Overproduction of L-Lysine from Methanol by 
*Methylobacillus glycogenes* Derivatives Carrying a Plasmid with a Mutated *dapA* Gene

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Methanol, a compound easily synthesized from natural gas, is an attractive raw material for microbial industries. Using methanol as a carbon source, production costs could be greatly reduced and purification and waste treatment processes could be simplified. A number of production processes for useful compounds with methylotrophs (methanol-utilizing microorganisms) have been studied. Production of single-cell protein derived from AL119. Enzymatic analysis revealed that aspar- 

Many attempts have also been made to use methanol in amino acid production; however, successful studies were limited. Lee et al. (9) reported the production of 47 g of L-lysine per liter by a gram-positive methylotroph, Bacillus methanoli-
cus. Izumi et al. (7) reported efficient conversion of glycine to L-serine by a gram-negative methylotroph, *Hyphomicrobium methylivororum*. Breeding of amino acid-producing mutants requires isolation of mutants desensitized in the feedback regulation of the biosynthetic enzymes for a desired amino acid and blocked in metabolic pathways of by-products. Isolation of mutants from methylotrophs is usually difficult, due to unknown reasons, preventing the use of methanol in amino acid production.

We isolated L-glutamic acid-hyperproducing mutants from the obligate methylotrophs *Methylobacillus glycogenes* ATCC 21276 and 21371 and subsequently derived L-lysine- and L-threonine-producing mutants from L-glutamic acid-producing mutants (13). An L-threonine-producing mutant, AL119, was isolated among mutants resistant to both DL-α-amino-β-hydroxy-valeric acid and L-lysine, and an L-lysine- and L-threo-

As is the case for other gram-negative methylotrophs, isolation of mutants from *M. glycogenes* was not easy. In order to efficiently enhance amino acid production, we tried to employ a recombinant technology. The *hom* and *thrC* genes, which encode homoserine dehydrogenase and threonine synthase in the L-threonine biosynthesis pathway, respectively, were cloned from *M. glycogenes* (15) and introduced into L-threo

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Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Phenotype</th>
<th>Source or reference</th>
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<tr>
<td>M. glycogenes ATCC 21276</td>
<td>i-Threonine-producing mutant isolated among AHV⁺ i-Lys⁺ mutants derived from ATCC 21276; AK⁺</td>
<td>Wild type</td>
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<tr>
<td>AL119</td>
<td></td>
<td>AHV⁺ i-Lys⁺</td>
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<tr>
<td>DHL122</td>
<td>i-Threonine- and i-lysine-coproducing mutant isolated from DHL⁺ mutants derived from AL119; AK⁺ DDPS⁺</td>
<td>AHV⁺ i-Lys⁺ DHL⁺</td>
<td>13</td>
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<td>E. coli AT997</td>
<td>dapa thi-1 relA spoT1</td>
<td>Dap⁻</td>
<td>20</td>
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<tr>
<td>DH5a</td>
<td>DH1 Δ(argF-lacZYA)U169 hsdR17 q80dlaccZΔM15 rec4; chromosomally integrated RP4 derivative</td>
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<td></td>
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<tr>
<td>S17-1</td>
<td></td>
<td>Pro⁻ Tp⁺</td>
<td>19</td>
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<td>Plasmids</td>
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<tr>
<td>pUC19</td>
<td>Vector, 2.7 kb</td>
<td>Amp⁺</td>
<td>Takara Shuzo</td>
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<td>pMFY42</td>
<td>Broad-host-range plasmid, 10.9 kb</td>
<td>Tc⁺ Km⁺</td>
<td>5</td>
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<td>pMW119</td>
<td>Vector, 4.2 kb</td>
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<td>Nippon gene</td>
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<td>pDYO1</td>
<td>Derivative of pUC19 containing 3.9-kb DNA fragment carrying the dapa gene cloned from M. glycogenes DHL122</td>
<td>Amp⁺ DDPS</td>
<td>This study</td>
</tr>
<tr>
<td>pDYO2</td>
<td>Derivative of pUC19 containing 1.7-kb EcoRI DNA fragment carrying the dapa gene</td>
<td>Amp⁺ DDPS</td>
<td>This study</td>
</tr>
<tr>
<td>pDYO4-1, pDYO4-2</td>
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<td>Amp⁺ DDPS</td>
<td>This study</td>
</tr>
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<td>pDYO6</td>
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<td>This study</td>
</tr>
<tr>
<td>pDYOM4-2</td>
<td>Derivative of pMFY42 containing 2.4-kb PstI DNA fragment carrying the dapa gene</td>
<td>Tc⁺ DDPS</td>
<td>This study</td>
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</table>

