

Overexpression of Wild-Type Aspartokinase Increases L-Lysine Production in the Thermotolerant Methylophilic Bacterium *Bacillus methanolicus*[∇]

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Aspartokinase (AK) controls the carbon flow into the aspartate pathway for the biosynthesis of the amino acids L-methionine, L-threonine, L-isoleucine, and L-lysine. We report here the cloning of four genes (*asd*, encoding aspartate semialdehyde dehydrogenase; *dapA*, encoding dihydrodipicolinate synthase; *dapG*, encoding AKI; and *yclM*, encoding AKIII) of the aspartate pathway in *Bacillus methanolicus* MGA3. Together with the known AKII gene *lysC*, *dapG* and *yclM* form a set of three AK genes in this organism. Overexpression of *dapG*, *lysC*, and *yclM* increased L-lysine production in wild-type *B. methanolicus* strain MGA3 2-, 10-, and 60-fold (corresponding to 11 g/liter), respectively, without negatively affecting the specific growth rate. The production levels of L-methionine (less than 0.5 g/liter) and L-threonine (less than 0.1 g/liter) were low in all recombinant strains. The AK proteins were purified, and biochemical analyses demonstrated that they have similar V_{max} values (between 47 and 58 $\mu\text{mol}/\text{min}/\text{mg}$ protein) and K_m values for L-aspartate (between 1.9 and 5.0 mM). AKI and AKII were allosterically inhibited by *meso*-diaminopimelate (50% inhibitory concentration [IC_{50}], 0.1 mM) and by L-lysine (IC_{50} , 0.3 mM), respectively. AKIII was inhibited by L-threonine (IC_{50} , 4 mM) and by L-lysine (IC_{50} , 5 mM), and this enzyme was synergistically inhibited in the presence of both of these amino acids at low concentrations. The correlation between the impact on L-lysine production in vivo and the biochemical properties in vitro of the individual AK proteins is discussed. This is the first example of improving L-lysine production by metabolic engineering of *B. methanolicus* and also the first documentation of considerably increasing L-lysine production by overexpression of a wild-type AK.

L-Lysine is an essential amino acid and is added to feed to meet the nutritional requirements of nonruminants, such as poultry, swine, and fish. L-Lysine is produced industrially by fermentation processes that mainly use the gram-positive bacterium *Corynebacterium glutamicum*. The gram-positive and thermotolerant methylophilic *Bacillus methanolicus* has been studied as an alternative producer of L-glutamate and L-lysine using methanol as the raw material (for a review, see reference 5). We have previously demonstrated that wild-type *B. methanolicus* produces 58 g/liter of L-glutamate (6), while mutants generated by random chemical mutagenesis have been reported to produce up to 37 g/liter of L-lysine (16, 23, 34). Favorable properties of *B. methanolicus*, such as a lack of sporulation at high temperatures, utilization of methanol as an energy and carbon source, a high methanol conversion rate, a high theoretical yield of L-lysine, and an optimal growth temperature of 50°C, indicate that this organism may represent a possible future noncarbohydrate substrate alternative for large-scale production of L-lysine (5).

L-Lysine is synthesized from L-aspartate as part of the as-

partate pathway, which also includes the biosynthetic pathways for L-methionine, L-threonine, and L-isoleucine (Fig. 1). The first step in the common pathway, phosphorylation of L-aspartate, is catalyzed by aspartokinase (AK) (ATP:4-L-aspartate-4-phosphotransferase; EC 2.7.2.4). The presence of three AK isozymes in *B. methanolicus* MGA3 has been predicted, and based on in vitro studies with crude cell extracts these proteins were proposed to be inhibited like the proteins of *Bacillus subtilis* (35). Only one of the genes encoding these *B. methanolicus* proteins, *lysC* encoding AKII, has been cloned previously (35). *B. subtilis* has three monofunctional AKs that are regulated in a distinct manner. AKI (encoded by *dapG*) is allosterically inhibited by *meso*-diaminopimelate (*meso*-DAP) (32), AKII (encoded by *lysC*) is inhibited by L-lysine (28), and AKIII (encoded by *yclM*, also designated *thrD*) is regulated by concerted feedback inhibition by L-lysine and L-threonine (13) (Fig. 1). In addition, L-lysine and L-threonine act as corepressors for *lysC* and *yclM* transcription, respectively (3, 13, 14, 21, 30). Transcription of *lysC* has been reported to be induced by L-methionine, while *yclM* transcription is induced by L-lysine (13, 40). The genetic organization of the *B. subtilis* DAP (*dap*) operon has been described previously (7). This operon includes *spoVFA* and *spoVFB* encoding the two subunits of dipicolinate synthase, *asd* encoding aspartate semialdehyde dehydrogenase, *dapG*, and *dapA* encoding dihydrodipicolinate synthase. All these enzymes are involved in the aspartate pathway (Fig. 1). During vegetative growth, the three distal genes,

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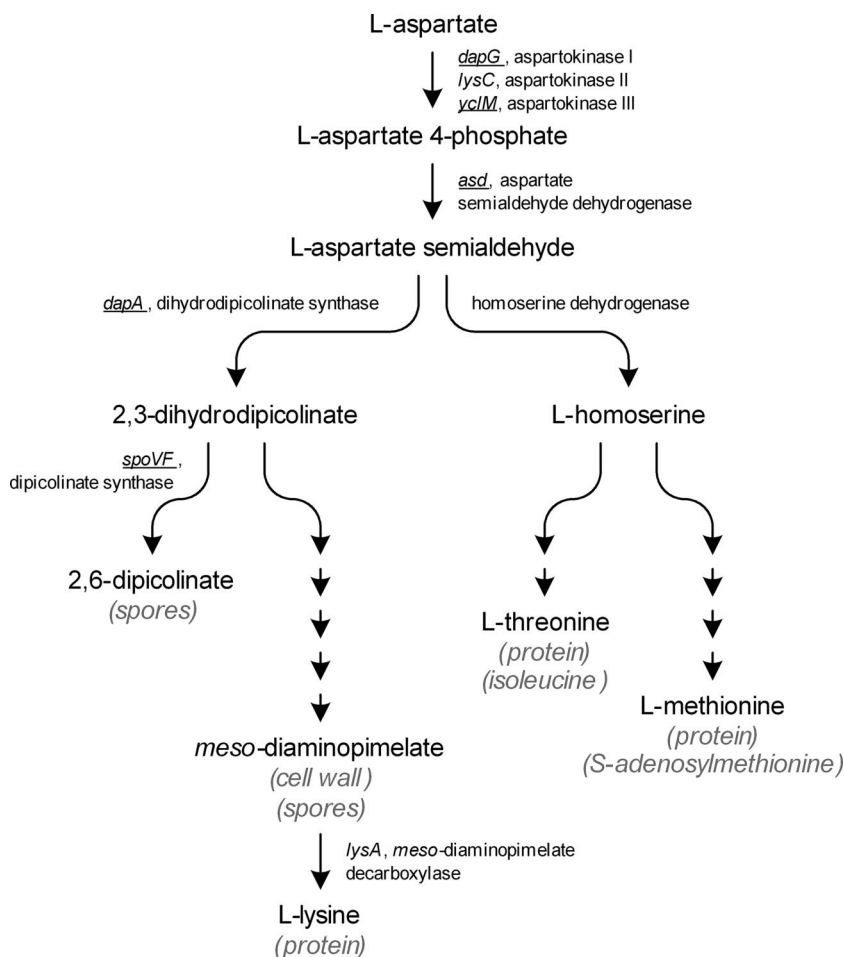


FIG. 1. General overview of the aspartate pathway in the genus *Bacillus* (30). The major metabolic functions of the end products are indicated in parentheses. *B. methanolicus* genes sequenced and described in this work are underlined.

asd, *dapG*, and *dapA*, are believed to be transcribed as one unit, while *spoVFA* and *spoVFB* expression occurs only after the onset of sporulation as part of a transcript comprising all five genes (7).

