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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Bacillus methanolicus pyruvate carboxylase and homoserine dehydrogenase I and II and their roles for L-lysine production from methanol at 50°C

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Abstract We here present the *pyc* gene encoding pyruvate carboxylase (PC), and the *hom-1* and *hom-2* genes encoding two active homoserine dehydrogenase (HD) proteins, in methylotrophic *Bacillus methanolicus* MGA3. In general, both PC and HD are regarded as key targets for improving bacterial L-lysine production; PC plays a role in precursor oxaloacetate (OAA) supply while HD controls an important branch point in the L-lysine biosynthetic pathway. The *hom-1* and *hom-2* genes were strongly repressed by L-threonine and L-methionine, respectively. Wild-type MGA3 cells secreted 0.4 g/l L-lysine and 59 g/l L-glutamate under optimised fed batch methanol fermentation. The *hom-1* mutant M168-20 constructed herein secreted 11 g/l L-lysine and 69 g/l of L-glutamate, while a sixfold higher L-lysine overproduction (65 g/l) of the previously constructed classical *B. methanolicus* mutant NOA2#13A52-8A66 was accompanied with reduced L-glutamate production (28 g/l) and threefold elevated *pyc* transcription level. Overproduction of PC and its mutant enzyme P455S in M168-20 had no positive effect on the volumetric L-lysine yield and the L-lysine yield on methanol, and caused significantly reduced volumetric L-glutamate yield and L-glutamate yield on methanol. Our results demonstrated that *hom-1* represents one key target for achieving L-lysine overproduction, PC activity plays an important role in controlling L-glutamate production from methanol, and that OAA precursor supply is not a major bottleneck for L-lysine overproduction by *B. methanolicus*.

Keywords Thermotolerant · Methylotrophy · Quantitative PCR · Anaplerotic · Fed batch fermentation · Metabolic engineering

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Introduction

The amino acids L-lysine and L-glutamate have numerous industrial applications and the current worldwide total production of these compounds is above 2 million tonnes per year (Pfefferle et al. 2003; Leuchtenberger et al. 2005; Sanchez and Demain 2008). The majority of the commercial L-lysine and L-glutamate production is gained by fermentations of Corynebacteria using sugar-based raw materials. Due to the large production scale of these amino acids, the price, abundance, and purity of the raw material is of great economical and ecological importance, and the one-carbon (C_1) compound methanol represents an attractive alternative to sugar (Schrader et al. 2009). Methylotrophs can utilize C_1 compounds for growth and energy generation (Anthony 1991), and the potential of the thermotolerant and methylotrophic *Bacillus methanolicus* for overproduction of L-lysine and L-glutamate from methanol has been reviewed by us (Brautaset et al. 2007).

L-glutamate is a side-product of the TCA cycle and L-lysine belongs to the aspartate family of amino acids, and thus L-glutamate and L-lysine are products of competing biochemical pathways from the common precursor metabolite oxaloacetate (OAA). For development of efficient L-lysine overproducers, it will be important to diminish wasteful L-glutamate production (Brautaset et al. 2003). Pyruvate carboxylase (PC) catalyses the anaplerotic carboxylation of pyruvate to form OAA (Fig. 1). Homoserine dehydrogenase (HD), on the other hand, controls a branch point in the L-lysine biosynthetic pathway, leading to synthesis of L-methionine and L-threonine (Fig. 1). Both PC and HD are regarded as key targets for improving bacterial L-lysine production (see below).

Only few bacteria use PC as the sole anaplerotic enzyme, while many bacteria use phosphoenol pyruvate carboxylase (PEPC) for this purpose. Some bacteria, like the important

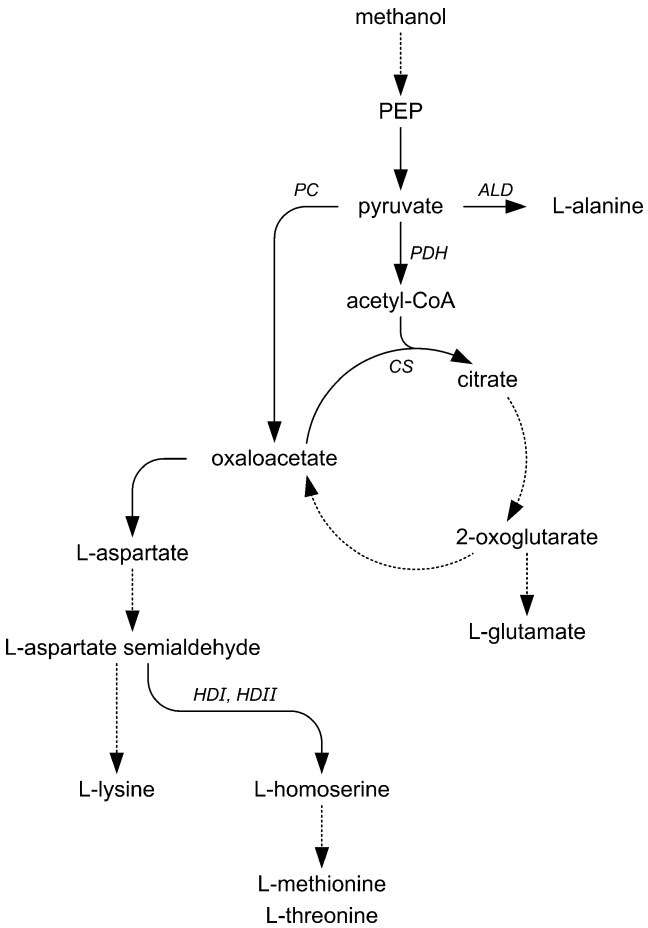

Fig. 1 Proposed biochemical routes for *B. methanolicus*. From pyruvate the carbon flow is split between entering the TCA cycle, conversion into L-alanine, or conversion into aspartate and its related amino acids. *Dotted lines* indicate multiple reaction steps. *PEP* phosphoenolpyruvate, *PC* pyruvate carboxylase, *ALD* alanine dehydrogenase, *PDH* pyruvate dehydrogenase, *CS* citrate synthase, *HD* homoserine dehydrogenase

L-lysine and L-glutamate producer *Corynebacterium glutamicum*, possess both PC and PEPC (Ikeda and Nakagawa 2003), while *Bacillus subtilis* and *Bacillus stearothermophilus* only have PC (Kondo et al. 1997). By ^{13}C-NMR and metabolite profiling analyses, it has been shown that PC is the major C_3-carboxylating anaplerotic enzyme in *C. glutamicum* (Koffas et al. 1998; Koffas et al. 2003; Sahm et al. 2000; Shirai et al. 2007; Hasegawa et al. 2008). In *B. methanolicus*, PC activity and no PEPC activity was detected in crude extracts in vitro (Brautaset et al. 2003), suggesting that this bacterium is similar to *B. subtilis* and *B. stearothermophilus* at this point. In general, *Bacillus* species possess one single HD gene. *Lactobacillus plantarum* has been reported to possess two HD genes, transcriptionally repressed by methionine and threonine. One of the HD proteins also has a low aspartokinase (AK) activity (Cahyanto et al. 2006).

