glutamicum GRLys1*ΔsugR*(pEKEx3-IolTBest) grew to lower biomass concentrations (3.2 ± 0.1 gCDW L^{-1}) than GRLys1(pEKEx3) (5.3 ± 0.1 gCDW L^{-1}) and produced more lactate (27 ± 2 mM) as by-product than GRLys1(pEKEx3) (4 ± 1 mM). Increased lactate formation is due to deletion of *sugR* and was also observed with *C. glutamicum* GRLys1*ΔsugR*(pEKEx3) that accumulated 22 ± 1 mM lactate. Since L-lactate is produced from pyruvate by NAD-dependent

Table 3 Growth parameters of *C. glutamicum* strains (A) WT(pEKEx3), WT(pEKEx3-ScoBest), and WT(pEKEx3-IolTbest) and (B) WT(pVWEx1), WT(pVWEx1-ScoBest) and WT(pVWEx1-IolTbest) in minimal medium with 2 % (*w*/*v*) of glucose

A	WT(pEKEx3)	WT(pEKEx3-ScoBest)	WT(pEKEx3-IolTbest)
Growth rate (h^{-1})	0.32 ± 0.01	0.35 ± 0.01	0.37 ± 0.01
Yx/s (g g^{-1})	0.46 ± 0.01	0.44 ± 0.02	0.41 ± 0.01
Qs (g g^{-1} h^{-1})	0.70 ± 0.02	0.79 ± 0.03	0.90 ± 0.02
L-lactate (mM)	6 ± 1	8 ± 1	9 ± 1
B	WT(pVWEx1)	WT(pVWEx1-ScoBest)	WT(pVWEx1-IolTbest)
Growth rate (h^{-1})	0.37 ± 0.00	0.40 ± 0.00	0.41 ± 0.01
Yx/s (g g^{-1})	0.45 ± 0.02	0.44 ± 0.01	0.43 ± 0.01
Qs (g g^{-1} h^{-1})	0.82 ± 0.03	0.91 ± 0.02	0.95 ± 0.02
L-lactate (mM)	6 ± 1	7 ± 1	8 ± 1

Means and standard deviations or triplicates are listed

L-lactate dehydrogenase LdhA (Toyoda et al. 2009b), *ldhA* was deleted in strain GRLys1*ΔsugR*(pEKEx3-IolTBest). The resulting strain GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest) did not accumulate L-lactate but produced L-lysine with a lower titer (31 ± 1 mM) and a lower volumetric productivity (0.21 ± 0.01 mM g L^{-1} h^{-1}) than the parental LdhA-positive strain (Fig. 5). Since GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest) and GRLys1*ΔsugR*(pEKEx3-IolTBest) grew to biomass concentrations of 3.2 ± 0.1 and 3.7 ± 0.1 gCDW L^{-1}, respectively, deletion of *ldhA* improved biomass formation rather than L-lysine production. To exclude vector-specific effects, pVWEx1-based vectors were also tested (Fig. S1). *C. glutamicum* GRLys1(pVWEx1) showed comparable growth rate, biomass yield, L-lysine titer, volumetric and specific productivity as GRLys1(pEKEx3) (Fig. 5). Moreover, the production parameters obtained with strains GRLys1*ΔsugR*(pVWEx1-IolTBest) and GRLys1*ΔsugRΔldhA*(pVWEx1-IolTBest), respectively, were comparable to those of GRLys1*ΔsugR*(pEKEx3-IolTBest) and GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest) (Fig. 5).

Engineering *C. glutamicum* for production of L-PA

In order to test how accelerated L-lysine production can be translated into production of a L-lysine-derived value-added chemical, strains overproducing the non-proteinogenic amino acid L-PA, a precursor of immunosuppressants, peptide antibiotics, or piperidine alkaloids, were constructed. L-PA is not synthesized by *C. glutamicum* which is unable to catabolize L-lysine (Vrljic et al. 1996). However, L-PA was rapidly accumulated from the growth medium by corynebacteria but did not accumulate under hyperosmolar conditions (Frings et al. 1993). To determine the response of *C. glutamicum* to L-PA, the effect of extracellularly added L-PA on growth and global gene expression was analyzed. Growth of *C. glutamicum* WT in 4 % (*w*/*v*) glucose minimal medium was hardly affected by addition of L-PA, and an inhibition constant (K_i) of about 3.5 M was extrapolated (Fig. 6a). The gene expression response to 200 mM L-PA added to the medium (Table 4) revealed increased expression of genes of carbon metabolism such as *rpe* (ribulose-5-phosphate-3-epimerase), *pck* (phosphoenolpyruvate carboxykinase), *vanR* (repressor of the vanillate utilization operon), and *cdaS* (cyclomaltodextrinase). On the other hand, *malP* coding for maltodextrin phosphorylase and *lldD* encoding quinone-dependent L-lactate dehydrogenases for L-lactate utilization (Stansen et al. 2005) showed decreased expression. The gene cg0569 encoding a cation transporting P-type ATPase, one of the 50 most abundant membrane proteins in *C. glutamicum* (Burkovski 2008), was highly expressed in the presence of L-PA. Neither the gene expression response nor the growth response to L-PA indicated that product inhibition may limit L-PA production by *C. glutamicum.*