Deregulation of AK has been reported to be the most important step in the development of commercial L-lysine production strains (10, 31). In the industrial production organism *C. glutamicum*, major increases in L-lysine production occurred as a result of introduction or amplification of allosterically feedback-resistant AK (9, 10, 18, 29). Although decreased feedback inhibition of AKI and AKII was demonstrated in *B. subtilis*, improved L-lysine production was not demonstrated (19, 40). Mutations in the 5' untranslated part of *lysC* mRNA decreased repression and increased L-lysine production in *B. subtilis*, but significant L-lysine production by such mutants (more than 1 g/liter) has not been reported (25, 26, 39). Until now, no mutation in any AKIII-encoding gene that leads to increased levels of L-lysine production in *Bacillus* species has been described. Dihydrodipicolinate synthase (Fig. 1), encoded by *dapA*, has been proven to be another key enzyme for microbial L-lysine production. It is thought that this enzyme is not feedback inhibited in either *C. glutamicum* or *B. subtilis*, but investigators have reported increased L-lysine production

when *dapA* expression is elevated in *C. glutamicum* (11). The last step in the L-lysine biosynthetic pathway is catalyzed by meso-DAP decarboxylase (Fig. 1), which is encoded by *lysA*. This enzyme has been shown to be inhibited by L-lysine in *B. methanolicus* with an inhibition constant of 0.9 mM as measured in vitro, suggesting a possible limiting step for efficient L-lysine production (27). In *B. subtilis*, *lysA* is repressed by L-lysine (1).

We have previously described the genetic organization of the *B. methanolicus* ribulose monophosphate pathway and metabolic engineering of this pathway (4, 17) leading to improved methylotrophic properties of this bacterium. To date, no recombinant work with the aim of increasing amino acid production in this organism has been reported, mainly due to the lack of suitable genetic tools, as well as limited relevant genetic knowledge. In this paper we report cloning and DNA sequencing of a partial putative *dap* operon including *asd*, *dapA*, and *dapG*, as well as the distant gene *yclM*. The four new genes should be interesting targets for manipulation of the aspartate pathway for increasing L-lysine production in this organism. *lysC*, *dapG*, and *yclM* encode a set of three different AK isozymes in *B. methanolicus* MGA3, and individual overexpression of each of the three AK isozymes resulted in in-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference(s) or source
<i>B. methanolicus</i> MGA3	Wild type	34
<i>E. coli</i> strains		
DH5 α	General cloning host	Bethesda Research Laboratories
ER2566	Expression host, carries chromosomal gene for T7 RNA polymerase	New England Biolabs
Plasmids		
pTB1.9mdhL	<i>E. coli</i> - <i>B. methanolicus</i> shuttle vector pTB1.9 carrying the <i>mdh</i> gene, Amp ^r Neo ^r	4
pHP13	<i>E. coli</i> - <i>B. methanolicus</i> shuttle vector, Clm ^r	15, 17
pHP13mp-dapG	pHP13 carrying the <i>dapG</i> coding region under control of the <i>mdh</i> promoter	This study
pHP13mp-lysC	pHP13 carrying the <i>lysC</i> coding region under control of the <i>mdh</i> promoter	This study
pHP13mp-yclM	pHP13 carrying the <i>yclM</i> coding region under control of the <i>mdh</i> promoter	This study
pET21a	<i>E. coli</i> expression vector, six-His tag, T7 promoter, Amp ^r	Novagen
pSB1	pET21a with <i>dapG</i> coding sequence under control of the T7 promoter and fused to six-His tag	This study
pSB3	pET21a with <i>lysC</i> coding sequence under control of the T7 promoter and fused to six-His tag	This study
pSB4	pET21a with <i>yclM</i> coding sequence under control of the T7 promoter and fused to six-His tag	This study

^a Amp^r, ampicillin resistance; Neo^r, neomycin resistance; Clm^r, chloramphenicol resistance.

creased L-lysine production in *B. methanolicus*. The purified AK proteins were characterized biochemically, and the correlation between their in vitro properties and their effects on L-lysine production in vivo is discussed below.

MATERIALS AND METHODS

Biological materials, DNA manipulation, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5 α was used as a standard cloning host, while *E. coli* strain ER2566 was used as a host for recombinant expression of the AK proteins. *E. coli* strains were generally grown at 37°C in liquid or solid Luria-Bertani (LB) medium supplemented with ampicillin (200 μ g/ml) or chloramphenicol (15 μ g/ml) when appropriate. Recombinant *E. coli* procedures were performed as described elsewhere (33). For production of AK proteins, overnight cultures of recombinant *E. coli* ER2566 cells growing at 37°C in LB medium were diluted 1:100 in 100 ml of 3 \times LB medium (with 3 \times tryptone, 3 \times yeast extract, and 1 \times NaCl), and cells were grown until the optical density at 600 nm (OD₆₀₀) was 0.6. Recombinant expression was induced by adding 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and then cells were grown for 2 h at 25°C before the growth temperature was changed to 16°C and cells were grown overnight. Cells were harvested by centrifugation (7,000 \times g, 15 min), and the pellets were stored at -20°C.

Transformation of *B. methanolicus* was performed by electroporation as previously described (17).

For shake flask cultures, *B. methanolicus* strains were grown at 50°C in 100 ml of MeOH₂₀₀ medium containing 200 mM methanol (17), and bacterial growth was monitored by measuring the OD₆₀₀.

