Effects of Deregulation of Methionine Biosynthesis on Methionine Excretion in Escherichia coli

Yoshihiro Usuda* and Osamu Kurahashi

Fermentation & Biotechnology Laboratories, Ajinomo To Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210-8681, Japan

Received 29 September 2004/Accepted 15 December 2004

Several regulators of methionine biosynthesis have been reported in Escherichia coli, which might represent barriers to the production of excess l-methionine (Met). In order to examine the effects of these factors on Met biosynthesis and metabolism, deletion mutations of the methionine repres sor (metJ) and threonine biosynthetic (thrBC) genes were introduced into the W3110 wild-type strain of E. coli. Mutations of the metJ gene encoding S-adenosylmethionine synthetase, which is involved in Met metabolism, were detected in 12 norleucine-resistant mutants. Three of the mutations in the metK structural gene were then introduced into metJ and thrBC double-mutant strains; one of the resultant strains was found to accumulate 0.13 g/liter Met. Mutations of the metA gene encoding homoserine succinyltransferase were detected in α-methylmethionine-resistant mutants, and these mutations were found to encode feedback-resistant enzymes in a 14C-labeled homoserine assay. Three metA mutations were introduced, using expression plasmids, into an E. coli strain that was shown to accumulate 0.24 g/liter Met. Combining mutations that affect the deregulation of Met biosynthesis and metabolism is therefore an effective approach for the production of Met-excreting strains.

1-Methionine (Met) is a sulfur-containing amino acid that is essential in mammals and is used both as a food additive and a medication (15). Met has a central role in the metabolism of sulfur-containing substances and is also involved in methyl group transfer via its derivative S-adenosylmethionine (SAM), which is an intermediate in the polyamine biosynthetic pathway (7).

Industrial Met is produced mainly from DL-methionine, which is widely used as a feed additive. This compound is chemically synthesized through the generation of N-acetyl-DL-methionine by the acetylation of DL-methionine, followed by enzymatic selective deacetylation of the N-acetyl-l-methionine. Industrial-scale microbial fermentation has not yet been developed for the production of Met. This is partly due to the complexity of the Met biosynthetic pathway and the strong metabolic regulation that results from its essential cellular functions.

Escherichia coli is one of the most important microorganisms used in the manufacture of amino acids (8). Met biosynthesis and metabolism have been well studied in this bacterium, and several regulatory factors have been identified (7). At the transcriptional level, the methionine repressor inhibits the Met biosynthetic genes metA, metB, metC, metE, and metF (Fig. 1). This activity is mediated by the MetJ repressor protein and its corepressor, SAM, which also acts as a methyl donor and a substrate for polyamine biosynthesis.

Homoserine succinyltransferase, which is the first enzyme in the Met biosynthetic pathway from homoserine, is encoded by the metA gene. This enzyme is inhibited by the combined activity of Met and SAM; the latter compound is synthesized from Met and ATP by S-adenosylmethionine synthetase (MetK), which is encoded by the metK gene. Mutations in metK lead to elevated levels of Met biosynthetic enzymes and defective feedback inhibition. It is well known that analogue resistance is effective in suppressing metabolic control (18). Selection for resistance to Met analogues, such as α-methylmethionine (α-MM) and norleucine, has therefore been suggested to lead to mutants with desensitized MetJ, homoserine succinyltransferase (MetA), and MetK (1). However, no nucleotide substitutions or corresponding amino acid exchanges have been identified so far in the metA and metK genes.

In this study, we attempted to deregulate the controls of Met biosynthesis and metabolism in the W3110 wild-type strain of E. coli. metK mutations were identified in norleucine-resistant mutants, and desensitization of the metA gene to Met and SAM inhibition was obtained through α-MM resistance.