It is well documented that mutations reducing or abolishing HD activity can cause L-lysine overproduction in *C. glutamicum* and other bacteria (Ohnishi et al. 2002; Ikeda et al. 2006; Sanches and Demain 2008). PC overproduction in a wild-type *C. glutamicum* genetic background in shake flasks resulted in reduced specific L-lysine productivity, sevenfold increased volumetric L-glutamate yield, and enhanced cell growth rate (Peters-Wendisch et al. 2001; Koffas et al. 2003). PC overproduction in an S-(2-aminoethyl)cysteine (AEC)-resistant L-lysine overproducing *C. glutamicum* genetic background resulted in 50% increased volumetric L-lysine yield and a sevenfold increase in volumetric L-glutamate yield under such conditions (Peters-Wendisch et al. 2001). Deletion of *pyc* caused significantly reduced L-glutamate production in wild-type *C. glutamicum* (Yao et al. 2009). A chromosomal point mutation, P458S, was identified in the *pyc* gene of an L-lysine overproducing classical *C. glutamicum* mutant. Chromosomal reintroduction of this *pyc* mutation in a wild-type genetic background had no positive effect on L-lysine production, while this mutation was beneficial for L-lysine production when introduced into an HD-deficient L-lysine overproducing *C. glutamicum* host (Georgi et al. 2005). When introduced into an HD plus AK mutant of *C. glutamicum*, a higher L-lysine production rate (3 g/l/h) and a slightly improved volumetric L-lysine yield (from 75 g/l to 80 g/l) was reported in high-cell-density cultivations (HCDC) (Ohnishi et al. 2002; Ikeda et al. 2006). Thus, PC is a key target for L-lysine overproduction by *C. glutamicum* mutants that have been deregulated in the L-lysine biosynthetic pathway.

By multiple cycles of classical mutagenesis and selection the AEC-resistant and auxotrophic L-lysine overproducing *B. methanolicus*, mutant NOA2#13A52-8A66 has been constructed (Lee et al. 1996; Hanson et al. 1996). We recently reported the first example of improved L-lysine production by metabolic engineering of *B. methanolicus*

(Jakobsen et al. 2009). In the present, report we clone three *B. methanolicus* genes; *pyc* encoding PC, and *hom-1* and *hom-2* both encoding HD proteins (Fig. 1); and the roles of these genes for L-lysine overproduction from methanol were investigated. We show that *hom-1* represents one major target for AEC resistance and concomitant L-lysine overproduction in this organism, while *hom-2* presumably plays a less important role. Our data showed that PC overproduction can affect L-lysine on L-glutamate production ratio from methanol. However, we were not able to demonstrate increased L-lysine production by PC manipulations in a *hom-1* mutant *B. methanolicus* host, which is different from results reported from analogous experiments in *C. glutamicum* (see above).

Materials and methods

Biological strains, transformation and growth conditions

Bacterial strains, plasmids, and phages used in this study are listed in Table 1. *Escherichia coli* DH5α was used as a standard cloning host and recombinant cells were grown at 37 °C in liquid or solid LB medium supplemented with ampicillin (100 μg/ml) or chloramphenicol (15 μg/ml) when appropriate. Transformation of *B. methanolicus* strains was performed by electroporation as described previously (Jakobsen et al. 2006), and recombinant strains were selected on solid regeneration plates supplemented with chloramphenicol (5 μg/ml). For small-scale amino acid production in liquid cultures, *B. methanolicus* cells were grown exponentially at 50 °C in $MeOH_{200}$ medium (100 ml) (Jakobsen et al. 2006) in baffled 500 ml shake flasks. Samples for amino acid measurements were collected during exponential and stationary growth phases.

Construction of PC expression vectors pHP13mp-pyc and pHP13mp-pycm, and HD expression vectors pTH1mp-hom1 and pTH1mp-hom2

pHP13mp-pyc 3.6 kb A DNA fragment was polymerase chain reaction (PCR)-amplified from MGA3 total DNA by using the primers pyc-Fa2: 5′-ttttacagtgAAAAGAAG CATTAACAAGGTTTTAGTAGC-3′ and pyc-fb: 5′-ACGACGTTGTAAAACGACGGCC-3′. The PCR product

Table 1 Bacterial strains, phages, and plasmids

Biological material	Description	Reference
B. methanolicus strains		
MGA3	Wild-type strain, ATCC 53907	Schendel et al. (1990)
NOA2#13A52-8A66	L-lysine overproducing NOA2 mutant	Hanson et al. (1996)
M168-20	1st generation AEC-resistant mutant of MGA3; L-lysine overproducer	This study
E. coli strains		
DH5α	General cloning host	BRL
XL-1 Blue MRA(P2)	Host for maintaining the gene library	Stratagene
Gif 102	Homoserine dehydrogenase mutant	Thèze et al. (1974)
Phages		
λ−DASH II	λ-vector	Stratagene
λ-PYC2	Recombinant λ-phage including the MGA3 *pyc* gene	This study
Plasmids		
pGEM-11zf	General cloning vector	New England Biolabs
pLITMUS28	General cloning vector	New England Biolabs
pGEM-T	General cloning vector	New England Biolabs
pPYC-1	960 bp PCR fragment from λ-PYC2 cloned into pGEM11-zf	This study
pHP13	*E. coli-B. subtilis* shuttle vector, Clm^r	Haima et al. (1989)
pHP13mp-lysC	pHP13 carrying *lysC* coding region under control of the *mdh* promoter	Jakobsen et al. (2009)
pHP13mp-pyc	pHP13 carrying *pyc* coding region under control of the *mdh* promoter	This study
pHP13mp-pycm	Similar as pHP13mp-pyc but with a P455S mutation in *pyc* coding sequence	This study
pTH1mp-lysC	Similar as pHP13mp-lysC but with *Pci*I site upstream *mdh* promoter removed	This study
pTH1mp-hom1	pHP13 carrying *hom-1* coding region under control of the *mdh* promoter	This study
pTH1mp-hom2	pHP13 carrying *hom-2* coding region under control of the *mdh* promoter	This study

Ap^r ampicillin resistance, *Clm^r* chloramphenicol resistance

was end-digested with *Pci*I (underlined in the primer) and *Sal*I, yielding fragment A. The pHP13 vector was digested with *Sal*I-*Pst*I; fragment B. The plasmid pHP13mp-lysC (Table 1) was digested with *Pst*I-*Pci*I and the 1.1 kb region including the *mdh* promoter region was isolated; fragment C. Fragments A, B, and C were ligated together, yielding plasmid pHP13mp-pyc. The latter vector has the *pyc* coding region cloned downstream of the *mdh* promoter region and in-frame with the *mdh* ribosome binding site (*rbs*).

pHP13mp-pycm A 1,412 bp fragment was PCR amplified from pHP13mp-pyc by using the primers P1_pyc_for: 5′-tttttctgcagGAAAGCAAGGACACAGGCACAGC-3′ and P2_pyc_rev: 5′-tttttgagctcCCTCTTTTAAGAACCGA TAGGCGAC-3′. The resulting DNA fragment was digested with *Pst*I and *Sac*I, respectively (restriction sites underlined in the primers), and ligated into the corresponding sites of pLITMUS28. The resulting construct was used as a template for QuickChange site-directed mutagenesis (Stratagene) in accordance with the manufacturer's instructions, and by using the following mutagenic oligonucleotide pair: P455S-1: 5'-TTTATCGA TACAACA**TCA**GAACTTTTCTTGTTCC-3' (sense) and P455S-2: 5'-GAACAAGAAAAGTTC**TGA**TGTTG TATCGATAAA-3' (antisense).