Fermentation was performed in Applikon 3-liter fermentors with an initial culture volume of 0.9 liter. The medium used, UMN1 medium, contained 4.09 g/liter K₂HPO₄, 1.30 g/liter NaH₂PO₄, 2.11 g/liter (NH₄)₂SO₄, 0.25 g/liter yeast extract (Difco), 6 mg/liter d-biotin, 0.01 mg/liter vitamin B₁₂, 1 mM MgSO₄, 1 ml/liter concentrated metal solution (23), and 150 mM methanol. Chloramphenicol (5 μ g/ml) was added when appropriate. Shake flask cultures in MeOH₂₀₀ medium were used as inocula and were harvested at an OD₆₀₀ of 1.1 to 1.3. The fermentors were inoculated with a culture volume equal to 75 ml divided by the OD₆₀₀ of the inoculum at the time of harvest (1.1 to 1.3). Fermentations were performed at 50°C with initial agitation at 400 rpm and an initial aeration rate of 0.5 vvm (volume of gas/volume of liquid/minute). The aeration rate was increased stepwise to 1.0 vvm, and the air was enriched stepwise until it contained 60% O₂, as the oxygen demand increased. At all times, the level of dissolved oxygen was maintained at 30% saturation by automatic adjustment of the agitation speed up to 2,000 rpm. The pH was maintained at 6.5 by automatic

addition of 12.5% (wt/vol) NH₃ (typically 200 to 250 ml). An antifoam agent (Sigma Antifoam 204) was added at an initial concentration of 0.005% (vol/vol) and then on demand throughout the fermentation (typically 3 ml). The methanol concentration in the fermentor was monitored by online analysis of the head-space gas with a mass spectrometer (Balzers Omnistar GSD 300 02). The head-space gas was transferred from the fermentor to the mass spectrometer in insulated stainless steel tubing with an inside diameter of 1/8 in. (heated to 60°C) at a flow rate of about 30 ml/min. The methanol concentration in the medium was maintained at 150 mM by automatic addition of MeOH feed solution on methanol demand. MeOH feed solution contained 50 ml of CKNFD trace metals per liter of methanol. CKNFD trace metals contained 344 mM MgCl₂, 78.5 mM FeCl₂, 50.5 mM MnCl₂, 1.53 mM CuCl₂, 1.60 mM CoCl₂, 1.57 mM Na₂MoO₄, 3.23 mM ZnCl₂, and 100 ml/liter HCl. Cell dry weight was calculated by using a conversion factor of 0.31 g (dry weight) of cells/liter per OD₆₀₀ unit (calculated as an average based on measurements of OD₆₀₀ and the dry weight of cells for the fermentation trials). Data for the dry weight of cells were determined by using multiple cell samples collected at OD₆₀₀ ranging from 65 to 107, and the standard deviation of the calculated conversion factor was \pm 0.02 (data not shown). The specific growth rate was calculated by linear regression analysis of semilogarithmic plots of biomass concentration versus time based on data points for the exponential growth period (biomass concentration, less than 15 g/liter). All fermentations were run until the CO₂ content of the exhaust gas was close to zero (no cell respiration).

Due to the significant increase in culture volume throughout fermentation, all the biomass and amino acid concentrations were corrected for the increase in volume and subsequent dilution by multiplying the measured concentration by the culture volume at the time of sampling divided by the original culture volume. The correction factors used for endpoint samples were between 1.5 and 1.7, and the actual concentrations of amino acids and biomass measured in the bioreactors were therefore accordingly lower.

Measurement of amino acids and ammonia. Amino acids were quantified as described by Skjerdal et al. (36), using a buffer containing 0.02 M sodium acetate and 2% tetrahydrofuran (pH 5.9). Ammonia was measured with a Spectroquant ammonium test kit (Merck) used according to the manufacturer's instructions (the samples were diluted 1:1,000 and 1:10,000 before analysis).

PCR-assisted cloning of *B. methanolicus* *asd*, *dapG*, *dapA*, and *lysC* genes. The putative AKI- and AKIII-encoding genes were PCR amplified from *B. methanolicus* MGA3 total DNA by using degenerate primers based on the DNA sequences of the *yclM*, *mlpA*, *asd*, *dapG*, and *ymfA* genes of *Bacillus licheniformis*, *Bacillus halodurans*, *Bacillus cereus*, *Listeria innocua*, *Listeria monocytogenes*, and *B. subtilis* (GenBank accession numbers AE017333, BA000004, NC_004722, AL592022, AL591824, and AL009126, respectively). The DNA fragments of

MGA3 covering *asd*, *dapG*, and *dapA* were PCR amplified as overlapping fragments by using primer mlpA-PPS-1F together with *asd*-PPS-1R (which yielded a 3.9-kb fragment) and primer *asd*-PPS-1F together with *yfmA*-PPS-1R (which yielded a 3.3-kb fragment). The fragments were sequenced by primer walking.

A central region of *yclM* was PCR amplified and partially sequenced by using degenerate primers based on conserved regions within *yclM*. Total DNA of MGA3 was digested with EcoRI, and this was followed by heat inactivation of the restriction enzyme. The material was diluted and ligated for 72 h at 4°C. Primers *yclM*-PPS-1F and *yclM*-PPS-1R, both pointing outward from the previously PCR-amplified *yclM* region, were used to PCR amplify a 3-kb DNA fragment using the ligation mixture as the template. This DNA fragment was sequenced by primer walking.

Construction of *B. methanolicus* AK expression vectors. DNA fragment A, including the methanol dehydrogenase (*mdh*) coding region, was PCR amplified from pTB1.9mdhL by using primers *mdh*-CDS-F1 and pTB1.9-R1. The putative *mdh* promoter region was PCR amplified using the same template and primers *mdh*-prom-F1 and *mdh*-prom-R1, which yielded DNA fragment B. pTB1.9mdhL was digested with PstI and BamHI, and the vector backbone fragment was ligated with BamHI/PciI-digested DNA fragment B and PciI/PstI-digested DNA fragment A. Two PciI sites were removed from the resulting vector by PCR amplification of the vector as two fragments (fragment 1 using primers mp-*mdh*-P2-F1 and mp-*mdh*-P2-R2 and fragment 2 using primers mp-*mdh*-P2-F2 and mp-*mdh*-P2-R1). The two fragments were end digested with SphI and KpnI and ligated to obtain pTB1.9mp-*mdh*, which had a PciI site between the *mdh* upstream and coding regions for simplified fusion of coding regions to the *mdh* promoter. Insertion of the PciI site changed the four nucleotides upstream of the *mdh* start codon from the original nucleotides AAGA to CATG.

The coding regions of *dapG*, *lysC*, and *yclM* were PCR amplified from *B. methanolicus* MGA3 total DNA by using primers *dapG*-CDS-F1 and *dapG*-CDS-R1, primers *lysC*-CDS-F1 and *lysC*-CDS-R1, and primers *yclM*-CDS-F1 and *yclM*-CDS-R1, respectively. The resulting PCR fragments, all of which had a PciI site partially overlapping a GTG start codon, were end digested with PciI and KpnI and used to replace the *mdh* coding region of pTB1.9mp-*mdh*, which yielded the vectors pTB1.9mp-*dapG*, pTB1.9mp-*lysC*, and pTB1.9mp-*yclM*, respectively. In this process, the original ATG start codons of *dapG* and *yclM* were changed to GTG. A PstI/EcoRI fragment of pTB1.9mp-*lysC* including the *mdh* promoter and the *lysC* coding region was inserted into the corresponding sites of pHP13, which yielded pHP13mp-*lysC* (7.3 kb). The *lysC* coding region of pHP13mp-*lysC* was exchanged with the *dapG* and *yclM* coding regions by inserting a PstI/KpnI fragment of pTB1.9mp-*dapG* and a SpeI/KpnI fragment of pTB1.9mp-*yclM* into the corresponding sites of pHP13mp-*lysC*, which yielded pHP13mp-*dapG* (7.1 kb) and pHP13mp-*yclM* (7.2 kb), respectively. To verify that PCR amplification was accurate, the *dapG*, *lysC*, and *yclM* coding regions and 600-bp upstream untranslated regions were sequenced. One base pair substitution in the putative *mdh* promoter region was discovered in all the constructs compared to the previously described sequence of *mdh* (GenBank accession number AY386314; T at position 15496 changed to C, 245 nucleotides upstream of the *mdh* start codon).