MATERIALS AND METHODS

Strains, plasmids, media, and cultivation. The W3110 strain of E. coli was used as the parent strain, and the JM109 strain (22) was used for plasmid preparation. Plasmids pUC18, pHSG298, pHSG398, pSTV28 (Takara Shuzo, Kyoto, Japan), and pMW118 (Nippon Gene, Toyama, Japan) were used for plasmid construction and gene amplification. Details of these strains and plasmids are summarized in Table 1. Cultivation for fermentative Met excretion was performed for 24 h (for W3110) or 48 h (for Thr- auxotrophic mutants) in a 500-ml Sakaguchi flask with a working volume of 20 ml at 37°C in MS medium containing (per liter of distilled water) 40 g glucose, 1 g MgSO₄ · 7H₂O, 16 g (NH₄)₂SO₄, 1 g KH₂PO₄, 2 g Bacto yeast extract, 0.01 g MnSO₄ · 4H₂O, 0.01 g FeSO₄ · 7H₂O, 0.5 g Thr (for Thr- auxotrophic mutants), and 30 g CaCO₃ (added after it was sterilized separately) (13). Growth was monitored by measuring the optical density at 600 nm. The Met concentration in the culture medium was evaluated by the analytical method for physiological fluids using an L-8500 amino acid analyzer (Hitachi, Tokyo, Japan). For mutant isolation, E. coli was grown at 37°C in a minimal medium based on the medium described by Davis and Mingioli (4), containing (per liter) 2 g glucose, 7 g K₂HPO₄, 3 g KH₂PO₄, 0.5 g Na₂ citrate · 2H₂O, 0.1 g MgSO₄ · 7H₂O, and 5 g (NH₄)₂SO₄. Luria-Bertani (LB) medium was used for all other manipulations.

* Corresponding author. Mailing address: Institute of Life Sciences, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210-8681, Japan. Phone: 81 44 210 5928. Fax: 81 44 244 4258. E-mail: yoshihiro_usuda@ajinomoto.com.
Chemicals. DL-[U-14C]homoserine (50 mCi/mmol) was synthesized by Muro-machi Chemical Industry (Tokyo, Japan). O-Succinylhomoserine and pyridoxal phosphate were purchased from Sigma-Aldrich (St. Louis, MO).

Gene cloning and DNA manipulations. General DNA manipulation procedures were performed as described previously (19). Sequencing was carried out using the dideoxy chain termination method with a Taq DyeDeoxy terminator cycle sequencing kit and a 377 DNA sequencer (Applied Biosystems, Foster City, CA). Genomic DNA was extracted using a genomic DNA purification kit (Advanced Genetic Technologies, Gaithersburg, MD). Gene replacement was performed using a temperature-sensitive origin of replication plasmid, pMAN997 (13), which was derived from plasmid pMAN031 (14). The plasmids for recombination were transformed and the integrants were selected at a nonpermissive temperature (42°C) in the presence of an antibiotic (ampicillin). The second recombination was performed at a permissive temperature (30°C) without ampicillin. The generation of recombinants was confirmed by the length of amplified PCR fragments.

Threonine-auxotrophic and metJ-deficient strains. To construct an L-threonine (Thr)-auxotrophic strain (Fig. 2A), the thrB region inside the thrABC operon was amplified by PCR with primers 5'/H11032-GGGGAATTCTGGCAGGAGGAACTGGCGCA-3'/H11032 (EcoRI site underlined) and 5'/H11032-GGGTCGACGCTCATTGACTGAA-3'/H11032 (SalI site underlined) and digested using EcoRI and SalI. The thrC region was amplified by PCR with primers 5'/H11032-GGGTCGACATCAGTAAAATCTATTCATT-3'/H11032 (SalI site underlined) and 5'/H11032-GGAAGCTTGCGTGAGATGGGGATTAACC-3'/H11032 (HindIII site underlined) and restricted using SalI and HindIII. Both of these amplified fragments were mixed, ligated into plasmid pMAN997, and digested using EcoRI and HindIII. The resultant plasmid, pMANthrBC, was used to obtain the thrBC-deficient mutant from W3110 by homologous recombination. The Thr-auxotrophic strain selected was designated W3110thrBC.