A synthetic pathway for conversion of L-lysine to L-PA was employed (Fig. 1). L-Lysine can be oxidatively deaminated by L-lysine 6-dehydrogenase to yield α-aminoadipic semialdehyde, which after spontaneous cyclization to 1-piperidine 6-carboxylic acid can be reduced by pyrroline 5-carboxylate reductase to L-PA. While *C. glutamicum* possesses pyrroline 5-carboxylate reductase ProC (Ankri et al. 1996), corynebacterial genomes lack genes encoding L-lysine 6-dehydrogenase (Neshich et al. 2013). Accordingly, a vector for overexpression of endogenous *proC* and for expression of heterologous L-lysine 6-dehydrogenase gene *lysDH* from *S. pomeroyi* under the control of the constitutive *C. glutamicum* promoter pTuf was constructed and used to transform GRLys1(pEKEx3), GRLys1*ΔsugR*(pEKEx3-IolTBest), and GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest). While the empty vector carrying control strain GRLys1(pEKEx3)(pSH1) produced L-lysine but not L-PA in 2 % (*w*/*v*) glucose minimal medium, the strains carrying pSH1-*lysDH-proC* accumulated L-PA besides L-lysine after 48 h (Fig. 6b).

Notably, the four strains accumulated L-lysine to comparable concentrations (about 20 mM), and L-PA was produced in addition to L-lysine by strains GRLys1(pEKEx3)(pSH1-

lysDH-proC), GRLys1*ΔsugR*(pEKEx3-IolTBest)(pSH1-*lysDH-proC*) and GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest)(pSH1-*lysDH-proC*) (Fig. 6b). This result may indicate that conversion of L-lysine to L-PA lowers the intracellular L-lysine concentration below a threshold relevant for genetic or allosteric regulation by L-lysine, as a similar metabolic pull effect was observed for the production of cadaverine employing L-lysine decarboxylase in *Bacillus methanolicus* (Naerdal et al. 2015). Indeed, the presence of pSH1-*lysDH-proC* in *C. glutamicum* WT(pEKEx3) reduced the growth rate from 0.32 ± 0.01 h^{-1} observed for WT(pEKEx3)(pSH1) to 0.17 ± 0.01 h^{-1} observed for WT(pSH1-*lysDH-proC*)(pEKEx3) (data not shown). While under these conditions L-PA was not produced, the availability of L-lysine for growth may have been perturbed.

The L-PA product titers achieved with GRLys1(pEKEx3)(pSH1-*lysDH-proC*), GRLys1*ΔsugR*(pEKEx3-IolTBest)(pSH1-*lysDH-proC*), and GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest)(pSH1-*lysDH-proC*), respectively, were 8 ± 1, 11 ± 1, and 14 ± 1 mM, respectively. Thus, while L-lysine could not be converted to L-PA completely, it is possible to produce L-PA in the grams per liter range using recombinant *C. glutamicum*.

Discussion

L-Lysine productivity of *C. glutamicum* on glucose media was improved here (a) by overexpressing genes for uptake and phosphorylation of glucose in addition to the endogenous PTS, (b) by relieving glycolysis genes from SugR-mediated repression, and (c) avoiding formation of L-lactate as by-product through disruption of *ldhA*. Subsequently, L-PA production was established.