Specific mutations introduced by using the mutated nucleotides are given in bold. Correctly mutated plasmid was identified by restriction analyses, and the entire insert was verified by DNA sequencing. From this plasmid, the 1,367 bp *Aat*II/*Nhe*I fragment (including the mutated region) was excised and cloned into the corresponding sites of the parental plasmid pHP13mp-pyc, yielding plasmid pHP13mp-pycm. The latter plasmid is identical with the parental plasmid pHP13mp-pyc, but harbours mutations corresponding to substitution mutation P455S in the cloned *pyc* gene (Table 1).

pTH1mp-lysC Plasmid pHP13 was digested with *Pci*I, and the resulting cohesive ends were blunted by using T4 DNA polymerase, to yield plasmid pTH1. Plasmid pHP13mp-lysC was digested with *Pst*I/*EcoR*I and the 2.6 kb fragment was isolated and ligated into the corresponding sites of pTH1, yielding the cassette cloning an expression vector pTH1mp-lysC. This vector is analogous to the plasmid pHP13mp-lysC (above) and has a unique *Pci*I site for one-step cloning of any coding gene downstream of the strong *mdh* promoter and in-frame with the *mdh* rbs region (Brautaset et al. 2004).

pTH1mp-hom1 and pTH1mp-hom2 DNA fragments with the coding regions for *hom-1* (1,353 bp) and *hom-2* (1,304 bp) were PCR amplified from *B. methanolicus* MGA3 total DNA by using the following primer pairs: hom1-F: 5′-ttttggtctcACATGTGGAAGCAATC CAAGTTGGTTT-3′ and hom1-R: 5′-TTTTTTATTCTTTA TAAGCTTCTAGAAGACC-3′. hom2-F: 5′-tttttggtctc ACATGTGTCAACCATTCA TATTGCACT-3′ and hom2-R: 5′-ttttttctagaTTTTAAAGGGGCTGTCTAGTCA-3′.

The PCR products were ligated into vector pGEM-T. The resulting plasmids were digested with *Eco31*I/*Xba*I and the inserts were individually ligated into the compatible *Pci*I/*Xba*I sites of pTH1mp-lysC (replacing the *lysC* gene), yielding expression plasmids pTH1mp-hom1 and pTH1mp-hom2 (Table 1).

DNA manipulations

Recombinant *E. coli* procedures were performed according to standard methods (Sambrook et al. 1989) and isolation of *B. methanolicus* total DNA was performed as described previously (Brautaset et al. 2004). The DNA fragment used for probe was labelled by using the Digoxigenin (DIG) kit from Roche Diagnostics, and used for the plaque hybridization analysis according to the manufacturer's instructions. Degenerated primers used for the PCR amplification of the *pyc* DNA probe, were as follows: pyc-F4: 5′-ttttctaGA(A/G) ATGTGGGGNG GNGCNACNTT(C/T)G-3′ and pyc-R3: 5′-ttttgcatGC CAT(A/G)TCNCCNACNAC(T/C)-3′. Underlined in the primers are restriction sites (*Xba*I and *Sph*I, respectively) for cloning of the resulting DNA fragment into pGEM11-zf. Primers used for PCR amplification of the complete *pyc* gene from *B. methanolicus* strains for the DNA sequencing were as follows: pycT7: 5′-GGGAGTGTGGG GAGTTAGC-3′ and pycT9: 5′-TCTCCCCGTTTTC GGTCG-3′. DNA sequencing was performed by using the BigDye kit from Applied Biosystems and MWG sequencing service, and sequence analysis was performed by using on-line programmes as described previously (Brautaset et al. 2004).

Construction of a λ-gene library

For construction of a λ-DASH gene library *B. methanolicus* MGA3 total-DNA was isolated as described previously (Brautaset et al. 2004) and partially digested with *Sau3*AI, and the resulting fragments were purified and cloned into the *BamHI* sites of Lambda DASH II vector according to the manufacturer's instructions (Stratagene). The size of the resulting library was experimentally determined to be 1.9×10^6 plaque forming units per milliliter.

Preparation of crude extracts and measurement of PC activity

Frozen ampoules of *B. methanolicus* cells harvested in exponential growth phase were prepared as described previously (Jakobsen et al. 2006). $MeOH_{200}$ medium (100 ml) was inoculated with 0.25 ml thawed cells from frozen ampoules, three replicates, and grown exponentially until OD_{600}=2.5-3.3. Cells were harvested by centrifugation, washed in 4 ml potassium phosphate buffer (50 mM, pH=7.5), and resuspended in 3.0 ml of the same buffer to give OD_{600}=9.0 and kept on ice. Cell lysis was performed by sonication with the conditions described previously (Jakobsen et al. 2006). Cell debris was removed by centrifugation (12,000×*g*, 20 min) and the supernatants were collected as crude extracts. PC assay was performed as follows. The 1 ml reaction mixture contained 25 mM Tris-HCl (pH=8.0), 0.02 mM fresh NADH, 0.5 mM fresh acetyl-CoA, four units of malate dehydrogenase, 80 mM $NaHCO_3$, 10 mM pyruvate, and 5 mM Mg-ATP. The reaction mixture was pre-warmed to 50 °C and the reaction was initiated by adding 50 µl of crude extract. The decrease in absorbance at 340 nm (A_{340}) due to reduction of NADH was monitored spectrophotometrically in quartz cuvette over a 3-min period at 50 °C. Reaction mixture without $NaHCO_3$, pyruvate, and Mg-ATP was used as blank. Background from the crude extract was measured in reaction mixture without pyruvate and acetyl-CoA and background from the reagents was measured by replacing crude extract with water. Total protein was assayed by using BioRad protein assay (BioRad) according to the manufacturer's protocol, with bovine serum albumin as the standard.

Mutagenesis and selection of AEC-resistant mutant M168-20

The L-lysine analogue S-(2-aminoethyl)cysteine can be used to select mutants deregulated in L-lysine feedback regulation and such mutants are useful model strains for targeted genetic manipulations affecting L-lysine production (Lee et al. 1996; Sahm et al. 1996; Peters-Wendisch et al. 2001; Koffas et al. 2002). Mutagenesis of MGA3 and selection of AEC-resistant mutant M168-20 was performed essentially as described previously (Hanson et al. 1996). After mutagenesis, 100 µl of exponentially growing cells were mixed with 3 ml of 0.6% MYtm soft agar, and the suspension was spread over solid MYtm plates. Next, 6-mm paper discs saturated with 4-8 µg of AEC were placed on the lawns prior to incubation at 37 °C, and colonies growing close to the paper discs were isolated and characterized further.

Isolation of total-RNA, cDNA synthesis, and quantitative PCR

These experiments were performed essentially as described previously (Jakobsen et al. 2006). Total RNA was isolated from exponentially (OD_{600}=1.0) growing *B. methanolicus* cells and treated with DNAse I to destroy any contaminating DNA. The DNA-free RNA was then used as template for cDNA synthesis which served as templates for the quantitative PCR experiments, and the primer pairs used were as follows: *pyc* coding region: pyc-RTA, 5′-ACCGATC GATGCCTACCTTG-3′; pyc-RTB, 5′-TAGCTG TGCCTGTGTCCTTG-3′. *orf-1*/*pyc* intergenic region: ylaT-F, 5′-TCGCAATCGGTTCAGGAGGA-3′; ylaT-R, 5′-GCCTATCATGCTCGAGATTC-3′, *hom-1* coding region: hom1-963F, 5′-GCGTCTTGGCGTGAATGG-3′; hom1-1031R, 5′-TCGTCCGGCCCTTTTAACTT-3′; *hom-2* coding region: hom2-859F, 5′-GCAGACATTGTCGGGAATATCA-3′; hom2-922R, 5′-CGCTTGCTGTCGGAAACAT-3′; 16S rRNA coding region: rRNA-1F, 5′-AAGATGGCTTCGGC TATCAC-3′; rRNA-1R, 5′-CGAACGGTACTTG TTCTTCC-3′. The quantitative PCR reactions were run by using the iTaq SYBR Green Supermix with Rox from BioRad and PCR products were detected with the Mx3000P cycler system from Stratagene. Amplification of 16 S rRNA was used for sample normalisation (Jakobsen et al. 2006), and the results of the quantitative PCR are expressed as stimulation indices (ratio of relative amounts of RNA obtained in cells growing under alternative conditions, methanol ($MeOH_{200}$) versus mannitol ($Mann_{10}$) media (Jakobsen et al. 2006), or ratio of relative amounts of RNA obtained in mutant versus wild-type cells growing under similar conditions). All experiments were run in triplicates and the mean values were calculated.