A 1-kb fragment containing the region of the metB gene was then amplified using primers 5'/H11032-GGGCATGCCAGGGAACTTCATCACATG-3'/H11032 (SphI site underlined) and 5'/H11032-GGGGAATTCTCATGGTTGCGGCGTGAGAG-3'/H11032 (EcoRI site underlined) and digested using SphI and EcoRI. The metJ gene region was amplified using primers 5'/H11032-GGAAGCTTGCGTGAGATGGGGATTAACC-3'/H11032 (HindIII site underlined) and 5'/H11032-GGGTAATTCCATGATCTGCTGCAGAG-3'/H11032 (EcoRI site underlined) and digested using SphI and EcoRI. The met gene region was amplified using primers 5'-GGGAAATTCCATGATCTGCTGCAGAG-3' (SphI site underlined) and 5'-GGGAAATTCCATGATCTGCTGCAGAG-3' (EcoRI site underlined) and digested using SphI and EcoRI. The met gene region was amplified using primers 5'-GGGAAATTCCATGATCTGCTGCAGAG-3' (SphI site underlined) and 5'-GGGAAATTCCATGATCTGCTGCAGAG-3' (EcoRI site underlined) and restricted using HindIII and EcoRI. Both of these amplified fragments were ligated into plasmid pMAN997, and digested using EcoRI and HindIII. The resulting plasmid, pMANthrBC, was used to obtain the thrBC-deficient mutant from W3110. The thrBC-deficient strain selected was designated W3110thrBC.

FIG. 1. Biosynthesis and regulation of methionine in E. coli. The boldface arrows indicate feedback inhibition, and the dotted arrows indicate repression. CoA, coenzyme A.

### TABLE 1. List of bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or gene</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild type</td>
<td>Our collection</td>
</tr>
<tr>
<td>WΔthrBC</td>
<td>thrBC</td>
<td>This study</td>
</tr>
<tr>
<td>WΔthrBCmetJ</td>
<td>thrBC metJ</td>
<td>This study</td>
</tr>
<tr>
<td>WΔthrBCmetJmetK2</td>
<td>thrBC metJ metK2</td>
<td>This study</td>
</tr>
<tr>
<td>WΔthrBCmetJmetK24</td>
<td>thrBC metJ metK24</td>
<td>This study</td>
</tr>
<tr>
<td>WΔthrBCmetJmetK32</td>
<td>thrBC metJ metK32</td>
<td>This study</td>
</tr>
<tr>
<td>pMW118</td>
<td>Plasmid vector, Amp’</td>
<td>Nippon Gene</td>
</tr>
<tr>
<td>pMWPhrmetA</td>
<td>Wild-type metA gene under the control of Pthr</td>
<td>This study</td>
</tr>
<tr>
<td>pMWPhrmetA4</td>
<td>metA4 gene under the control of Pthr</td>
<td>This study</td>
</tr>
<tr>
<td>pMWPhrmetA5</td>
<td>metA5 gene under the control of Pthr</td>
<td>This study</td>
</tr>
<tr>
<td>pMWPhrmetA9</td>
<td>metA9 gene under the control of Pthr</td>
<td>This study</td>
</tr>
<tr>
<td>pMWPhrmetA4+9</td>
<td>metA gene with combined mutation of metA4 and metA9 under the control of Pthr</td>
<td>This study</td>
</tr>
<tr>
<td>pMWPhrmetA5+9</td>
<td>metA gene with combined mutation of metA4 and metA5, and metA9 under the control of Pthr</td>
<td>This study</td>
</tr>
</tbody>
</table>
these 12 strains using primers 5'-H11032-GGAAGCTTAAGCAGAGATGCAGAGTG (Fig. 2B). 100 g/ml of norleucine on minimal medium plates.

ThrBC

The resultant plasmid was designated pMAN997, and the resultant plasmid was designated pMANmetJ-PmetJ-metB. Using this plasmid, a metJ-deficient strain and a strain with the promoter replaced were derived from W3110 and W3110 and designated W3110 and W3110, respectively (Fig. 2B).

Isolation of metK mutants. W3110 cells were cultivated for 24 h in LB medium. The cells from 1 ml of culture were collected by centrifugation, washed twice in 0.9% NaCl, inoculated into Davis-Mingioli minimal medium plates containing 100 μg/ml of norleucine, and incubated for 5 days. Colonies were then isolated, and 12 norleucine-resistant strains were confirmed again to be resistant to 100 μg/ml of norleucine on minimal medium plates.