In nature, *C. glutamicum* rarely encounters the high glucose concentrations prevalent in the early phases of biotechnological batch and fed-batch fermentations. While a high-affinity system for glucose utilization such as the PTS is favorable under most natural conditions, its capacity may limit glucose utilization under conditions of industrial L-lysine production. In line with this notion, replacing the endogenous PTS under low glucose conditions was achieved with the high-affinity glucose permease of *S. coelicolor* A3(2) ($GlcP^{Sco}$), which constitutes the major glucose uptake system of this organism (van Wezel et al. 2005). Intracellular glucose was phosphorylated by GlcK from *B. subtilis*, a high-affinity glucokinase with a Km for glucose of 0.24 mM (Mesak et al. 2004). Coupling of *iolT2* overexpression with overexpression

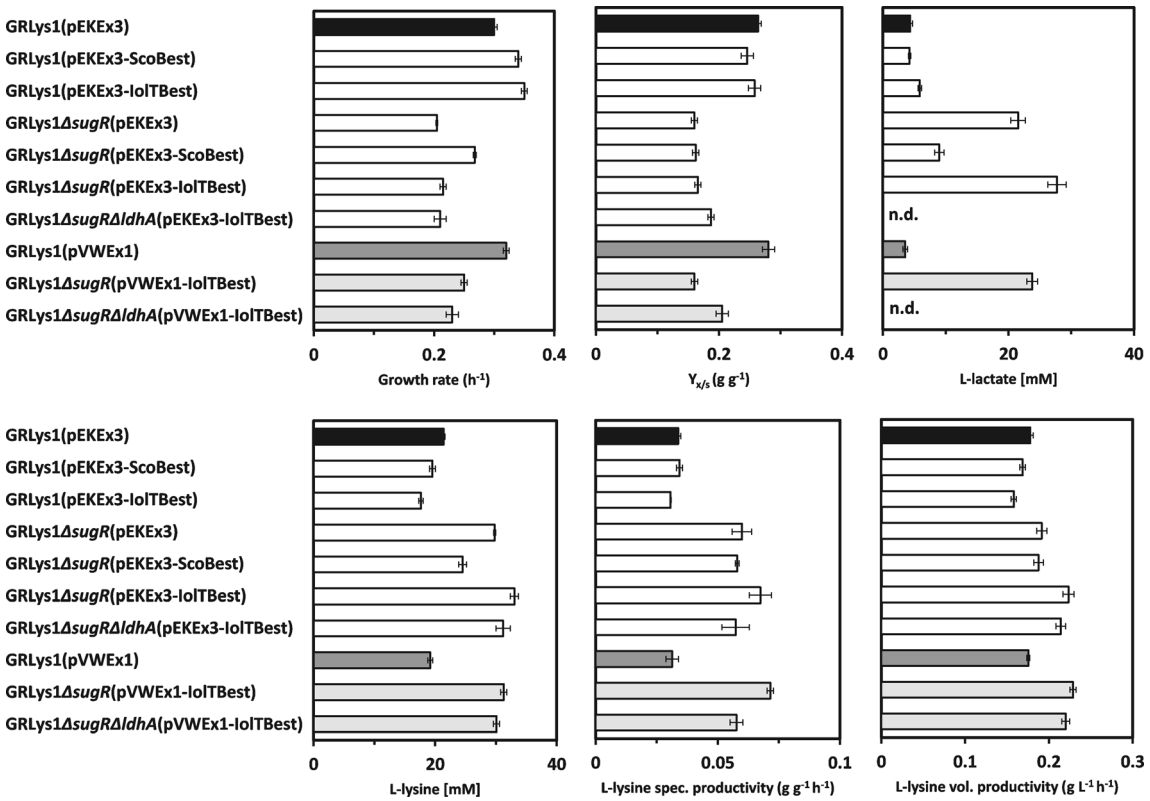

Fig. 5 Growth and L-lysine production of various strains derived from *C. glutamicum* GRLys1. Growth rates, biomass yields ($Y_{x/s}$), L-lactate titers, L-lysine titers, L-lysine volumetric productivities, and L-lysine-specific productivities are given as means and standard deviations of three replicates. The parameters of the control strain GRLys1(pEKEx3) carrying empty vector pEKEx3 are highlighted in *black*. The parameters of the control strain GRLys1(pVWEx1) carrying empty vector pVWEx1 are highlighted in *dark gray*. Parameters obtained for the strains that carry pEKEx3-ScoBest or pEKEx3-IolTBest are shown in *white*, while data for strains that carry pVWEx1-ScoBest or pEKEx3-IolTBest are shown in *light gray*. *nd* not detected