Construction of vectors for expression of six-His-tagged AK proteins in *E. coli*.

(i) **pSB1 for expression of six-His-tagged AKI.** The *dapG* coding region was PCR amplified from *B. methanolicus* MGA3 total DNA by using PCR primers *dapG*-fwd1 and *dapG*-rev1. The resulting 1,259-bp PCR product was end digested with NdeI and XhoI (restriction sites are indicated in Table 2), and ligated into the corresponding sites in frame with the six-His tag sequence of plasmid pET21a(+), which yielded plasmid pSB1.

(ii) **pSB3 for expression of six-His-tagged AKII.** The *lysC* coding region was PCR amplified from *B. methanolicus* MGA3 total DNA by using PCR primers *lysC*-fwd1 and *lysC*-rev1. The resulting 1,257-bp PCR product was end digested with SalI and partially digested with NdeI (restriction sites are indicated in Table 2), and the resulting DNA fragment was ligated into the NdeI and XhoI sites in frame with the six-His tag sequence of plasmid pET21a(+), which yielded plasmid pSB3.

(iii) **pSB4 for expression of six-His-tagged AKIII.** The *yclM* coding region was PCR amplified from *B. methanolicus* MGA3 total DNA by using PCR primers *yclM*-fwd1 and *yclM*-rev1. The resulting 1,385-kb PCR product was end digested with NdeI and XhoI (restriction sites are indicated in Table 2) and ligated into the corresponding sites in frame with the six-His tag sequence of plasmid pET21a(+), which yielded plasmid pSB4.

All the vectors constructed were verified by DNA sequencing before transformation into the expression host *E. coli* ER2566.

Affinity purification of AK proteins. Frozen pellets of recombinant *E. coli* ER2566 cells were thawed on ice and resuspended in native binding buffer (50 mM Na₂H₂PO₄, 0.5 M NaCl, 10 mM imidazole, 1 mM dithiothreitol; pH 8.0), and cells were disrupted by sonication as described elsewhere (6). Recombinant

TABLE 2. PCR primers used in this study

Primer	Sequence (5'-3') ^a
mlpA-PPS-1FTCTACCTTCGTTGAGGAAGA
asd-PPS-1RCACTCCTGAACGGTTAATCC
asd-PPS-1FTGAGCAGACAAGAGCGATTA
yfmA-PPS-1RATAGATCGCTCCGATATGGT
yclM-PPS-1FCCTGTGATCGGAATTGCAAGTGATAAAGG ATTCTG
yclM-PPS-1RATCTTCGTTCCAGGAGCCGATGGATTATT GGTGT
mdh-CDS-F1TCGACATGTGACAACAACTTTTTTC
pTB1.9-R1ACGCATACCATTTTGAACGATGACC
mdh-prom-F1GCCGGATCTCGAGTTTCATTAAGAG CAGC
mdh-prom-R1CGCGACATGTACTACCTCCTATTTATG
mp- <i>mdh</i> -P2-F1CGCGCATGCGTTTCAATGAAGATCC
mp- <i>mdh</i> -P2-R1TTAAGCATGCAAAAGGCCAGGAACCG
mp- <i>mdh</i> -P2-F2TTTTGGTACCCGCATAGGCTAGAG
mp- <i>mdh</i> -P2-R2GGGCGGTACCTTATTCTTTAGTCTATC
lysC-CDS-F1CCGAACATGTGGGATTAATTGTCC
lysC-CDS-R1TTCCGGTACCCAGCAAATTGAACAGC
dapG-CDS-F1GCGCACATGTGAAAATTATCGTTCAAAA TTCGG
dapG-CDS-R1GCTAGGTACCGCTCCTCCTATTCTATC
yclM-CDS-F1GCGCACATGTGAAAGTAGCGAAGTTTG AGGTTCTTC
yclM-CDS-R1GCTAGGTACCGAGTGTTCACACCCAAA TTCG
dapG-fwd1AAAGGTACCCATATGAAAATTATCGTTCA AAAATTCGG
dapG-rev1AACTCGAGTTCTATCCGTTCAAACCTCCAG
lysC-fwd1CTACATGTCCATATGGGATTAATTTGTCCA AAAGTTTGG
lysC-rev1CGTGAAGTCGACGCGTTCCGATTTAACAG CGGATC
yclM-fwd1ATCGCTATACATATGAAAGTAGCGAAGTT TGGAGGTTTC
yclM-rev1AACTCGAGAACATTTACAGGAACCTGGAGC

^a The underlined nucleotides are restriction sites used for simplified cloning of PCR products.

proteins were purified by a one-step purification procedure under native conditions by using an Ni-nitrilotriacetic acid agarose kit (Qiagen) according to the manufacturer's instructions. AK proteins were eluted by using 10 ml of native elution buffer (similar to native binding buffer but with 250 mM imidazole) and finally dialyzed against dialysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol; pH 8.0) at 4°C overnight. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33), and the purity of each AK protein was estimated by visual inspection of the resulting images. Protein concentrations were determined spectrophotometrically by using the Bradford assay at 595 nm (Bio-Rad) and bovine serum albumin as a reference. The purified enzymes were immediately subjected to biochemical analyses or, alternatively, were snap frozen in liquid N₂ and stored at -80°C.

Preparation of *B. methanolicus* crude cell extracts and concomitant measurement of AK activity. Crude cell extracts were prepared by using a protocol based on the protocol described by Brautaset et al. (4). *B. methanolicus* cells were grown in MeOH₂₀₀ medium to exponential phase (OD₆₀₀, 1.9 to 2.1), and 20 ml of the cell culture was harvested by centrifugation (3,200 × g, 10 min, 10°C). The supernatant was discarded, and the cells were resuspended in 20 ml high-salt buffer (the salt buffer used for MeOH₂₀₀ medium at a concentration of 1×; pH 7.2). Three milliliters of the resuspended culture was centrifuged (3,200 × g, 10 min, 10°C), the supernatant was discarded, and the pellet was frozen and stored at -20°C. The cells were thawed on ice and resuspended in 3 ml AK assay buffer (50 mM potassium phosphate, 10 mM MgSO₄; pH 7.5), lysozyme (1 mg/ml) was added, and the cells were sonicated for 3 min (Branson Sonifier 250; output control 3; 30% duty cycle). Cell debris was removed by centrifugation (3,200 × g, 20 min, 4°C), and the supernatant was collected as crude cell extract and stored on ice for subsequent enzyme activity and total cell protein analyses. AK activity was determined by using the formation of aspartyl hydroxamate from hydroxylamine (2). The reaction mixture contained 400 μl reaction buffer (0.5 M Tris-