The chromosomal DNA was extracted, and the metK gene was amplified from these 12 strains using primers 5'-GGAAGCTTAAATTTTTATGACTAGTACCAAATACATTTAACAAATATGCACTAAAGGGACAGTGAATC-3' (HindIII and SphI sites underlined, respectively), which were synthesized, annealed, and digested using HindIII and SphI.

Three fragments and plasmid pHSG298 digested with EcoRI were ligated. These fragments were excised from pHSGmetJ-PmetJ-metB and inserted into pMAN997, and the resultant plasmid was designated pMANmetJ-PmetJ-metB. Using this plasmid, a metJ-deficient strain and a strain with the promoter replaced were derived from W3110 and W3110, respectively (Fig. 2B).

Isolation of metK mutants, W3110 cells were cultivated for 24 h in LB medium. The cells from 1 ml of culture were collected by centrifugation, washed twice in 0.9% NaCl, inoculated into Davis-Mingioli minimal medium plates containing 1 g/liter α-MM, and cultured for 3 days. The culture was then diluted and spread on minimal medium plates containing 1 g/liter α-MM. Grown colonies were subsequently isolated, and they were confirmed to be resistant to 1 g/liter α-MM on minimal medium plates again.

The metK fragment was amplified using primers 5'-GGTGCAGTTCGAGTC-3' (SphI site underlined) and 5'-GAGGATCTTAATCAGATCCGATCCGACGACCGGCCGACA-3' (SphI site underlined) and cloned into plasmid pHSG398 at the SphI and SalI sites. Sequencing of wild and mutated metA genes was performed using the internal primers 5'-TGTGCGGTGCCGGTCA-3' and 5'-AGAGGATTTCGCGTGTC-3', respectively.

For the enzyme assays, cells were suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. The suspension was then centrifuged at 18,000 × g for 30 min, and the supernatant was desalted in a Sephadex G-50 column (Amersham-Pharmacia Biotech, Tokyo, Japan) to obtain the crude enzyme extract. To measure the MetA activity, 5 μl of the crude enzyme extract was added to a reaction mixture (final volume, 50 μl) containing 0.1 M potassium phosphate (pH 7.5), 1 mM succinyl coenzyme A, and 100 μg/ml of norleucine. The reaction was followed for 30 min, and the amount of phosphorylated MetA was determined by measuring the increase in absorbance at 280 nm. The MetA activity was expressed as the amount of phosphorylated MetA per cell per hour.

Enzyme assays. For the enzyme assays, cells were suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. The suspension was subjected to cell disruption treatment using sonication. The sonicated suspension was then centrifuged at 18,000 × g for 30 min, and the supernatant was desalted in a Sephadex G-50 column (Amersham-Pharmacia Biotech, Tokyo, Japan) to obtain the crude enzyme extract. To measure the MetA activity, 5 μl of the crude enzyme extract was added to a reaction mixture (final volume, 50 μl) containing 0.1 M potassium phosphate (pH 7.5), 1 mM succinyl coenzyme A, and 100 μg/ml of norleucine. The reaction was followed for 30 min, and the amount of phosphorylated MetA was determined by measuring the increase in absorbance at 280 nm. The MetA activity was expressed as the amount of phosphorylated MetA per cell per hour.
A (Sigma-Aldrich), 0.2 mM L-homoserine, and 2 mM ta-[U-14C]homoserine. The mixture was incubated at 30°C for 10 min. Subsequently, 1 ml of the reaction mixture was spotted onto a cellulose plate (Merck, Whitehouse Station, NJ) and developed with a mixed solvent containing acetonitrile, butanol, water, and diethylamine at a ratio of 10:10:5:2. After the plate was air dried, autoradiography was performed using a BAS2000 image analyzer (Fuji Photo Film, Tokyo, Japan). The conversion of [14C]homoserine to [14C]O-succinylhomoserine was used to calculate MetA activity. To measure the cystathionine γ-synthase (MetB) activity, 100 μl of the crude enzyme extract was added to a reaction mixture (final volume, 1 ml) containing 0.2 M Tris-HCl (pH 8.0), 5 mM O-succinylhomoserine (Sigma-Aldrich), and 0.25 mM pyridoxal phosphate (Sigma-Aldrich). The mixture was incubated at 37°C for 20 min and then cooled with ice. The amount of pyridoxal phosphate-dependent reduction of O-succinylhomoserine was used to calculate the MetB activity.