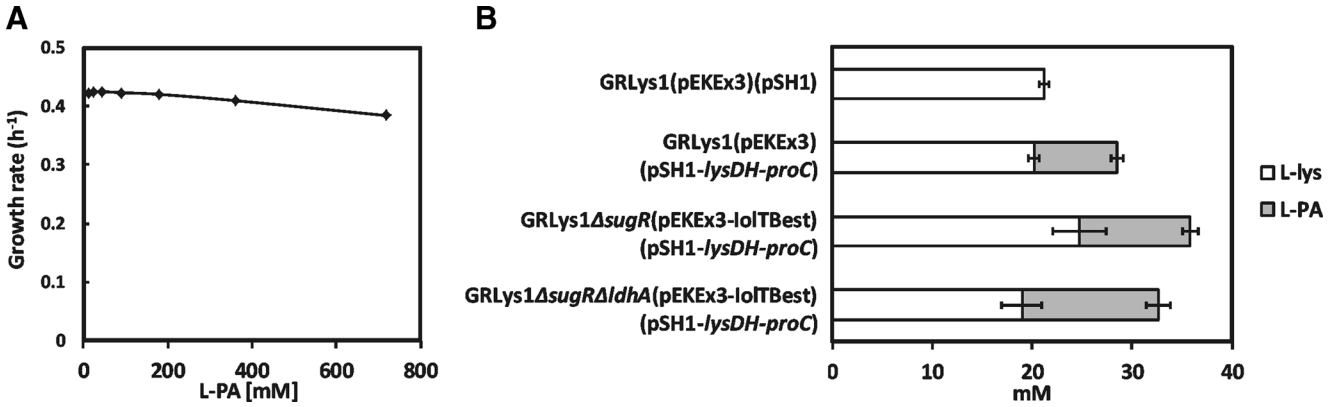

Fig. 6 Influence of L-PA on growth of *C. glutamicum* WT (**a**) and L-lysine and L-PA titers produced by various strains derived from *C. glutamicum* GRLys1 (**b**). **a** L-PA was added to the medium before inoculation. **b** Means and standard deviations of three replicates are given

of endogenous *glk* or *ppgK* was less efficient (Fig. 3), which may be explained by lower affinities of the endogenous glucokinases and commensurates with earlier findings that e.g., deletion of *ppgK* only entails a growth disadvantage at high glucose concentrations (Lindner et al. 2010).

At 4 % (*w*/*v*) glucose, however, overexpression of the low affinity permease IolT2 in combination with overexpression of *glcK* from *B. subtilis* proved beneficial as a 10 % higher growth rate than the control strain *C. glutamicum* WT(pEKEx3) was reached (Fig. 3). This was accompanied by a higher specific glucose utilization rate (Qs), lower final biomass concentrations, and higher titers of L-lactate as by-product (Table 3). The problem of L-lactate formation was enhanced when *sugR* was deleted. This effect may be called overflow metabolism and is often observed when glycolysis capacity exceeds other parts of metabolism, in *E. coli* e.g., the TCA cycle (Veit et al. 2007) or the respiratory chain (Vemuri et al. 2006). This limitation may be due to transcriptional

Table 4 Genes differentially expressed in *C. glutamicum* WT grown in glucose minimal medium in the presence of 200 mM L-PA as compared to sodium chloride

Gene ID[ab]	Gene name[b]	Gene description[b]	Ratio of mRNA levels (L-PA/NaCl)[c]
cg0569	–	Cation transporting P-type ATPase	8.0
cg0684	*papA*	Prolyl aminopeptidase A	3.5
cg1012	*cdaS*	Cyclomaltodextrinase	4.1
cg1740	–	Putative nucleoside-diphosphate-sugar epimerase	2.4
cg1801	*rpe*	Ribulose-5-phosphate-3-epimerase	2.4
cg2615	*vanR*	Repressor of *vanABK* operon	3.5
cg3169	*pck*	Phosphoenolpyruvate carboxykinase	3.6
cg3272	–	Putative membrane protein	3.9
cg0136	–	Putative membrane protein	0.2
cg0834	–	Extracellular solute-binding protein	0.4
cg0844	–	Type II restriction enzyme, methylase subunit	0.2
cg1227	–	Putative membrane protein	0.4
cg1332	–	Putative secreted hydrolase	0.2
cg1479	*malP*	Maltodextrin phosphorylase	0.4
cg1761	*sufS*	Cysteine desulfhydrase	0.3
cg1811	*ihf*	Probable integration host factor cIHF	0.3
cg1825	*efp*	Translational elongation factor P	0.3
cg3011	*groEL*	Chaperonin GroEL	0.2
cg3227	*lldD*	Quinone-dependent L-lactate dehydrogenase	0.4
cg3254	–	Putative membrane protein	0.4