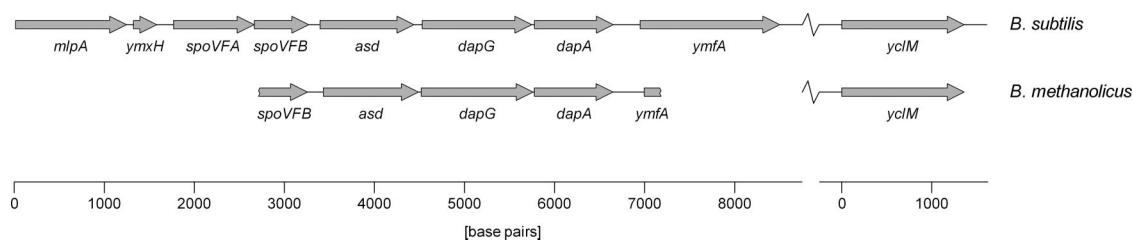


FIG. 2. Genetic organization of the partial *dap* operon and *yclM* of *B. methanolicus* MGA3 compared to the corresponding genes of *B. subtilis*.

HCl, 2 M KCl; pH 8.0), 200 μ l hydroxylamine solution (2 M hydroxylamine, pH 8.0), 100 μ l AAM solution (0.1 M L-aspartic acid, 0.1 M ATP, 0.1 M MgCl₂, 0.2 M Tris-HCl; pH 8.0), and 300 μ l sample diluted in AK assay buffer. The reaction mixture was incubated at 50°C for 20 min before the reaction was terminated by addition of 1 ml of an Fe solution (10% [wt/vol] FeCl₃ and 3% [vol/vol] trichloroacetic acid in 0.7 M HCl, sterile filtered before use). Formation of aspartyl hydroxamate was measured immediately at 540 nm using a spectrophotometer (Shimadzu, UV1700). Assays in which the sample was replaced by aspartyl hydroxamate standards were performed to correlate absorbance with aspartyl hydroxamate concentration. One unit of AK activity was defined as the amount of enzyme needed to produce 1 μ mol aspartyl hydroxamate per min under the conditions described above (2). Protein concentrations were determined by the method of Bradford (Bio-Rad), using bovine serum albumin as a standard. AK assays were done in triplicate, and analyses of the protein concentration were done in quadruplicate. The uncertainty for AK specific activity was calculated using the general formula for error propagation (37) based on average values and standard deviations of measured AK activities and protein concentrations.

Biochemical analyses of purified AK proteins. The purified AK proteins were used in kinetic experiments by performing the AK assay essentially as described above. The ATP concentration was maintained at a saturating level (10 mM), while the concentration of L-aspartate was varied (0.5 to 20 mM) for determination of the values for the K_m for L-aspartate ($K_{m,ASP}$) and V_{max} . Assays were performed at 50°C, and the time points used for measurement of activity were 0, 1, 5, 10, and 20 min for AKI and 0, 1, 2, 3, and 5 min for AKII and AKIII. By using 1 μ g of AK enzyme in the reaction mixtures, the slopes for activity versus time were linear in each period measured. $K_{m,ASP}$ and V_{max} values were calculated by using nonlinear regression with the Microsoft Excel solver-tool to fit the measured data to the Michaelis-Menten equation. The values obtained from the regression were then compared to the values obtained from Lineweaver-Burk and Hanes-Woolf plots to ensure that the global minimum, not a local minimum, had been found.

For the AK inhibition studies the proteins were initially assayed for AK activity in the presence of a fixed concentration (total concentration, 5 mM) of the potential inhibitor compounds *meso*-DAP, L-lysine, L-isoleucine, L-methionine, L-threonine, and L-lysine plus L-threonine. In cases where significant allosteric inhibition was observed, the experiments were extended by using various concentrations (0.01 to 10 mM) of the relevant inhibitors. Based on the data obtained in the latter experiments, 50% inhibitory concentrations (IC₅₀) were determined, which were the concentrations of inhibitor that decreased the rate of the AK-catalyzed reaction by 50% (8).

Nucleotide sequence accession numbers. The DNA sequences of *yclM* and the partial *dap* operon including *asd*, *dapG*, and *dapA* of *B. methanolicus* MGA3 reported in this paper have been deposited in the GenBank nucleotide sequence database under accession numbers FJ485943 and FJ485942, respectively.

RESULTS

***yclM* encodes a putative AKIII in *B. methanolicus*.** Previous AK inhibition studies with *B. methanolicus* cell extracts suggested that there were three AK isozymes that were inhibited like those of *B. subtilis* (35). So far, only *lysC* encoding AKII has been cloned and sequenced, so the two remaining AK-encoding genes had to be cloned to fully investigate the role of AK in L-lysine production in *B. methanolicus*. A putative *yclM* gene was successfully PCR amplified from MGA3 total DNA using a set of degenerate primers (see Materials and Methods).

A 2,012-bp DNA fragment was sequenced and shown to contain the expected *yclM* coding region and 605 bp of upstream sequence (Fig. 2). The deduced primary sequence (455 amino acids) of the *yclM* gene product exhibited the highest overall level of identity to the *B. licheniformis* AKIII sequence (74%) and was 71% identical to the *B. subtilis* strain 168 AKIII sequence, whose AK activity was experimentally verified previously (20). It had low levels of primary sequence identity to both AKI and AKII of *B. subtilis* (23 and 25%, respectively). Together, these data suggest that the *B. methanolicus yclM* gene encodes a putative AKIII isozyme.

***asd*, *dapG*, and *dapA* encoding a putative aspartate semialdehyde dehydrogenase, AKI, and dihydrodipicolinate synthase, respectively, are organized in a putative *dap* operon in *B. methanolicus*.** In *B. subtilis*, *asd*, *dapG*, and *dapA* are located in the *dap* operon (7). By aligning known sequences of several related species (see Materials and Methods), we noted that the organization of genes upstream of, inside, and downstream of the *dap* operon is conserved, and we hypothesized that this genetic organization was similar in *B. methanolicus*. By using degenerate primers based on conserved regions within *mlpA*, *asd*, *dapG*, and *ymfA* (Fig. 2), we PCR amplified overlapping DNA fragments covering a partial, putative MGA3 *dap* operon. Altogether, a 4,465-bp DNA region was sequenced, which comprised the putative genes *asd*, *dapG*, and *dapA* in addition to parts of the upstream putative *spoVFB* gene and downstream putative *ymfA* gene (Fig. 2). The deduced gene products of *asd*, *dapG*, and *dapA* (351, 413, and 290 amino acids, respectively) displayed the highest levels of primary sequence identity to aspartate semialdehyde dehydrogenase, AKI, and dihydrodipicolinate synthase of *Bacillus* sp. strain NRRL B-14911 (76, 85, and 79%, respectively). The deduced *dapG* gene product was 68% identical to AKI of *B. subtilis* strain 168, whose AK activity was experimentally verified (7), while the levels of primary sequence identity to AKII and AKIII of *B. subtilis* were low (38 and 24%, respectively). This observation suggests that the *B. methanolicus dapG* gene encodes a putative AKI isozyme.