Fed batch fermentations

Fed-batch fermentations were performed in UMN1 medium in Applikon 3-l fermentors with an initial volume of 0.75 l essentially as described previously (Jakobsen et al. 2009). The flow rate of the exhaust gas from the fermentor vessel was measured by a flow metre (Bronkhorst High-Tech El-flow F-111B), and its CO_2-content was measured by a CO_2-analyzer (Fisher-Rosemount Binos 100). These online measurements were continuously logged by a computer and used to calculate the CO_2 evolution rate of the culture. Chloramphenicol (5 µg/ml) was added to the batch growth medium for all recombinant strains. Unless otherwise stated, the methanol concentration in the medium was maintained at a set point of 150 mM by automatic addition of MeOH feed solution (Jakobsen et al. 2009) on methanol demand. Recombinant M168-20 strains were fed a solution of 150 mM DL-methionine at one third of the feed rate of the MeOH feed solution. Mutant NOA2#13A52-8A66 was fed a solution of 200 mM L-threonine, 90 mM L-leucine, and 40 mM DL-methionine at one third of the feed rate of the MeOH feed solution. Inoculum preparation protocol and fermentation

conditions were as described previously (Jakobsen et al. 2009), except that 4% (*v*/*v*) Sigma Antifoam 204 was added to the MeOH feed solution (leading to a total antifoam addition of typically 15 ml in one fermentation trial). All fermentations were run until the CO_2 content of the exhaust gas was close to zero (no cell respiration). Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}). Dry cell weight was calculated using a conversion factor of one OD_{600} unit corresponding to 0.24 g dry cell weight per litre (calculated based on multiple measurements of dry cell weight and OD_{600} during the fermentation trials). Due to the significant increase in culture volume throughout the fermentation, biomass and amino acid concentrations were corrected for the increase in volume and subsequent dilution as described previously (Jakobsen et al. 2009). Samples for determination of volumetric amino acid yields were collected from early exponential phase and into the stationary phase (10-48 h). Product yields on methanol are given on a gram carbon per gram carbon basis and were calculated as the amount of carbon in the product (present in the growth medium and samples removed from the fermentor) divided by the amount of carbon in the consumed methanol (fed to the fermentor). Product yields on methanol were calculated for various time-points throughout the fermentation trial (from time of inoculation to a chosen time), based on interpolation of measured data using a cubic spline function. The carbon content of the biomass was here assumed to be 50%, and biomass carbon was assumed not to become available for new biosynthesis upon cell lysis. In addition to L-lysine and L-glutamate for which individual product yields on methanol were presented, total yield on methanol of L-aspartate, L-methionine, L-threonine, and L-alanine was calculated and was typically 0.03-0.05 g/g on a carbon/carbon basis at the end of the fermentation. Undetected biomass production due to simultaneous cell growth and cell lysis may contribute to the unaccounted carbon in the total mass balance, in addition to other potential products not detected in our analyses.

Analysis of plasmid stability in recombinant *B. methanolicus* cells

For testing of plasmid stability, recombinant *B. methanolicus* cells collected from fermentation trials were diluted appropriately and streaked on regeneration plates (Cue et al. 1997) and incubated at 50 °C for 18-24 h. Single colonies were picked and transferred to fresh regeneration plates with and without chloramphenicol, and the resulting colony numbers on selective versus non-selective plates were compared.

Measurement of amino acid concentrations

Of the cell cultures, 1.8 ml was centrifuged (13,000×*g*, 10 min) and 0.8 ml of the supernatant was mixed with 0.2 ml sulfosalicylic acid (10%). The mixture was diluted appropriately with 100 mM sodium phosphate buffer (pH 7.0) and analysis of amino acid concentration was performed by HPLC as described previously (Brautaset et al. 2003; Jakobsen et al. 2009).

Sequence submissions

The *pyc*, *hom-1* and *hom-2* sequences of *B. methanolicus* MGA3 have been submitted to Genbank and appear with accession number DQ025534, GU377284, and GU377285, respectively.

Results

Cloning, expression, and transcriptional analysis of the *B. methanolicus* MGA3 *pyc* gene encoding PC activity at 50 °C

A putative PC encoding gene, denoted *pyc*, was cloned by screening of a *B. methanolicus* MGA3 gene library (see "Materials and methods" section). The deduced *pyc* gene product is a 1,147 amino acid polypeptide with a calculated molecular weight of 128.4 kDa. PC shared 82% and 46% primary sequence identity to the α-homotetrameric PC proteins of *B. stearothermophilus* (AC number BAA12072) and *C. glutamicum* (AC number NP_599921), respectively. We then constructed plasmid pHP13mp-pyc which has the *pyc* coding region placed under transcriptional control of the strong *mdh* promoter (Brautaset et al. 2004; Jakobsen et al. 2009). The plasmid was introduced into the wild-type *B. methanolicus* MGA3, and the recombinant cells were cultivated in methanol medium in shake flasks, and samples collected for preparation of crude extracts and concomitant PC assays. The results of these experiments showed that MGA3 (pHP13mp-pyc) displayed about sevenfold (490 nmol/min/mg protein) elevated specific PC activity compared to the control strain MGA3 (pHP13) (70 nmol/min/mg protein). These data confirmed that *pyc* encodes an active PC protein, representing the first PC encoding gene cloned and characterised from any methylotrophic bacterium.

Immediately upstream of *pyc* another open reading frame, *orf-1*, is located on the same strand, encoding a putative gene product with unknown function (data not shown). To test if *orf-1* and *pyc* are co-transcribed, we used quantitative PCR and two PCR primer pairs were designed as follows; primers pyc-RTA and pyc-RTB for the amplification of a 211 bp 5′-terminal region of the *pyc* coding sequence, and primers ylaT-F and ylaT-R for the amplification of a 379 bp DNA fragment including the 5′-terminal coding region of *pyc* and the 3′-terminal region of *orf-1* (see "Materials and methods" section). The purpose was to quantify any transcription through the *orf-1/pyc*

intergenic region caused from a distant upstream promoter. A similar strategy has been used to analyse for co-transcription of methylotrophy genes in *Methylobacterium extorquens* (Zhang and Lidstrom 2003), and in *B. methanolicus* (Jakobsen et al. 2006). The cDNA prepared from MGA3 cells growing exponentially on methanol, was used as a template in this experiment, and the result shows that the level of transcript representing the *orf-1/pyc* intergenic region was below 6% compared to the *pyc* transcript level. This result indicates that *pyc* is transcribed from an endogenous promoter and into a monocistronic mRNA molecule in *B. methanolicus*, similar as reported for *C. glutamicum* (Peters-Wendisch et al. 1998).

It has been reported that the carbon source used for growth can modulate bacterial *pyc* expression (Peters-Wendisch et al. 1997; Koffas et al. 2002). Previous data have shown that the MGA3 growth rate on mannitol and methanol is similar, and comparative analyses of cells growing on these two different C-sources have proven useful to understand regulation of methylotrophic pathways in this organism (Jakobsen et al. 2006). Therefore, we decided to analyse *pyc* transcription in cell upon growth on these two carbon sources. The result of this experiment showed that *pyc* mRNA levels in *B. methanolicus* MGA3 cells consuming methanol and mannitol are similar (relative transcription levels 1.0±0.2 for both conditions).