Abbreviations: AHV⁺ i-Lys⁺, resistance to both DL-α-amino-β-hydroxy-valeric acid and i-lysine; AK⁺, aspartokinase insensitive to the feedback inhibition by i-lysine and i-threonine; Amp⁺, ampicillin resistance; Dap⁺, diaminopimelic acid auxotrophy; DDPS⁺, conferring the dihydrodipicolinate synthase activity; DDPS⁻, dihydrodipicolinate synthase partially insensitive to the feedback inhibition by i-lysine; DHL⁺, resistance against 2,6-diamino-4-hexenoic acid hydrochloride; Km⁺, kanamycin resistance; Pro⁺, proline auxotrophy; Tc⁺, tetracycline resistance; Tp⁺, trimethoprim resistance.

Materials and Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. M. glycogenes ATCC 21276 and the derived mutants were cultivated as described previously (13). Methanol was used as a sole carbon source. Escherichia coli cells were cultivated as described previously (10). M9S5 medium (M9 medium [10] supplemented with 50 mg each of 19 amino acids [except i-lysine] per liter) was used for complementation testing of E. coli AT997. Tetracycline (10 mg/liter) or ampicillin (100 mg/liter) was used to supplement liquid or agar media to cultivate M. glycogenes strains containing plasmids.

DNA manipulation. DNA restriction enzyme digestion, separation of DNA fragments by gel electrophoresis, and transformation of E. coli strains were performed by standard methods as described previously (10). Restriction enzymes and DNA ligase were supplied by Takara Shuzo Co. Ltd. (Kyoto, Japan). Southern hybridization and colony hybridization were done with a DNA labeling and detection kit (Boehringer Mannheim) according to the method recommended by the supplier. Chromosomal DNAs of M. glycogenes strains were prepared by the method described by Marmur (11).

Cloning of the dapa gene. The dapa gene of DHL122 was isolated by complementation of E. coli AT997 with a lesion in dapa as follows. The chromosomal DNA of M. glycogenes ATCC 21276 was partially digested with Sau3AI and separated by agarose gel electrophoresis. The 2- to 6-kb DNA fragments purified from the gel were ligated into the BamHI site of pUC19 to construct the gene library and transform E. coli AT997. The cells were plated onto L broth (10) supplemented with 100 mg of ampicillin per liter, and the resulting colonies were examined by colony hybridization with the 1.7-kb EcoRI fragment containing the dapa gene of DHL122. Plasmids isolated from the positive colonies were analyzed.

Subcloning of plasmids. The 1.7-kb EcoRI fragment of pDYO1 was introduced into the EcoRI site of pUC19 to construct pDYO2. The 2.4-kb PstI fragment of pDYO1 was inserted into the PstI site of pUC19 to form pDYO4-1 and pDYO4-2, in both orientations. pDYO6 was formed by the self-ligation of the 6.3-kb EcoRV fragment of pDYO1. The 2.4-kb PstI fragment of pDYO2 was inserted into the PstI site of pMFY42 to construct pDYOM4-2.

Conjugation. Introduction of plasmids from E. coli S17-1 to M. glycogenes by conjugation was done as described previously (16).

DNA sequencing and analysis. DNA sequencing was performed by the dideoxy chain termination method on both strands with an Applied Biosystems model 373A sequencer. DNA sequences analyses and homology alignments of amino acid sequences were carried out using GENETYX MAC version 9.0 (Software Development Co. Ltd., Tokyo, Japan).

Analysis. Bacterial growth was measured by the increase in absorbance at 660 nm, and amino acids in culture supernatants were measured by high-pressure liquid chromatography as described previously (13). Cell extracts of M. glycogenes transconjugants were prepared and DDPS activities were measured as described previously (14).

Nucleotide sequence accession number. The nucleotide sequence of the 1.7-kb EcoRI fragment encoding DDPS of M. glycogenes ATCC 21276 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under accession no. AB038266.