Construction of a cassette cloning and expression system for *B. methanolicus*. We previously observed high transcription levels of *mdh* encoding methanol dehydrogenase in *B. methanolicus* (4, 17). We therefore constructed a cassette expression system as a tool for simplified gene overexpression in *B. methanolicus* based on *mdh* and the *E. coli*-*B. methanolicus* shuttle vector pHP13. By introducing a unique restriction site partially overlapping the *mdh* start codon and unique restriction sites downstream of the coding region, this cassette system provided

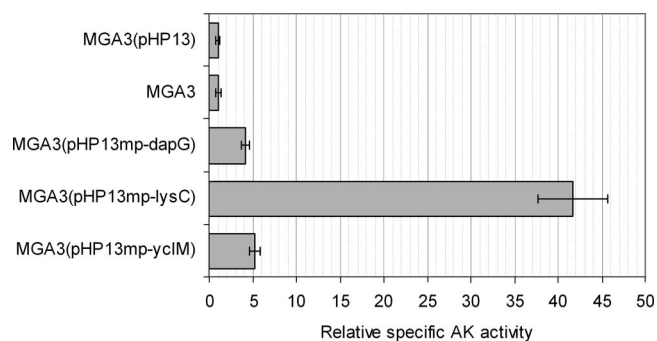


FIG. 3. Total AK specific activity in crude extracts of shake flask cultures of *B. methanolicus* MGA3 and recombinant strains overexpressing *dapG*, *lysC*, and *yclM*. For each strain, the AK activity is given relative to the activity of control strain MGA3(pHP13) (which was defined as 1), which had a measured total AK specific activity of 0.05 U/mg protein.

a one-step cloning system for in-frame fusion of any coding region to the *mdh* promoter region and ribosome binding site.

Recombinant expression of *dapG* and *yclM* confirmed that they encode AK activity. For overexpression of AK genes in *B. methanolicus*, we constructed the expression vectors pHP13mp-*dapG*, pHP13mp-*lysC*, and pHP13mp-*yclM*, in which *dapG*, *lysC*, and *yclM* were under expression control of the *mdh* promoter and its ribosome binding site. Thus, in these constructs, the AK-encoding genes were released from any original transcription regulation by-products of the aspartate pathway. These expression vectors were introduced into wild-type strain MGA3, and we used MGA3(pHP13) as a control strain. We compared the total specific AK activities in crude extracts prepared from MGA3 and the recombinant strains grown in shake flasks in defined methanol medium. The results (Fig. 3) show that crude extracts of the recombinant strains overexpressing putative AKI and AKIII (encoded by *dapG* and *yclM*, respectively) and AKII (encoded by *lysC*) exhibited 4- to 40-fold-higher AK activities in vitro than crude extracts of the control strain. These results confirmed the deduced biochemical function of the *dapG* and *yclM* gene products. As expected, wild-type strain MGA3 and control strain MGA3(pHP13) expressed similar AK activities. Interestingly, *lysC* overexpression resulted in 10-fold-higher AK activity than overexpression of *dapG* and *yclM*. For all samples, similar in vitro AK activities could be obtained for different dilutions of the crude extract (data not shown), indicating that under the conditions tested, feedback regulation did not affect the results of the enzyme assay.

Overexpression of *dapG*, *lysC*, and *yclM* leads to increased L-lysine production in *B. methanolicus* MGA3. In order to evaluate the effect of increased expression of *dapG*, *lysC*, and *yclM* on L-lysine production in wild-type *B. methanolicus*, we conducted high-cell-density fed-batch fermentation trials with defined methanol medium and the established recombinant strains. A fed-batch process with a controlled methanol concentration (150 mM) in the growth medium prevented toxic effects on cells that could have resulted from methanol concentrations that were too high or too low (17). Extracellular amino acid production was monitored throughout the fermentations. To compare different bioreactor trials, the biomass concentrations and amino acid production values reported

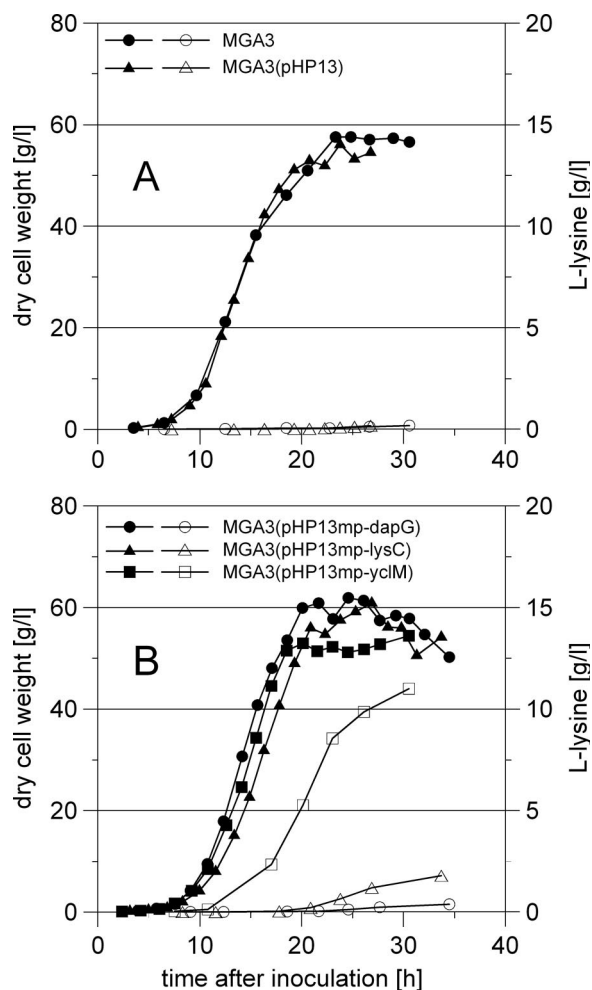


FIG. 4. Growth and L-lysine production in fermentation trials for (A) wild-type strain MGA3 and control strain MGA3(pHP13) and (B) recombinant MGA3 strains overexpressing *dapG*, *lysC*, or *yclM*. Filled symbols, cell dry weight; open symbols, L-lysine production (volume corrected). Throughout the fermentations, the methanol level in the medium was maintained at 150 mM by automatic addition of methanol.

here were also volume corrected to compensate for dilution caused by feeding throughout the fermentation (see Materials and Methods).