The *hom-1* and *hom-2* genes encode active HD proteins and they are transcriptionally repressed by L-threonine and L-methionine, respectively

Classical AEC resistant *B. methanolicus* mutants typically require supply of methionine and threonine in the growth media (Hanson et al. 1996; Lee et al. 1996; Brautaset et al. 2007), suggesting that they are HD mutants. However, genetic evidence supporting such assumptions has been lacking. Interestingly, in silico screening of the recently acquired *B. methanolicus* MGA3 genome sequence (Brautaset, unpublished) revealed the presence of two distantly located putative HD genes, designated *hom-1* and *hom-2*. Primary sequence comparison of the deduced gene products demonstrated that they share 32% overall identity with each other, and they shared 71% and 35% overall primary sequence identity, respectively, to the monofunctional HD from *B. subtilis* (Parsot and Cohen 1988). To experimentally confirm whether *hom-1* and *hom-2* encoded active HD proteins, a *hom*-negative *E. coli* mutant Gif 102 (Thèze et al. 1974), not able to grow on minimal medium unless added 1.5 mM of both L-threonine and L-methionine, was used for complementation experiments. The *hom-1* and *hom-2* expression vectors pTH1mp-hom1 and pTH1mp-hom2 were constructed (Table 1) and individually transformed to *E. coli* Gif 102. The resulting recombinant strains Gif102 (pTH1mp-hom1) and Gif 102 (pTH1mp-hom2) both grew well on minimal agar plates without added threonine and methionine. As a negative control we established Gif102 (pHP13) and this recombinant strain could not grow under these conditions. Together these results confirmed that *B. methanolicus* possesses two genes *hom-1* and *hom-2* both encoding active HD proteins.

We next analysed transcriptional repression of *hom-1* and *hom-2* in cells growing on methanol and supplemented with 10 mM of relevant amino acids (Table 2). The data showed that *hom-1* transcription is reduced 33-fold by L-threonine and twofold by L-methionine, while *hom-2* transcription was reduced about twofold by L-threonine and 14-fold by L-methionine. L-lysine had no significant effect on transcription levels of these two genes.

Optimisation of HCDC for reliable evaluation of amino acid production by *B. methanolicus* strains

Yield depression as a consequence of high methanol concentrations in batch cultures of methylotrophs has been reported (Hamer et al. 1979; Brooke et al. 1989), and we therefore investigated the impact of different methanol concentration (75-500 mM) on cell growth and amino acid production of wild-type *B. methanolicus* MGA3. Reported biomass and amino acid concentrations are volume corrected (see "Materials and methods" section) to make direct comparisons between fermentation trials with different required feeding profiles (and thereby different dilution) possible, as described previously (Jakobsen et al. 2009). The results demonstrated that at methanol concentrations of 75 mM and 150 mM, the volumetric production yields and yields on methanol of biomass, L-glutamate, L-aspartate, and L-alanine were similar (Fig. 2 and Table 3). At a methanol concentration of 300 mM significant lower volumetric production yields and yields on methanol of L-glutamate and L-alanine were observed, and these numbers were further reduced at 500 mM methanol (Fig. 2 and Table 3). We noticed that the fraction of unaccounted carbon (37%) based on a total carbon balance for the fermentation trial conducted at 500 mM was significantly

Table 2 Transcriptional repression of *hom-1* and *hom-2* genes

Gene	Relative transcription level in the presence of amino acids[a]			
	None	Lysine	Methionine	Threonine
hom-1	1.0	1.2	0.6	0.03
hom-2	1.0	1.0	0.07	0.43

[a] Exponentially growing cells were added 10 mM of amino acids and cultivated another 1 h before cell harvesting and rt-PCR experiments (see "Materials and methods" section)

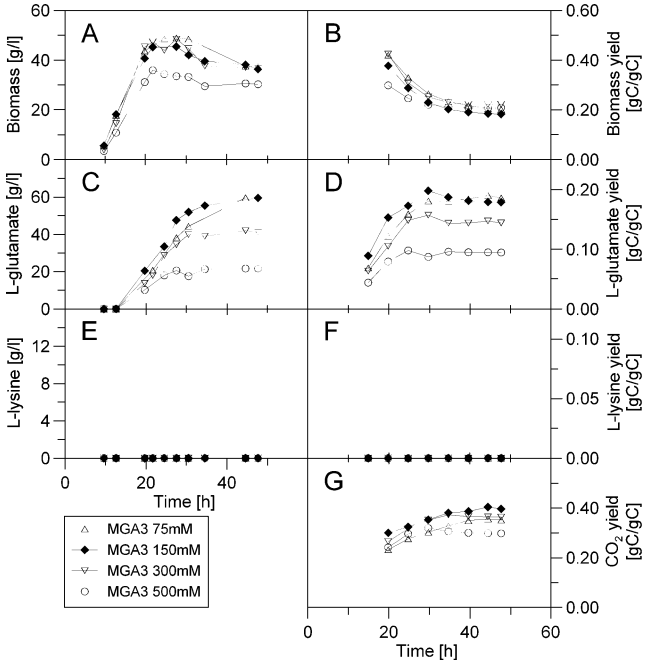

Fig. 2 Growth (**a**) and production of L-glutamate (**c**) and L-lysine (**e**) by the wild-type strain MGA3 in defined methanol medium with varying methanol concentrations (without any amino acid feeding). Throughout the fermentation trials, the methanol concentration was maintained approximately at set-point by automatic feeding of methanol to the fermentor. Biomass and amino acid concentrations have been volume corrected as described in "Materials and methods" section. Additionally, yields of biomass (**b**), L-glutamate (**d**), L-lysine, (**f**) and CO_2 (**g**) on methanol are presented on a carbon/carbon basis as described in "Materials and methods" section

higher than for the cultivations conducted at 75 mM and 150 mM (20%). Possibly, undetected biomass production due to cell lysis may contribute to the unaccounted carbon, in addition to non-measured products. However, the latter remained to be experimentally tested. In any case, all further HCDC were performed by maintaining a methanol concentration of 150 mM in the growth medium throughout the fermentation trials, in agreement with recent report (Jakobsen et al. 2009).

L-lysine overproduction (65 g/l) and reduced L-glutamate production (28 g/l) of classical mutant NOA2#13A52-8A66 is accompanied with a threefold elevated *pyc* transcription level

We chose to analyse the previously constructed classical AEC-resistant *B. methanolicus* mutant NOA2#13A52-8A66 under optimised HCDC (see "Materials and methods" section), and supplemented with DL-methionine+L-threonine+L-leucine feeding as described previously (Hanson et al. 1996; Lee et al. 1996). The results showed that this strain can produce 65 g/l of L-lysine and only 28 g/l of L-glutamate (volume-corrected values) and reached a maximum CDW of 34 g/l (Table 3) under such conditions. Even without volume correction the volumetric L-lysine yield of this strain was 47 g/l which is well above the previously reported production level (37 g/l) for this mutant (Hanson et al. 1996; Lee et al. 1996). We also noticed that L-alanine levels

Table 3 Maximum biomass concentrations, specific growth rates and volumetric amino acid yields of *B. methanolicus* strains

B. methanolicus strain	CDW	μ[a]	Glu[b]	Lys[b]	Asp[b]	Ala[b]
	(g/l)	(1/h)	(g/l)	(g/l)	(g/l)	(g/l)
Effects of different methanol concentrations						
MGA3 75 mM	49	0.49	60	ND	1.1	13
MGA3 150 mM	45	0.50	59	ND	1.6	12
MGA3 300 mM	48	0.51	42	ND	ND	8.8
MGA3 500 mM	36	0.42	22	ND	ND	5.6
Evaluation of recombinant and mutant strains at 150 mM methanol						
MGA3 (pHP13)	45	0.49	59	0.4	1.1	12
M168-20 (pHP13)	31	0.36	32	1.9	0.6	4.1
M168-20 (pHP13)+M	51	0.46	69	11	1.7	10
MGA3 (pHP13)+M	71	0.53	38	0.2	3.4	11
MGA3 (pHP13mp-pyc)	36	0.43	31	0.3	0.6	6.6
M168-20 (pHP13mp-pyc)+M	57	0.42	52	11	0.9	9.1
M168-20 (pHP13mp-pycm)+M	55	0.41	43	11	0.8	8.9
NOA2 #13A52-8A66+MTL	34	0.36	28	65	0.2	5.6

CDW cell dry weight, *Glu* L-glutamate, *Lys* L-lysine, *Asp* L-aspartate, *Ala* L-alanine, *ND* not detected. *+M* the fermentation trial was run with DL-methionine feeding, *+MTL* DL-methionine+L-threonine + L-leucine feeding (see "Materials and methods" section).