Results

Cloning of the dapa gene of M. glycogenes DHL122. We attempted to isolate the dapa gene of M. glycogenes DHL122 by complementation of an E. coli DDPS-deficient mutant, E. coli AT997, which has a lesion in dapa and requires i-diamin-
L-lysine in the culture supernatants increased two- to threefold, whereas the accumulation of L-threonine and L-glutamic acid was reduced. We thought that the growth reduction of the strains with the \( \text{dapA} \) gene might be caused by a shortage of cofactors, such as ATP and NADPH, the compounds required for both L-lysine biosynthesis and microbial growth. The enhanced L-lysine biosynthesis caused by the elevation of the DDPS activity might consume a considerable amount of cofactors. As sufficient amounts of the cofactors might not be generated due to the limited oxygen supply in test tube cultures, this might cause the growth to be reduced.

To circumvent the drawback of test tube cultures, we exam-
ined the effect of the *dapA* gene by cultivating the strains in 5-liter jar fermentors, where the oxygen supply was more favorable than in test tube cultures. Figure 2 shows the time courses of cultivation of the AL119 and DHL122 strains with or without the *dapA* gene. The growth of the strains with the *dapA* gene was comparable to that of the strains without the *dapA* gene in 5-liter jar fermentors. L-Lysine accumulation was greatly enhanced by the introduction of the *dapA* gene and was accompanied by the reduction of L-threonine accumulation as in test tube cultures. DHL122 containing pDYOM4-2 and AL119 harboring pDYOM4-2 accumulated 5.3 and 8 g of L-lysine per liter, respectively, after 72 h of cultivation. All of the transconjugants constructed produced more than 30 g of L-glutamic acid per liter, which was much higher than the accumulation of L-lysine and L-threonine, in 5-liter jar fermentors.

**Table 2. Effects of the *dapA* gene on amino acid production by the *M. glycogenes* mutants in test tube cultures**

<table>
<thead>
<tr>
<th>Host</th>
<th>Plasmid</th>
<th>Insert</th>
<th>Optical density at 660 nm</th>
<th>DDPS sp act&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Production (g/liter) of the following amino acid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td>DHL122</td>
<td>pMFY42</td>
<td></td>
<td>12.7</td>
<td>0.030 (1.0)</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>pDYOM4-2</td>
<td><em>dapA</em></td>
<td>6.3</td>
<td>0.580 (19.3)</td>
<td>4.7</td>
</tr>
<tr>
<td>AL119</td>
<td>pMFY42</td>
<td></td>
<td>8.7</td>
<td>0.055 (1.0)</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>pDYOM4-2</td>
<td><em>dapA</em></td>
<td>5.8</td>
<td>1.200 (21.0)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were cultivated in test tubes at 30°C for 48 h.

<sup>b</sup> Specific activities of DDPS expressed as change in A<sub>540</sub> per minute per milligram of protein. Numbers in parentheses show relative activities.

**Analysis of the nucleotide sequence of *dapA* and the location of the mutation in DHL122.** The 1.7-kb *EcoRI* fragment of pDY02 was sequenced on both strands, and a single open reading frame (ORF) was found (Fig. 3). The ORF encodes a predicted peptide with an *M*<sub>r</sub> of 30,664, initiating at the ATG codon (nucleotides 472 to 474) and terminating at the TGA codon (nucleotides 1342 to 1344). Neither distinct promoter-like nor distinct terminator-like sequences were found in the upstream and downstream regions of the ORF, respectively. To identify the mutation in DHL122, the 1.7-kb *EcoRI* fragments containing the *dapA* gene were cloned from the wild type, ATCC 21276, and AL119 and sequenced. The nucleotide sequences of the 1.7-kb *EcoRI* fragments from ATCC 21276 and AL119 were identical to that from DHL122 except at nucleotide 733. Nucleotide 733 is C in ATCC 21276 and

**FIG. 2. Time courses of amino acid production in 5-liter jar fermentors.** (A) DHL122/pMFY42; (B) DHL122/pDYOM4-2; (C) AL119/pMFY42; (D) AL119/pDYOM4-2. ◆, optical density (OD) at 660 nm; ■, Lys; □, Thr; ×, Glu.
AL119, whereas it is T in DHL122, which altered the amino acid residue L88 in ATCC 21276 and AL119 to F88 in DHL122. The mutation in this amino acid residue was considered to cause the partial desensitization of DDPS of DHL122 to feedback inhibition by L-lysine (14).