RESULTS

Disruption of the metJ and thrBC genes. As Thr is synthesized from homoserine, which is a common substrate for Met biosynthesis (Fig. 1), a Thr-auxotrophic strain was expected to be effective in inducing Met excretion. In addition, the growth of such a strain can be controlled by the addition of Thr to the medium. We therefore produced a Thr-auxotrophic strain by deleting the thrBC genes of W3110 through homologous recombination (Fig. 2A). Auxotrophy was confirmed through growth on minimal medium, and the resultant disruption mutant was designated WΔthrBC.

The metJ gene encodes an aporepressor that mediates the suppression of the Met biosynthetic genes metA, metB, metC, metE, metF, metH, and metL (Fig. 1). We attempted to disrupt the metJ repressor and the adjacent metBL promoter region with PstI simultaneously using the temperature-sensitive origin of replication plasmid (Fig. 2B). We obtained a metJ-deficient mutant from WΔthrBC and mutants WΔthrBCΔmetJ and WΔmetJ from W3110. To confirm derepression of the Met biosynthetic genes, we measured MetA and MetB activities. The level of MetA activity in the presence of Met in minimal medium was significantly higher in the WΔmetJ strain (126 mmol/min/mg protein) than in the W3110 strain (0.3 mmol/min/mg protein). Furthermore, the MetB activity of WΔmetJ was 1,300 mmol/min/mg protein, compared with 140 mmol/min/mg protein in W3110. These results clearly demonstrated that there was successful derepression of the Met biosynthetic genes.

Isolation of MetK mutations. MetK catalyzes the formation of SAM from Met and ATP, and it has been suggested that metK is an essential gene (21). Resistance to the Met analogues norleucine and ethionine was reported to lead to the formation of Thr-auxotrophic mutants; these mutations were designated metA and metK (10). However, the nucleotide sequence information that is involved in these mutations remains unknown. Here, we obtained six spontaneous α-MM-resistant mutants from W3110 in independent experiments. The nucleotide sequences of the mutants were determined using PCR-amplified metA gene fragments. No mutations were detected in one strain; point mutations were observed in three strains, in which the mutant genes were designated metA4, metA5, and metA9; and an IS2 transposition (6) with a duplication of five nucleotides (586ATCTC) was found at the same position in the remaining two strains, in which the mutant genes were designated metA7 and metA8. As a consequence of the mutations in metA7 and metA8, the amino acid sequence from 298Pro onward was altered to 298Arg-Leu-Ala-Pro-STOP (Table 4).

Crude extracts from strains containing metA4, metA5, and metA9 were prepared, and their MetA activities were measured using chemically synthesized ta-[14C]homoserine as a substrate. The results showed that the metJ deletion had a significant effect on Met excretion in Thr-auxotrophic mutants. Furthermore, the metK mutations, the metK24 mutation clearly had the greatest impact on Met production.

Desensitization of MetA. MetA is the first unique enzyme in the Met biosynthetic pathway, which catalyzes the succinylination of homoserine. Its activity is reported to be inhibited by the combined effects of Met and SAM (11, 20). In Salmonella enterica serovar Typhimurium, α-MM-resistant mutants are known to have feedback-resistant mutations in the metA gene (10). However, the nucleotide sequence information that is involved in these mutations remains unknown. Here, we obtained six spontaneous α-MM-resistant mutants from W3110 in independent experiments. The nucleotide sequences of the mutants were determined using PCR-amplified metA gene fragments. No mutations were detected in one strain; point mutations were observed in three strains, in which the mutant genes were designated metA4, metA5, and metA9; and an IS2 transposition (6) with a duplication of five nucleotides (586ATCTC) was found at the same position in the remaining two strains, in which the mutant genes were designated metA7 and metA8. As a consequence of the mutations in metA7 and metA8, the amino acid sequence from 298Pro onward was altered to 298Arg-Leu-Ala-Pro-STOP (Table 4).