[a] Specific growth rates are maximum values calculated from the exponential growth period

[b] Amino acid concentrations are maximum values and volume corrected (see "Materials and methods" section)

were reduced to about 50% compared to the wild-type strain (Table 3). The high volumetric L-lysine yield may be due to more optimised cultivation conditions such as the growth medium, and the feeding strategy of methanol, amino acids, and the antifoam (see "Materials and methods" section). A maximum L-lysine productivity of 1.6 g/l/h (volume corrected value) was obtained at 36-42 h after inoculation (with a corresponding volumetric L-lysine yield of 56-65 g/l in this period). To our knowledge, this result represents the highest volumetric L-lysine yield reported by any methylotroph (see Brautaset et al. 2007), and it also confirms that the optimised HCDC should be valuable for reliable evaluation of amino acid production by recombinant *B. methanolicus* strains (see below).

We previously showed that in vitro PC activity in crude extracts prepared from NOA2#13A52-8A66 was threefold higher than that of the wild-type strain (Brautaset et al. 2003). To unravel whether this elevated PC activity was due to mutations affecting PC catalytic activity or *pyc* expression level, we PCR amplified and sequenced a 4.0 kb DNA fragment including the *pyc* gene and 421 bp upstream region using NOA2#13A52-8A66 total DNA as a template. No mutations were found. We next performed real-time PCR with cDNA prepared from total RNA isolated from exponentially growing NOA2#13A52-8A66 cells. The results showed that *pyc* transcription in mutant NOA2#1 3A52-8A66 was threefold higher than that of the wild-type strain under such conditions (relative transcription level 3.0 ±0.2 versus 1.0±0.2, respectively), indicating that *pyc* is transcriptionally regulated in *B. methanolicus*. Moreover, these data suggested that the L-lysine on L-glutamate ratio from methanol may be increased by overproducing PC in *B. methanolicus*.

First generation AEC-resistant mutant M168-20 requires DL-methionine for rapid growth and can produce 11 g/l of L-lysine and 69 g/l of L-glutamate

In *C. glutamicum*, it has been shown that manipulations with PC can positively affect L-lysine production if the host strain has been deregulated in L-lysine biosynthesis (Peters-Wendisch et al. 2001; Ohnishi et al. 2002; Ikeda et al. 2006; Georgi et al. 2005). Bacterial resistance to the L-lysine analogue AEC has previously been shown to be directly associated with overproduction of L-lysine (Sahm et al. 1996; Peters-Wendisch et al. 1997; Koffas et al. 2003). Therefore, to be in possession of a relevant *B. methanolicus* host strain for investigating effects of PC manipulations on L-lysine production, we constructed the first generation classical AEC-resistant MGA3 mutant M168-20. M168-20 and its wild-type strain MGA3 were initially analyzed and compared for cell growth and amino acid production under optimised HCDC. To obtain data directly relevant for further recombinant experiments, the empty expression vector pHP13 (Table 1) was introduced into both host strains MGA3 and M168-20. MGA3 and MGA3 (pHP13) displayed similar maximum specific growth rates, respiration, and volumetric production yields and yields of biomass and amino acids on methanol (Table 3 and Fig. 4). These data confirmed that maintenance of plasmid pHP13 confers no negative effects on *B. methanolicus* cell growth, in agreement with previous studies (Jakobsen et al. 2009). Both the specific growth rate (0.36 h^{-1}) and the maximum biomass concentration (31 g/l) of M168-20 (pHP13) were significantly reduced compared to MGA3 (pHP13). However, supplementation with DL-methionine was sufficient to compensate these phenotypes (Table 3 and Fig. 3) and all further HCDC experiments were conducted with DL-methionine feeding for M168-20 strains and without DL-methionine feeding for MGA3 strains.

The maximum volumetric L-lysine yield of M168-20 (pHP13) was 11 g/l (Table 3; Fig. 3e) which is above 20-fold higher than that of MGA3 (pHP13) (Table 3; Fig. 4e). The maximum volumetric L-glutamate yield of M168-20 (pHP13) was also high compared to MGA3 (pHP13) (69 g/l

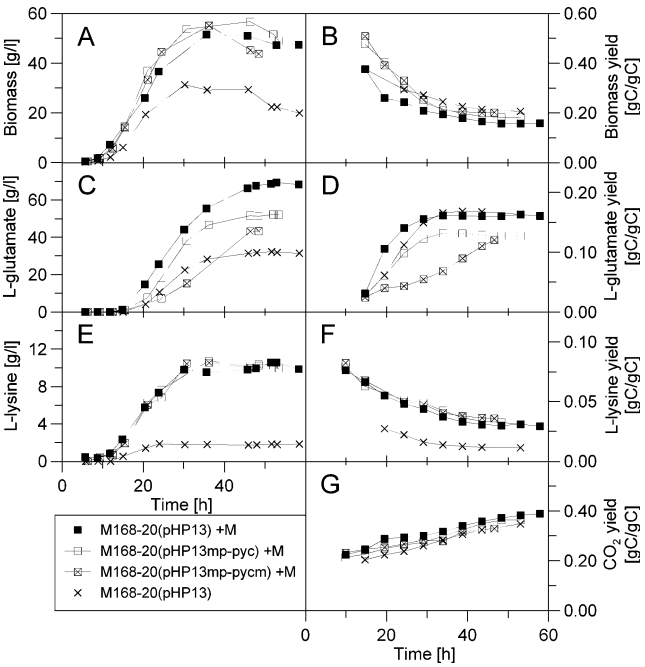

Fig. 3 Growth (**a**) and production of L-glutamate (**c**) and L-lysine (**e**) by the reference strain M168-20 (pHP13) with and without DL-methionine feeding, and the recombinant strains M168-20 (pHP13mp-pyc) and M168-20 (pHP13mp-pycm) with DL-methionine feeding, overexpressing the genes encoding PC and its mutant P455S, respectively. Biomass and amino acid concentrations have been volume corrected as described in "Materials and methods" section. Additionally, yields of biomass (**b**), L-glutamate (**d**), L-lysine (**f**), and CO_2 (**g**) on methanol are presented on a carbon/carbon basis as described in "Materials and methods" section. *+M* indicates DL-methionine feeding (see "Materials and methods" section)