Under the conditions tested, the maximum biomass concentration of wild-type strain MGA3 was 58 g/liter after 23 h with an initial specific growth rate of 0.49 h^{-1} . The final concentration of L-lysine produced by MGA3 was 0.18 g/liter, in agreement with previous results (6, 34). Control strain MGA3(pHP13) was similar to the wild type with respect to the specific growth rate, the maximum cell density, and L-lysine production (Fig. 4 and Table 3). Interestingly, all the recombinant strains overexpressing *dapG*, *lysC*, or *yclM* produced more L-lysine than the control strain, and they also retained similar specific growth rates (Table 3). The most dramatic effect on L-lysine production was observed with strain MGA3(pHP13mp-*yclM*), which produced more than 60-fold more L-lysine (11 g/liter, volume corrected) than the control strain (Fig. 4 and Table 3). The recombinant strains overexpressing *dapG* and *lysC* showed 2- and 10-fold in-

TABLE 3. Initial specific growth rates, maximum dry weights of cells, and L-lysine production for *B. methanolicus* wild-type strain MGA3 and recombinant strains overexpressing *dapG*, *lysC*, and *yclM*

Strain	Specific growth rate (h ⁻¹)	Dry wt of cells (g/liter) ^a	L-Lysine production (g/liter) ^a	L-Lysine concn in growth medium (g/liter) ^b
MGA3	0.49	58	0.18	0.12
MGA3(pHP13)	0.49	56	0.18	0.12
MGA3(pHP13mp-dapG)	0.50	62	0.38	0.23
MGA3(pHP13mp-lysC)	0.46	61	1.8	1.1
MGA3(pHP13mp-yclM)	0.50	54	11	7.0

^a The biomass and L-lysine production values were corrected for dilution caused by feeding throughout the fermentation in order to compare results from different bioreactor trials (see Materials and Methods).

^b L-Lysine concentration in growth medium (no volume correction).

creases in L-lysine production, respectively. All strains were similar with respect to L-glutamate production (48 to 52 g/liter), and the levels of other relevant aspartate pathway products remained low; the concentration of L-methionine was <0.5 g/liter, and the concentration of L-threonine was below the detection limit (0.1 g/liter). To verify the reproducibility of the results, fermentation trials with both MGA3(pHP13) and MGA3(pHP13mp-yclM) were conducted twice. The amino acid concentration and biomass concentration (as monitored by optical density) varied less than 10% for the parallel fermentation trials at any sampling point, and the calculated specific growth rate did not vary more than ± 0.02 h⁻¹.

Kinetic properties of the purified AK proteins. In order to better understand the in vivo data obtained after AK overexpression in *B. methanolicus* (see above), the three AK isozymes were recombinantly expressed in *E. coli*, purified, and kinetically characterized. SDS-PAGE analysis indicated that all of the proteins were expressed at high levels, and the proteins were purified by affinity chromatography to >90% purity, as judged by inspection of SDS-PAGE gels (data not shown). The purified AK proteins (1 μ g) were then subjected to kinetic analyses in vitro with various aspartate concentrations, and the results are summarized in Table 4. Interestingly, both the $K_{m,ASP}$ values (1.9 to 5.0 mM) and the V_{max} values (47 to 58 μ mol/min/mg protein) were in the same ranges for all three AK isozymes under these conditions. The V_{max} of the *B. subtilis* AKII protein has been reported to be 30 μ mol/min/mg protein (19), the V_{max} of AKIII has been reported to be 0.62 μ mol/min/mg protein (20), and to our knowledge the V_{max} of the *B. subtilis* AKI protein has not been determined. The $K_{m,ASP}$ values for *B. subtilis* are 3.0 mM for AKI (38), 10 mM for AKII (19), and 20 mM for AKIII (20). However, since the previous analyses with *B. subtilis* were carried out under different conditions, a direct comparison must be made with care.

Thus, the kinetic data could not provide a direct explanation for the different in vivo effects that the overexpression of each of the three AK isozymes had on L-lysine production in *B. methanolicus*. We therefore analyzed the purified AK proteins to examine allosteric regulation in vitro.

Allosteric inhibition of the purified AK proteins. To determine whether there was any allosteric regulation of the purified AK proteins, AK assays were initially performed in the

TABLE 4. Kinetic parameters for the *B. methanolicus* AKI, AKII, and AKIII proteins

Enzyme	$K_{m,ASP}$ (mM)	V_{max} (μ mol/min/mg protein)
AKI	5.0	47
AKII	1.9	58
AKIII	3.2	49

presence of 5 mM of each of the potential inhibitor compounds L-isoleucine, L-methionine, L-threonine, L-lysine, and *meso*-DAP (Table 5). L-Isoleucine and L-methionine had no inhibitory effect on the catalytic activity of any of the AK isozymes under these conditions. AKI and AKII were strongly inhibited by *meso*-DAP and by L-lysine, respectively, and the data were in agreement with analogous data reported for *B. subtilis* (28, 32). AKIII was inhibited to some extent by L-threonine and also by L-lysine, and synergistic inhibition was observed when these two amino acids were both present in the assay mixture. The latter result is similar to results reported previously for AKIII of *B. subtilis* (13). We also noticed that AKIII catalytic activity increased 1.3-fold in the presence of added L-isoleucine or L-methionine and increased 1.5-fold in the presence of *meso*-DAP (Table 5). The biological impact of the latter observations is unknown.

IC_{50} s were calculated by using individual inhibitor concentrations between 0.01 and 10 mM, as described elsewhere (8). The results (Table 5) showed that AKI and AKII had high affinities for the inhibitors *meso*-DAP and L-lysine (IC_{50} s, 0.1 and 0.3 mM, respectively). AKIII, on the other hand, had a considerably lower affinity for the allosteric inhibitors L-threonine (IC_{50} , 4 mM) and L-lysine (IC_{50} , 5 mM) when they were tested individually. To further investigate the observed synergistic inhibition described above, an additional series of assays were performed with AKIII in the presence of both L-lysine and L-threonine at various individual concentrations ranging from 0.01 to 10 mM (data not shown). The results of these experiments demonstrated that AKIII activity was strongly inhibited (the activity was less than 20% of the activity without inhibitors) when these two amino acids were both present at individual concentrations of 0.25 mM or higher.

DISCUSSION

Four genes (*asd*, encoding aspartate semialdehyde dehydrogenase; *dapA*, encoding dihydrodipicolinate synthase; *dapG*, encoding AKI; and *yclM*, encoding AKIII) of the aspartate pathway in *B. methanolicus* MGA3 were cloned and sequenced. Three of these genes, *asd*, *dapG*, and *dapA*, were organized as a *dap* operon similar to the operon in *B. subtilis* (7), while *yclM* was localized elsewhere on the *B. methanolicus* chromosome (Fig. 2). Together with the previously described AKII gene *lysC* (35), *dapG* and *yclM* form a set of three putative AK genes, and here we investigated the role of these three AK genes in overproduction of L-lysine in *B. methanolicus*. First, a cassette expression system based on the *B. subtilis*-*E. coli* shuttle plasmid pHP13 (15) was constructed, and the *dapG*, *lysC*, and *yclM* coding regions could be individually placed under transcriptional and translational control of the methanol dehydrogenase (*mdh*) promoter and ribosome

TABLE 5. Results of inhibition studies for purified AKI, AKII, and AKIII proteins

Enzyme	AK activity in the presence of inhibitors (%) ^a							IC ₅₀ ^c	
	None	Ile	Met	Thr	Lys	<i>meso</i> -DAP	Lys + Thr ^b	Inhibitor	Concn (mM)
AKI	100	115	95	95	110	15	95	DAP	0.1
AKII	100	105	90	105	5	95	4	Lys	0.3
AKIII	100	130	125	35	50	150	0.8	Thr	4
AKIII								Lys	5

^a The values are the percentages of remaining AK activity; 100% activity was defined as the activity of the AK protein obtained without any inhibitor compound. The AK assays were performed in the presence of the various inhibitor compounds at a concentration of 5 mM. Ile, L-isoleucine; Met, L-methionine; Thr, L-threonine; Lys, L-lysine.