Crude extracts from strains containing metA4, metA5, and metA9 were prepared, and their MetA activities were measured using chemically synthesized ta-[14C]homoserine as a substrate. The results showed that the metJ deletion had a significant effect on Met excretion in Thr-auxotrophic mutants. Furthermore, the metK mutations, the metK24 mutation clearly had the greatest impact on Met production.

Deregulation of Methionine Biosynthesis in E. coli

TABLE 2. metK mutations found in norleucine-resistant strains

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Change in nucleotide</th>
<th>Change in amino acid(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>metK24</td>
<td>907T → A</td>
<td>309Ile → Leu</td>
</tr>
<tr>
<td>metK2</td>
<td>554T → A</td>
<td>188Val → Glu</td>
</tr>
<tr>
<td>metK32</td>
<td>1135C deletion</td>
<td>397Arg-Asp-Ala-Gly-Leu-Lys-stop → 378Ala-Met-Leu-Pro-Val-stop</td>
</tr>
</tbody>
</table>

* Each value is the mean ± standard deviation of the mean for two replicate cultures.

* ND, not detected.
substrate (Table 5). The MetA activity in these extracts showed significant desensitization against α-MM, Met, and SAM, although the specific activities were reduced by approximately one-quarter. No MetA activity was detected in the pMWP

metA9 extract as a result of the amino acid modification caused by the IS2 insertion. In both metA4 and metA5 extracts, the inhibition caused by SAM alone and that caused by SAM and Met together were reduced to the level reported previously by Lawrence (10).

Met excretion caused by the introduction of desensitized metA genes. In order to examine the effects of metA gene mutations that were feedback resistant to Met and SAM inhibition, we constructed expression plasmids carrying the metA4, metA5, and metA9 mutations as follows: pMWPthrmetA4, pMWPhrmetA5, and pMWPhrmetA9 harbored single mutations; pMWPhrmetA4+9 and pMWPhrmetA5+9 harbored double mutations; and pMWPhrmetA4+5+9 harbored all three mutations. These plasmids were introduced into host strain W3110ΔthrBCΔmetJmetK32, which showed the greatest effect on Met excretion, and cultivated in MS medium.

As shown in Table 6, the metA4, metA5, and metA9 single mutations all had notable effects on Met excretion. Furthermore, combinations of these mutations (metA4 plus metA9, metA5 plus metA9, and metA4 plus metA5 plus metA9) increased the amount of Met excretion. These findings indicate that inhibition of MetA is critical for Met biosynthesis. All of the mutations investigated during our research had significant effects on Met excretion levels.

### DISCUSSION

Many regulators of methionine biosynthesis and metabolism have been reported in *E. coli* (7). In order to determine the effects of factors predicted to be important for Met production by fermentation, we deregulated several of the known controls in the Met biosynthetic and metabolic pathways of *E. coli*. Introduction of deletion mutations of *metI* and *thrBC* into the W3110 strain had a significant effect on the amount of Met excreted into the medium. In addition, three mutations of the *metK* structural gene, which were obtained from norleucine-resistant mutants, also significantly increased Met excretion levels when they were introduced into the *metI* and *thrBC* double-mutant strain.

Mutations of the *metA* structural gene that were isolated from α-MM-resistant mutants were found to encode feedback-resistant enzymes using a 14C-labeled homoserine assay. A strain containing three *metA* mutations, which were introduced using expression plasmids, was shown to accumulate 0.24 g/liter Met in the medium. The *metA9* mutation showed only a slight effect on MetA inhibition (Table 5) and little effect on Met excretion (Table 6). The *metA9* mutation with Met excretion affected the double-mutant strain. The *metA9* mutation exhibited significant effects with high Met concentrations (compare 1 mM Met and 1 mM Met + 1 mM SAM in Table 5). The influence of the *metA9* mutation might therefore appear only in combination with other mutations. The 27Arg residue that was replaced in the protein encoded by the *metA9* mutant gene was located in a region near the amino terminus; this residue is wel conserved across the bacteria. Similarly, the 289Pro residue that was replaced in the protein encoded by the *metA5* gene, which was located in an area near the carboxy terminus of the protein, is also conserved across many species. By contrast, the 204Ile residue that was replaced in the protein encoded by the *metA4* gene, which was located in an area near the carboxy terminus, is conserved only in closely related bacteria, such as *Salmonella* (accession no. P37413), *Shigella* (Q7UBA4), and *Yersinia* (Q8ZAR4). Combinations of these amino- and carboxy-terminal mutations had additive effects on Met excretion (Table 6), which suggests that both regions are important for the feedback inhibition of MetA.