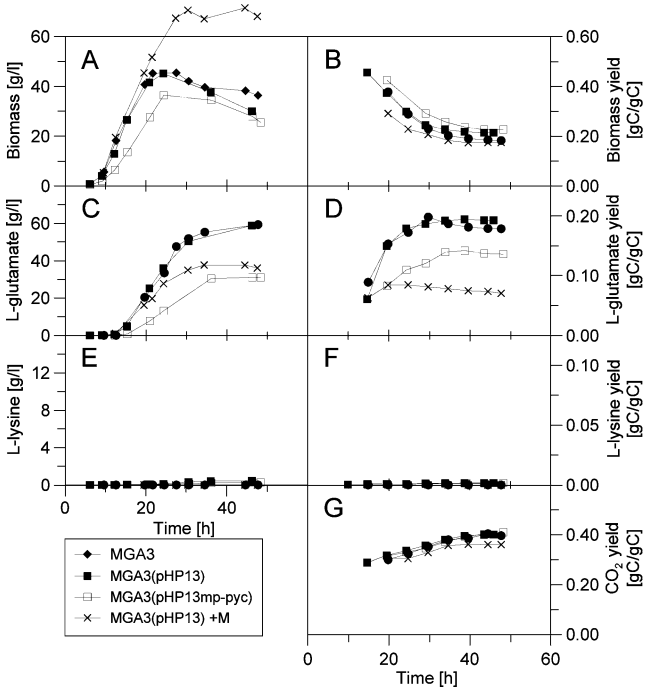

Fig. 4 Growth (**a**) and production of L-glutamate (**c**) and L-lysine (**e**) by the wild-type strain MGA3 without DL-methionine feeding, the reference strain MGA3 (pHP13) with and without DL-methionine feeding, and the recombinant strain MGA3 (pHP13mp-pyc) without DL-methionine feeding. Biomass and amino acid concentrations have been volume corrected as described in "Materials and methods" section. Additionally, yields of biomass (**b**), L-glutamate (**d**), L-lysine (**f**), and CO_2 (**g**) on methanol are presented on a carbon/carbon basis as described in "Materials and methods" section. *+M* indicates DL-methionine feeding (see "Materials and methods" section)

versus 59 g/l; Table 3) while the L-glutamate yield on methanol (0.16 g L-glutamate-carbon / g methanol-carbon; Fig. 3d) was slightly lower to that of MGA3 (pHP13) (0.19 g/g; Fig. 4d). M168-20 (pHP13) with DL-methionine feeding and MGA3 (pHP13) produced significant amounts of L-alanine (up to 10-12 g/l respectively, while the volumetric L-aspartate yield was below 2 g/l for both strains (Table 3). Volumetric L-threonine and DL-methionine yields were close to zero for both strains (data not shown).

We noticed that the maximum volumetric production yields of L-lysine, as well as L-glutamate and L-alanine, were significantly increased in M168-20 (pHP13) cells when grown with DL-methionine feeding compared to without such feeding (Table 3). In *C. glutamicum*, L-methionine represses HD activity leading to reduced synthesis of the AK inhibitor L-threonine, eventually resulting in increased L-lysine production (Vrljic et al. 1995). To unravel if this is similar in *B. methanolicus*, we also conducted a fermentation trial of MGA3 (pHP13) with DL-methionine feeding. Both the specific growth rate and maximum biomass production increased (Table 3), while the volumetric L-lysine yield was not affected under these conditions. This result confirmed that the feeded DL-methionine does not exert any significant effect on L-lysine production level in *B. methanolicus* MGA3. In contrast to the data obtained with M168-20 (pHP13), the volumetric L-glutamate yield and yield of L-glutamate on methanol dramatically decreased with DL-methionine feeding in MGA3 (pHP13) (Table 3 and Fig. 4), and the biological reason for this remained unknown.

M168-20 and NOA2#13A52-8A66 both possess chromosomal *hom-1* mutations and *hom-1* encodes the major HD in *B. methanolicus*

Genetic analyses revealed that mutant strain M168-20 had two point mutations in the *hom-1* coding region (G in position 357 substituted with A, and G in position 1188 substituted with T, causing substitutions D120N and V397F in the *hom-1* gene product, respectively), and no mutations in the *hom-2* gene including upstream regions. To experimentally analyse the impact of the *hom-1* mutations on L-lysine production, the recombinant strains M168-20 (pTH1mp-hom1) and M168-20 (pTH1mp-hom2) were established (Table 1) and tested for L-lysine production in shake flasks (see "Materials and methods" section). MGA3 (pHP13) and M168-20 (pHP13) were included as control strains. The maximum L-lysine production by M168-20 (pHP13) was 24-fold higher than that of MGA3 (pHP13) (170 mg/l versus 7 mg/l) under these conditions, and these results were in good agreement with the analogous data obtained under HCDC (see above). Interestingly, M168-20 (pTH1mp-hom1) displayed L-lysine production close to wild-type levels (10 mg/l), while L-lysine production of M168-20 (pTH1mp-hom2) was only slightly reduced (140 mg/l) compared to that of the control strain M168-20 (pHP13). We also unravelled that mutant NOA2#13A52-8A66 had a point mutation in the *hom-1* coding region (G in position 784 substituted with T leading to amino acid substitution V262F in the corresponding gene product), and no mutations in the *hom-2* gene. Together, these results should imply that *hom-1* encodes the major HD and represents one key target for AEC resistance in *B. methanolicus*.

Mutant M168-20 has no chromosomal *pyc* mutations and is not affected in *pyc* transcription level

Genetic characterization revealed that M168-20 has no chromosomal *pyc* mutations. Quantitative PCR analysis showed that the *pyc* transcription level of this mutant strain, in contrast to NOA2#13A52-8A66, is similar to that of the wild-type strain (relative transcription level 1.3±0.2 versus 1.0±0.2, respectively). Moreover, specific PC activity of strain M168-20 (pHP13) upon growth in shake flasks was 90 nmol/min/mg protein, which is also similar

to that of the analogous strain MGA3 (pHP13) (see above). Altogether, M168-20 is a *hom-1* mutant with wild-type level *pyc* transcription and PC activity, and it should thus represent a suitable host strain for experimentally testing the effects of PC manipulations on L-lysine production in *B. methanolicus*.

Overproduction of PC and its P455S mutant in M168-20 had no positive effect on L-lysine production and resulted in reduced L-glutamate production

Recombinant strain M168-20 (pHP13mp-pyc) was then established and this strain had about fivefold higher specific PC activity (410 nmol/min/mg protein) than the control strain M168-20 (pHP13) in shake flasks (see above), confirming PC overexpression. M168-20 (pHP13mp-pyc) was analysed for amino acid production under HCDC, and the results showed that both maximum volumetric L-lysine yield (Fig. 3e; Table 3) and L-lysine yield on methanol (Fig. 3f) were similar to those of M168-20 (pHP13), indicating that PC overproduction has no positive effect on L-lysine production yield in this strain.

A specific *pyc* point mutation, P458S, has been reported to mediate enhanced L-lysine production when introduced into an L-lysine overproducing genetic background of *C. glutamicum* (Ohnishi et al. 2002). To our knowledge, no experimental data on the effects of this mutation on the biochemical properties of PC has been described in the scientific literature. This mutated proline residue is conserved in the *B. methanolicus* PC protein, P455, and we therefore used site-directed mutagenesis to introduce substitution mutation P455S into the *B. methanolicus pyc* coding region. The resulting mutated *pyc* gene was then substituted with the wild-type *pyc* gene of plasmid pHP13mp-pyc, yielding vector pHP13mp-pycm (Table 1). Recombinant strain M168-20 (pHP13mp-pycm) was established and shake flask experiments confirmed a specific PC activity (420 nmol/min/mg protein) about fivefold higher than that of control strain M168-20 (pHP13) and similar to that of M168-20 (pHP13mp-pyc). As for M168-20 (pHP13mp-pyc), analyses under HCDC showed that both the maximum volumetric L-lysine yield and the L-lysine yield on methanol were similar to that of M168-20 (pHP13) (Fig. 3e, f; Table 3). Together, these data implied that L-lysine production in M168-20 cannot be increased by manipulating *pyc* transcription or PC activity, suggesting that OAA precursor supply is not a major limitation for further improved L-lysine production by this mutant.