Comparison of the predicted amino acid sequences of the *dapA* genes. The predicted amino acid sequence of the *dapA* gene of *M. glycogenes* was compared with those from other organisms (Fig. 4). Extensive amino acid sequence homology was found between *M. glycogenes* and other organisms. The amino acid sequence of *M. glycogenes* has identities of 52.4% (153 of 292 amino acid residues), 47.2% (137 of 290 amino acid residues), 35.5% (107 of 301 amino acid residues), 29.4% (96 of 326 amino acid residues), and 25.3% (96 of 380 amino acid residues) with those from *E. coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Nicotiana sylvestris*, and *Zea mays*, respectively.

**DISCUSSION**

In our previous study we derived an L-lysine and L-threonine coproducer, DHL122, from an L-threonine producer, AL119 (13). However, DHL122 could produce only a small amount of L-lysine. We report here that the introduction of the *dapA* gene into DHL122 and AL119 greatly elevated the specific activity of DDPS and L-lysine production with concomitant reduction of L-threonine accumulation. This suggests that the carbon flow from L-aspartic acid to L-lysine was limited at the conversion of L-aspartic acid-β-semialdehyde to dihydrodipicolinate in both strains and was liberated by the amplification of the *dapA* gene with the redirection of the carbon flow from toward L-threonine to toward L-lysine. We found that DDPS of DHL122, from which the *dapA* gene was cloned, was partially desensitized to the feedback inhibition by L-lysine (14). The altered regulatory property of the enzyme was thought to con-
FIG. 4. Homology between the amino acid sequences of the DDPSs of *M. glycogenes* ATCC 21276 and those of other organisms. Conserved amino acid residues found in more than four species are shaded. Asterisks indicate the putative active sites of the DDPS. Amino acid residue 88, which was found to be mutated in DHL122, is indicated as F(DHL122) above the sequence. The amino acid residues whose mutations were reported to cause the desensitization of DDPSs of other organisms are indicated by plus signs: *E. coli*, A81^3^V and H118^3^Y (8); *Z. mays*, S157^3^N, E162^3^K, and A166^3^T,V (17); and *N. sylvestris*, N104^3^I (6). MG, *M. glycogenes*; EC, *E. coli*; BS, *B. subtilis*; CG, *C. glutamicum*; NS, *N. sylvestris*; ZM, *Z. mays*. 

MG 1-MLGGLVIVTPEMLDDSLH1IQLRLSDIDH1EIQSKDGIYV 42
EC 1-MLGGLVIVTPEMLDDSLH1IQLRLSDIDH1EIQSKDGIYV 42
BS 1-MLGGLVIVTPEMLDDSLH1IQLRLSDIDH1EIQSKDGIYV 42
CG 1-MLGGLVIVTPEMLDDSLH1IQLRLSDIDH1EIQSKDGIYV 42
NS 7-HLPMLRESNVVRFAKKKLRLLSKIKFLPDRLFLEAYTVNLQIENGAGVGYG 66
ZM 61-YLPMLRESNVVRFAKKKLRLLSKIKFLPDRLFLEAYTVNLQIENGAGVGYG 120

MG 43-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 102
EC 43-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 102
BS 44-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 102
CG 55-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 102
NS 67-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 126
ZM 121-GGDGPDYDVDPDEHALILRHDPSAGPCRPHV1AGTAPIK1SEAI1RRKAKKDLR1ACLL 180

MG 103-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 161
EC 103-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 161
BS 104-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 161
CG 115-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 161
NS 127-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 173
ZM 181-VYHNLRESGDQQYQQCIPKGA1VQ16YQGZQLNTRVQV1AAIKK-N1V1GK 183

MG 162-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 220
EC 162-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 220
BS 162-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 220
CG 174-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 220
NS 184-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 230
ZM 238-VALNIEGTGDLRLRAPAFGAIYSG-DIASLALLLLROGQIYQG1TSA1AFLR-HDMCTA 239