[a] Genes showing increased or reduced RNA levels are sorted according to their identifiers

[b] Gene ID, name and description are according to BX927147 and www.coryneregnet.de

[c] Differential gene expression. Values listed were selected for $P < 0.05$ and at least twofold mRNA level

control by global regulators such as ArcA (Perrenoud and Sauer 2005). Overexpression of genes encoding glycolytic enzymes as applied in *E. coli* (Xie et al. 2014; Seol et al. 2015), *B. subtilis* (Liu et al. 2014), or *S. coelicolor* (Borodina et al. 2008) was also used for *C. glutamicum*, e.g., to improve the production of high value compounds like amino acids (Yamamoto et al. 2012; Reddy and Wendisch 2014), alcohols (Jojima et al. 2015; Yamamoto et al. 2013), or the organic acid D-lactate (Tsuge et al. 2015). Under these conditions or when a repressor gene of glycolysis genes was deleted, i.e., that of the DeoR-type transcriptional regulator SugR, high titers of L-lactate were formed as side effect of faster glucose utilization (Engels and Wendisch 2007; Engels et al. 2008; Teramoto et al. 2011). Likewise, *sugR* deletion accelerated L-valine production, but at the expense of increased L-lactate formation (Blombach et al. 2009a). Similarly, deletion of *sugR* in the L-lysine-producing strain GRLys1 increased L-lactate formation (22 ± 1 mM as compared to 4 ± 1 mM; Fig. 5). Consequently, the fermentative L-lactate dehydrogenase gene *ldhA* was deleted in GRLys1Δ*sugR*(pEKEx3-IolTBest) and L-lactate formation was abolished. This entailed more biomass formation rather than more L-lysine production since the final cell dry weight increased by 15 % while the L-lysine titer was slightly reduced. However, the volumetric L-lysine productivity remained almost unchanged (Fig. 5). Taken together, GRLys1Δ*sugR*Δ*ldhA*(pEKEx3-IolTBest) and GRLys1Δ*sugR*Δ*ldhA*(pVWEx1-IolTBest) are suitable strains for fast production of L-lysine and L-lysine derivatives from glucose without formation of L-lactate as by-product.

L-PA production from glucose using recombinant *C. glutamicum* strains was established here (Fig. 6). Chemical methods to obtain optically pure L-PA (and derivatives thereof) have been described and include diazotation of L-lysine or electrochemical oxidation of protected L-lysine, but also conversion of protected L-aspartic acid or L-glutamic acid derivatives to L-PA molecules substituted at the 4- or 5-position (Couty 1999). However, these reaction sequences are often long, e.g., 11 steps from L-glutamic acid to 4-oxopiperidine, and show low yields even for the one-step conversion of L-lysine to L-PA using disodium nitrosylpentacyanoferrate(II) as oxidant (Couty 1999). L-PA can be generated from racemic pipecolic acid via resolution by fractional crystallization of diastereomeric salts or via dynamic kinetic resolution by stereoselective amidases (Eichhorn et al. 1997), but it is difficult to achieve 100 % enantiomeric excess. In nature, L-PA occurs in L-lysine-catabolizing bacteria, e.g., in *S. pomeroyi*, and its biosynthesis is known to be induced in a L-lysine-dependent manner under high salt conditions (Neshich et al. 2013). L-PA accumulated intracellularly (to about 0.6 μg mg^{-1} protein) when *E. coli* BL21 expressing the L-lysine 6-dehydrogenase gene from *S. pomeroyi* was grown in L-lysine-containing medium (Neshich et al. 2013). Biotransformation of L-lysine to L-PA has been developed using *E. coli* overproducing LAT (about 3.9 g L^{-1} of optically pure L-PA (Fujii et al. 2002). Additional overexpression of the *E. coli* gene for lysine uptake, *lysP*, accelerated L-PA formation from L-lysine about five fold (Fujii et al. 2002). Biotransformation of racemic DL-lysine with *E. coli* overproducing L-lysine α-oxidase from *S. japonicus* yielded high titers (about 45 g L^{-1}) of optically pure L-PA (Tani et al. 2015). The functionality of L-lysine cyclodeaminase from *Actinoplanes friuliensis* has been shown in *C. glutamicum* (Wagner et al. 2010), and the enzyme from *Streptomyces. pristinaespiralis* has been used in biotransformations of L-lysine to L-PA with recombinant *E. coli* (Tsotsou and Barbirato 2007). However, since L-lysine cyclodeaminases show low turnover numbers (about 0.6 s^{-1}), only 4.5 mM L-PA was produced per hour using 1 g L^{-1} pure enzyme (Tsotsou and Barbirato 2007). All biotransformations with isolated enzymes or whole cells use L- or DL-lysine as substrate for conversion to L-PA, while to the best of our knowledge, a fermentative route for production of L-PA from glucose is presented here for the first time.