^b The concentration of each compound was 5 mM.

^c The IC₅₀ values were determined when significant inhibition was observed, as follows: for AKI, with various *meso*-DAP concentrations; for AKII, with various Lys concentrations; and for AKIII, with various Thr and Lys concentrations (see Materials and Methods).

binding site, respectively (17). Recombinant *B. methanolicus* MGA3 cells harboring these expression vectors displayed 4- to 40-fold-higher AK specific activities, confirming that the cloned *dapG* and *yclM* genes encode active AK proteins.

Analyses of the recombinant *B. methanolicus* strains under high-cell-density conditions demonstrated that they all displayed increased L-lysine production (Table 3) compared to the wild type (0.18 g/liter, volume corrected). Overexpression of *dapG* resulted in only a twofold increase in L-lysine production (0.38 g/liter, volume corrected), and this result is in good agreement with the in vitro biochemical data showing that there was strong inhibition of AKI catalytic activity by *meso*-DAP (IC₅₀, 0.1 mM). Like the *B. subtilis* enzyme (30, 32), *B. methanolicus* AKI is allosterically inhibited by *meso*-DAP at concentrations lower than the concentration needed for efficient conversion of this intermediate into L-lysine by diaminopimelate decarboxylase. Interestingly, the *K_m* of *B. methanolicus* diaminopimelate decarboxylase for *meso*-DAP has been reported to be 0.8 mM (27).

Overexpression of *lysC* resulted in a ~10-fold increase in L-lysine production (1.8 g/liter, volume corrected). This result was surprising, and it could not be predicted based on the in vitro biochemical data showing that AKII is strongly inhibited by L-lysine (IC₅₀, 0.3 mM), similar to findings for AKII of *B. subtilis* (28). However, the total AK specific activity measured in crude extracts of MGA3(pHP13mp-lysC) was about 40-fold higher than that for the wild-type strain (Fig. 3), indicating that there was high-level recombinant expression of AKII. Accordingly, high intracellular L-lysine concentrations may be needed to effectively inhibit all AKII activity in the recombinant cells. Moreover, purified AKII retained about 5% of its catalytic activity (Table 5) in vitro even when relatively high L-lysine concentrations were added (5 mM). Possibly, the lack of complete allosteric inhibition by L-lysine, together with a high level of expression, may partially explain the observed 10-fold-higher level of L-lysine in recombinant strain MGA3(pHP13mp-lysC). Finally, we could not rule out the possibility that the in vivo intracellular L-lysine concentration was low compared to the extracellular concentration, as predicted for, e.g., *C. glutamicum* (12). Interestingly, the high total AK specific activity measured in crude extracts of MGA3(pHP13mp-lysC) compared to the corresponding data obtained for strains MGA3(pHP13mp-dapG) and MGA3(pHP13mp-yclM) was difficult to explain based on the overall similar kinetic parameters of the corresponding AK

isozymes that were overexpressed (Table 4). The biological reason for this presumed discrepancy is unclear.

Overexpression of *yclM* had by far the most positive effect in vivo on L-lysine production in *B. methanolicus* (11 g/liter, volume corrected). The concomitant in vitro analyses demonstrated that AKIII was inhibited by L-threonine and by L-lysine, and the inhibition was particularly strong when these two amino acids were present simultaneously (Table 5). The catalytic activity of *B. subtilis* AKIII was reduced to about 25% with 20 mM L-lysine in vitro (13) when the L-threonine concentration was greater than 1 mM. Our results may indicate that the synergistic inhibition by L-lysine and L-threonine of AKIII catalytic activity in *B. methanolicus* is strong, as individual inhibitor concentrations of only 0.25 mM were sufficient to cause inhibition of >80% of the AKIII activity in vitro. Therefore, the in vitro kinetic data do not explain the elevated lysine levels obtained with AKIII overexpression in this organism. The observed low level of extracellular production of L-threonine by *B. methanolicus* strains (below the detection limit of 0.1 g/liter) suggests that a combination of AKIII overexpression with a low intracellular L-threonine level may have led to the observed 60-fold increase in L-lysine production in MGA3(pHP13mp-yclM).

To our knowledge, a substantial increase in L-lysine production upon overexpression of wild-type AK has not been reported previously. Overexpression of wild-type AK in a *Brevibacterium flavum* mutant resistant to the L-lysine analogue S-(2-aminoethyl)cysteine resulted in a marginal (33%) increase in L-lysine production (24). AK overexpression had no effect on L-lysine production and was detrimental to *C. glutamicum* growth in defined medium (9, 22). Interestingly, overexpression of the AK proteins presumably had no negative effects on the specific growth rate of the *B. methanolicus* cells under these conditions (Fig. 4).

We noticed that all the L-lysine-overproducing recombinant *B. methanolicus* strains produced low levels of both L-methionine and L-threonine. It has been suggested that in *C. glutamicum* a difference in the kinetic properties of dihydrodipicolinate synthase and homoserine dehydrogenase controls the split between the L-lysine branch and the L-methionine/L-threonine branch of the aspartate pathway (11) (Fig. 1). It has been suggested that in *B. subtilis* mRNA secondary structures in the *asd-dapG* intergenic region reduce *dapG* and *dapA* translation relative to *asd* translation (7). In silico analysis of the *B. meth-*

anolicus asd-dapG intergenic region did not identify any probable mRNA secondary structure (data not shown). Without such a mechanism, *B. methanolicus* may be unable to efficiently downregulate dihydrodipicolinate synthase expression when L-lysine is abundant, possibly partially explaining the exclusive L-lysine overproduction obtained here. Moreover, no homoserine dehydrogenase gene has been cloned from *B. methanolicus*.

The kinetic parameters determined for the three *B. methanolicus* AK isozymes indicated that these proteins are relatively similar with respect to both their $K_{m,ASP}$ values and their V_{max} values (Table 4). These parameters could therefore not explain the different effects that overexpression of these three enzymes had on levels of L-lysine production in *B. methanolicus*. The levels of L-lysine production observed in the recombinant strains seemed to be largely determined by the differences in allosteric regulation of the AK enzymes.

The present work provided further insight into AK regulation and function and also provided valuable information for engineering L-lysine-producing *B. methanolicus* strains. The recombinant strains and genetic constructs described here may provide a basis for future metabolic engineering to obtain further increases in L-lysine production in this methylotrophic bacterium.

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