MG 221-ALAGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 279
EC 221-ALAGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 279
BS 223-ALAGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 279
CG 223-ALAGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 279
NS 240-AlAGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 292
ZM 294-AGDTKARRSAKAFKIQHKEVLVEANP1PKKVWLQGQ1L1Q-GG1R1CLLV1LSSQFHD 346

MG 280-TLSAMKQAAL- 290
EC 281-TLSAMKQAAL- 290
BS 282-TLSAMKQAAL- 290
CG 290-TLSAMKQAAL- 290
NS 293-TPKIVKDDGRENFGISEDFQV1LDDND1LGVRY 326
ZM 347-TPKIVKDDGRENFGISEDFQV1LDDND1LGVRY 380
tribute to the increased production of L-lysine. However, we believe that this effect was partial. DHL122, which had mutated DDPS, produced more L-lysine than AL119, which had wild-type DDPS, but the production of L-lysine by DHL122 was less than that of L-threonine, suggesting that the partial desensitization of DDPS was not enough to change the carbon flow from L-threonine to L-lysine (13) (Fig. 2A and C). The introduction of the dapA gene-containing plasmid drastically enhanced the enzyme activity, and the production of L-lysine greatly exceeded that of L-threonine (Fig. 2B and D). We speculate that the elevated DDPS activity mainly caused the overproduction of L-lysine in the strains with the dapA-containing plasmid.

AL119 and DHL122 were derived from an L-glutamic acid producer, iA111 (13). The transconjugants bearing the dapA gene produced much more L-glutamic acid than L-lysine and L-threonine. The metabolic flow from methanol is possibly limited at some steps in the biosynthesis of L-aspartic acid and directed toward the formation of L-glutamic acid. To further enhance L-lysine production, it is necessary to reduce the metabolic flow toward L-glutamic acid and direct it toward L-aspartic acid. In the course of breeding L-lysine-producing mutants from a C. glutamicum L-glutamic acid producer, pyruvate kinase deficiency (18), reduction of citrate synthase activity, and desensitization of phosphoenolpyruvate carboxylase (21) were reported to be effective to reduce the production of L-glutamic acid and enhance that of L-aspartic acid and L-lysine. These strategies may also be applicable for M. glycogenes. The isolation of mutants with lesions in the enzymes of the tricarboxylic acid cycle and those altered in the regulation of the enzymes of the biosynthesis of L-aspartic acid, together with the amplification of biosynthetic genes of L-aspartic acid, should be considered.

The dapA gene was cloned from M. glycogenes and sequenced, and the amino acid sequence was found to have extensive homology with those from other organisms. In E. coli (12) and N. sylvestris (2), DDPS was shown to consist of homotetramers by X-ray crystallography studies. The homology throughout the amino acid sequence between DDPSs from M. glycogenes and those from E. coli and N. sylvestris suggests that DDPSs of M. glycogenes has a similar structure. Blickling and Knoblein (3) proposed that in E. coli and other organisms K161, Y133, and R138 (numbering refers to the E. coli and M. glycogenes sequences) are involved in significant roles in catalysis—the formation of Schiff bases with pyruvate, proton shuttling during amine formation and transamination, and interaction with the carboxy group of L-aspartic acid-β-semialdehyde, respectively. These residues are conserved in M. glycogenes and might have the same roles in catalysis as in other organisms. It was also suggested that in E. coli the residues H53, H56, Y106, Y107, N80, and E84 were involved in the interaction with an allosteric effector, l-lysine (3). Most of the mutations that have been reported to cause the desensitization of E. coli and plant DDPSs (marked by plus signs in Fig. 4) were found in the region between amino acid residues 79 and 88. The mutation in the DDPSs of DHL122 (F88) is also located in this region, suggesting that the mutation that occurred in this region might alter the structure of DDPSs, prevent the efficient interaction between L-lysine and other amino acid residues (possibly N80 and E84), and lead to the partial desensitization of DDPS to L-lysine. DDPSs of DHL122 was shown to be inhibited by high concentrations of L-lysine (14). The DDPS might be further desensitized by alteration of other amino acid residues that were found to be effective for the desensitization of other DDPSs. The availability of more desensitized DDPSs constructed in such ways will be an important tool to improve L-lysine production from methanol by M. glycogenes.

ACKNOWLEDGMENTS

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REFERENCES