Surprisingly, the maximum volumetric L-glutamate yield of M168-20 (pHP13mp-pyc) was reduced to 75% compared to M168-20 (pHP13) (Fig. 3c; Table 3), and a similar reduction (to 79%) were observed for the L-glutamate yield on methanol (Fig. 3d). The maximum volumetric L-glutamate yield and L-glutamate yield on methanol for M168-20 (pHP13mp-pycm) were reduced to 62% and 75%, respectively, compared to M168-20 (pHP13) (Fig. 3c, d; Table 3). The biomass- and CO_2-yields on methanol of all these three recombinant strains were similar (Fig. 3b, g) indicating that the genetic manipulations did not cause any metabolic burden to the cells.

Overproduction of PC in wild-type MGA3 host resulted in reduced production levels of L-glutamate and L-alanine

To unravel if observed effects of *pyc* overexpression might be specifically related to the mutant M168-20 genetic background, recombinant strain MGA3 (pHP13mp-pyc) was analysed under HCDC. The maximum volumetric L-glutamate yield and L-glutamate yield on methanol was only 53% and 70% of those of the control strain MGA3 (pHP13), respectively (Fig. 4c, d; Table 3). As expected, *pyc* overexpression had no positive effect on volumetric L-aspartate and L-lysine yield in the wild-type genetic background, and the volumetric L-alanine yield was about 50% reduced compared to the control strain (Table 3). As observed when using host M168-20 (see above), the yields of biomass and CO_2 on methanol were similar for MGA3 (pHP13mp-pyc) and its control strain MGA3 (pHP13) (Fig. 4b, g). We noticed that PC overproduction reduced the volumetric biomass yield in MGA3 host strain (Fig. 4a), and the biological reason for this is unclear.

To verify HCDC reproducibility, fermentation trials of MGA3 (pHP13) and MGA3 (pHP13mp-pyc) were run as duplicates. For both strains, volumetric biomass, L-lysine, and L-glutamate yields in addition to methanol utilisation did not vary more than maximum 11% between the biological replicates (data not shown). Next, to rule out any problems related to plasmid loss under the conditions tested, cell samples from the various recombinant cell cultures were collected and analysed for plasmid stability (see "Materials and methods" section). The overall results verified that the various plasmids were maintained stable and no significant plasmid loss was detected (data not shown). In conclusion, these data indicated that elevated *pyc* expression level or altered PC catalytic activity have no significant positive effect on L-lysine production, while it reduced L-glutamate and L-alanine production, both in wild-type and AEC resistant mutant *B. methanolicus* strains under the conditions tested.

Discussion

Three genes with assumed important roles for overproduction of L-lysine from methanol were cloned from *B.*

methanolicus; *pyc* encoding PC involved in OAA precursor supply and *hom-1* and *hom-2* encoding HD proteins controlling a branch point in the L-lysine biosynthetic pathway (Fig. 1). To our knowledge, this is the first reported *Bacillus* species with two functional HD genes, and we show that *hom-1* and *hom-2* are strongly repressed by L-threonine and L-methionine, respectively, similar as in *L. plantarum* (Cahyanto et al. 2006). We showed that *pyc* is transcribed from an endogenous promoter and into a monocistronic mRNA molecule encoding a catalytically active PC protein, similarly as described for *C. glutamicum* (Peters-Wendisch et al. 1998). Two structurally different forms of PC proteins, α-homotetrameric and heterooctameric, are found among prokaryotes (Dunn et al. 1996; Yong-Biao et al. 2004), and the *B. methanolicus* PC protein is presumably α-homotetrameric based on primary sequence comparisons.

We took advantage of classical *B. methanolicus* mutant strains to investigate the roles of these three genes for L-lysine overproduction by *B. methanolicus*. The previously constructed *B. methanolicus* classical mutant NOA 2#13A52-8A66 displayed above 150-fold increased L-lysine production compared to the wild-type (65 g/l versus 0.4 g/l) under optimised HCDC, while the volumetric L-glutamate and L-alanine yields were low compared to the wild-type (28 g/l versus 59 g/l and 5.6 g/l versus 12 g/l, respectively). These altered amino acid production levels were accompanied with a threefold elevated *pyc* transcription level in the cells, and this strain had chromosomal mutations in *hom-1*, and none in *pyc* and *hom-2*. We therefore speculated that PC overproduction may be beneficial for L-lysine production in a relevant *B. methanolicus* host strain, in particular by reducing the L-glutamate levels (see Fig. 1). To be in possession of a suitable *B. methanolicus* host to experimentally analyse the impact of PC on L-lysine production, the AEC-resistant *hom-1* mutant M168-20 was constructed. M168-20 (pHP13) produced 11 g/l of L-lysine under HCDC while the analogous wild-type strain MGA3 (pHP13) produced only 0.4 g/l under such conditions. In contrast to mutant NOA2#13A52-8A66, M168-20 retained a high maximum volumetric L-glutamate yield and a high L-glutamate yield on methanol. Interestingly, both the *pyc* transcription and the specific PC activity of M168-20 were at wild-type levels, and as for NOA2#13A52-8A66 this strain had chromosomal mutations in *hom-1* and none in *pyc* and *hom-2*. Overproduction of PC and its mutant P455S in M168-20 resulted in significantly reduced maximum volumetric L-glutamate yield and L-glutamate yield on methanol (Fig. 3). However, these manipulations had no positive effects on maximum volumetric L-lysine yield and L-lysine yield on methanol, indicating that PC activity is not a major bottleneck for improved L-lysine production in M168-20 host under the conditions tested. L-lysine production by M168-20 is presumably not limited by OAA supply but likely by other enzymes of the aspartate pathway (see Jakobsen et al. 2009). These results were different from results reported from analogous experiments in *C. glutamicum* (Peters-Wendisch et al. 2001; Koffas et al. 2003).

One possible explanation for the reduced L-glutamate production observed upon PC overproduction in *B. methanolicus* could be an imbalance in the generation of OAA versus acetyl-CoA in the cells, eventually leading to low metabolite entry into the TCA cycle catalysed by citrate synthase (Fig. 1). It has been shown that a balanced partitioning of the carbon flux from pyruvate towards OAA and acetyl-CoA is important for efficient L-glutamate production by *C. glutamicum* (Hasegawa et al. 2008). Moreover, the low PDH activity together with the high PC activity of *B. methanolicus* mutant NOA2#13A52-8A66 (Brautaset et al. 2003) may indicate a reduced conversion of pyruvate into acetyl-CoA in these cells (see Fig. 1). The latter correlates well with the relative low volumetric L-glutamate yield by this mutant compared to the wild-type (see Table 3), and possibly this reduced L-glutamate production exerts a positive effect on the high volumetric L-lysine yield by this mutant strain.

We previously showed that mutation in the citrate synthase II gene *citY* (Fig. 1) almost completely abolished L-glutamate production in *B. methanolicus*; however, the mutant cells grew very poorly unless supplied with excess L-glutamate in the medium (Brautaset et al. 2003). It has been demonstrated that PC and PEPC operates simultaneously in balancing OAA formation in *C. glutamicum* (Peterson et al. 2000). In contrast, *B. methanolicus* lacks PEPC activity, has a low overall TCA activity, and its intracellular OAA concentrations are high (450 mM; Brautaset et al. 2003), indicating that OAA supply is not a major limitation for L-lysine overproduction. It may thus seem as if these two organisms differ considerably with respect to enzymes and regulation around the anaplerotic node. Possibly, upregulating PC is only beneficial for L-lysine production in an appropriate *B. methanolicus* genetic background, for example in combination with upregulated AK activity (Jakobsen et al. 2009).

In conclusion, our results unravelled that *hom-1* is one key target for AEC resistance and concomitant L-lysine overproduction by *B. methanolicus*, while the biological role of *hom-2* was less clear. Together with previous results (Jakobsen et al. 2009) our data should indicate that metabolic engineering for further improved L-lysine production should primary focus on the L-lysine biosynthetic pathway from aspartate. In addition, reducing L-glutamate synthesis by PC overproduction may also be important to maximise L-lysine production and yield.

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