The two other mutations detected in the *metA* structural gene were both insertions at the same position, which caused a frameshift in the carboxy-terminal region after 295Pro. Interestingly, some bacteria, such as *Bacillus subtilis* (accession no. P54167) and *Lactobacillus plantarum* (Q88UF5), lack this region. Although we were unable to measure the activity of the frameshifted enzyme (probably due to a lower specific activity compared with the enzymes with point mutations), these findings suggest that the carboxy-terminal region of MetA is essential for its regulation.

Thr auxotrophy is an important trait for Met excretion in *E. coli*. LB medium was enough for basal growth of W3110ΔthrBC and its derivatives. However, addition of 0.5 g/liter of Thr to MS medium was appropriate for Met excretion. We could not detect the effects of wild-type *metA* amplification (Table 3) and *metI* deletion (data not shown) on W3110. A Thr auxotroph might be useful for restricting the biomass of *E. coli* and for blocking off the branching pathway for the formation of by...
products. For the latter reason, an L-lysine (Lys) auxotroph could also be effective in promoting Met excretion.

The metK gene has been proposed to be an essential gene (21), and we were unable to achieve complete metK deletion in this study. However, we did obtain norleucine-resistant MetK mutants. When the wild-type metA gene was amplified, the metK24 mutant was found to be most effective for Met production in a thrBC metA background (Table 3). However, when the feedback-resistant MetA was amplified in the same background, the metK32 mutation resulted in the greatest effects on Met excretion (Table 6 and data not shown). These findings imply that the extent of the attenuation of MetK activity might vary depending on the genetic background of the strain. Clarification of the relationship between various levels of MetK activity and the amount of Met excretion is very important and will be the subject of further investigation.

Nakamori and colleagues (17) reported a MetJ mutant with replacement of 35Ser by Asn, which accumulated Met in the feedback-resistant MetA was amplified in the same background (Table 6). However, we did obtain norleucine-resistant MetK (21), and we were unable to achieve complete MetK inactivation, using a combination of gene disruption and amplification, we produced strains that could excrete increased amounts of Met into the growth medium. Further studies are necessary to identify additional factors that are essential for the realization of large-scale Met production by fermentation using E. coli.

ACKNOWLEDGMENTS

We thank H. Kojima and K. Hashiguchi for helpful advice. We are also grateful to T. Inamura for amino acid measurements and to S. Murata for technical assistance.

REFERENCES


<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Optical density at 600 nm</th>
<th>Excreted Met conc (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA</td>
<td>6.5 ± 0.4</td>
<td>0.023 ± 0.002</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA4</td>
<td>7.4 ± 0.3</td>
<td>0.158 ± 0.008</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA5</td>
<td>6.9 ± 0.3</td>
<td>0.108 ± 0.007</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA9</td>
<td>7.3 ± 0.2</td>
<td>0.131 ± 0.006</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA4+9</td>
<td>8.1 ± 0.2</td>
<td>0.206 ± 0.021</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA5+9</td>
<td>8.6 ± 0.7</td>
<td>0.207 ± 0.017</td>
</tr>
<tr>
<td>WΔthrBCΔmetJmetK32</td>
<td>pMWPmetmetA4+5+9</td>
<td>9.5 ± 0.3</td>
<td>0.236 ± 0.007</td>
</tr>
</tbody>
</table>

* Each value is the mean ± standard deviation of the mean for two replicate cultures.