L-PA production from glucose proceeded with a yield of 0.09 ± 0.01 g g^{-1}, and a volumetric productivity of 0.04 ± 0.01 g L^{-1} h^{-1} by the recombinant strains developed here (Fig. 6). Metabolic engineering often targets transport of products and/or by-products, which pertains to export of L-PA, re-uptake of L-PA, and export of L-lysine here. L-PA has been described to rapidly accumulate from the growth medium by corynebacteria; however, L-PA did not accumulate under hyperosmolar conditions (Frings et al. 1993). Avoiding L-PA re-uptake may not be of priority for L-PA production by *C. glutamicum* since the L-PA concentration did neither diminish once it was produced nor when added extracellularly (data not shown). On the other hand, improving L-PA export may be relevant to increase volumetric productivity since, for instance, lysine producing strains such as DM1933 and GRLys1 contain multiple copies of the lysine export gene *lysE* (Unthan et al. 2015). It is tempting to speculate that L-PA is exported by L-proline export systems since both heterocyclic secondary amino acids only differ in ring size. Genes and proteins for L-proline import are known (the L-proline-specific uptake system PutP, the proline/ectoine uptake system ProP, and the ectoine/proline/glycine betaine carrier EctP (Peter et al. 1998), but L-proline export has only been characterized biochemically. Osmoregulated channel(s) are responsible for efflux of various solutes upon sudden decrease in osmotic pressure and were shown to preferentially mediate efflux of the compatible solutes glycine betaine and L-proline (Ruffert et al. 1997). L-Proline and glycine betaine export occurred even in the absence of the genes *mscL* and *yggB*, which encode mechanosensitive channels (Nottebrock et al. 2003). YggB functions as major L-glutamate export system, its C-terminal domain is important for gating hysteresis in a voltage-dependent manner (Nakayama et al. 2016), and

certain amino acid exchanges in YggB proved beneficial for L-glutamate export (Nakamura et al. 2007). By contrast, a genetic strategy to improve L-proline export is not evident as long as proteins/genes important for L-proline export remain unknown.

Full conversion of L-lysine to L-PA was not observed. Since conversion of L-lysine to L-PA occurs intracellularly, L-PA production suffers from L-lysine export by GRLys1 that overexpresses *lysE* from two chromosomal copies. Export of L-lysine is well studied and is due to a single protein, LysE (Vrljic et al. 1996). Thus, deletion of *lysE* in the L-PA-producing strain may be of advantage for L-PA production. The sum of L-lysine production and L-PA production was higher than production of L-lysine alone, which may be explained by a metabolic pull effect. Export of L-lysine and conversion of L-lysine to L-PA may lower the intracellular L-lysine concentration below a threshold relevant for negative genetic or allosteric regulation by L-lysine. A similar metabolic pull effect was observed for the production of cadaverine from L-lysine employing L-lysine decarboxylase in methylotrophic *B. methanolicus* (Naerdal et al. 2015). On the other hand, strains with increasing L-lysine productivity, i.e., GRLys1(pEKEx3), GRLys1*ΔsugR*(pEKEx3-IolTBest), and GRLys1*ΔsugRΔldhA*(pEKEx3-IolTBest), showed increasing L-PA production after transformation with pSH1-*lysDH-proC* (8 ± 1 mM, 11 ± 1 mM, and 14 ± 1 mM, Fig. 6), thus, supply of L-lysine as precursor positively influenced L-PA production. It can be expected that transformation of industrial L-lysine-producing strains with pSH1-*lysDH-proC* is a viable strategy to reach higher L-PA titers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ehical approval The research performed did not involve human participants and/or animals.

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