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

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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 repressor (*metJ*) and threonine biosynthetic (*thrBC*) genes were introduced into the W3110 wild-type strain of *E. coli*. Mutations of the *metK* 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 ^{14}C-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.

L-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*, *metBJ*, *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 $MgSO_4 \cdot 7H_2O$, 16 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 2 g Bacto yeast extract, 0.01 g $MnSO_4 \cdot 4H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g Thr (for Thr-auxotrophic mutants), and 30 g $CaCO_3$ (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 K_2HPO_4, 3 g KH_2PO_4, 0.5 g Na_2 citrate $\cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 5 g $(NH_4)_2SO_4$. Luria-Bertani (LB) medium was used for all other manipulations.

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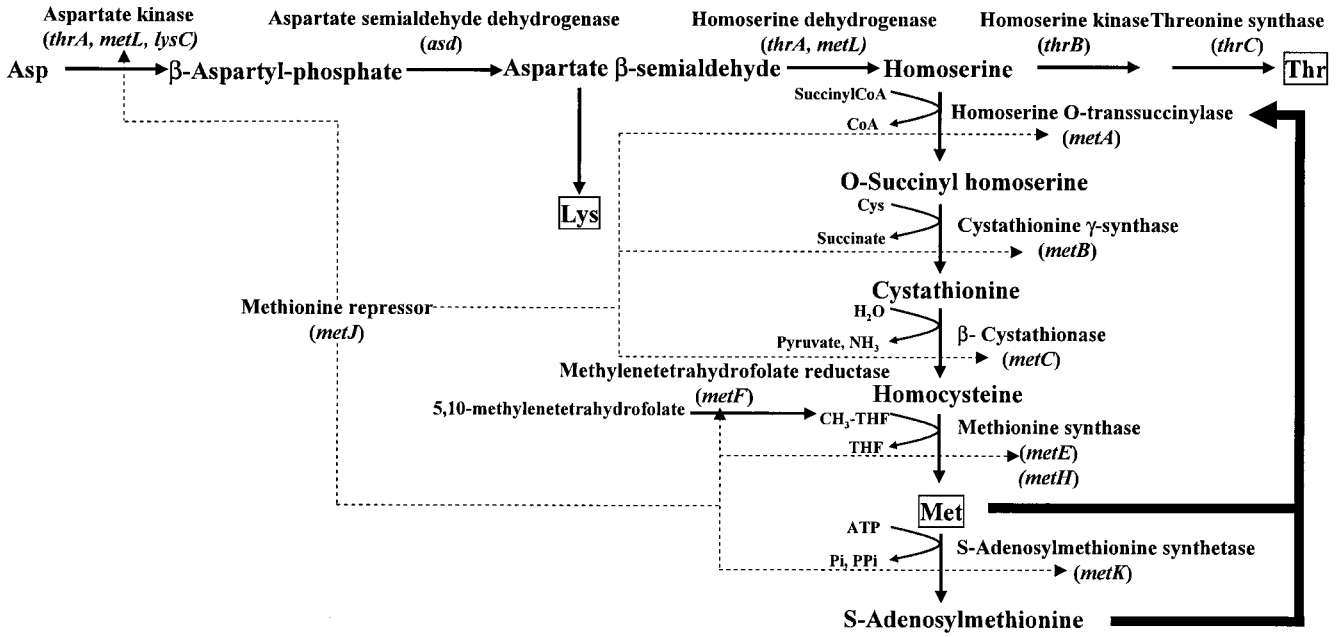

FIG. 1. Biosynthesis and regulation of methionine in *E. coli*. The boldface arrows indicate feedback inhibition, and the dotted arrows indicate repression. CoA, coenzyme A.

Chemicals. DL-[U-^{14}C]homoserine (50 mCi/mmol) was synthesized by Muromachi 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′-GGGAATTCTGGCAGGAGG AACTGGCGCA-3′ (EcoRI site underlined) and 5′-GGGTCGACGCTCATA TTGGCACTGGAAG-3′ (SalI site underlined) and digested using EcoRI and SalI. The *thrC* region was amplified by PCR with primers 5′-GGGTCGACAT CAGTAAAATCTATTCATT-3′ (SalI site underlined) and 5′-GGAAGCTTGC CCGAGGGAAAGATCTGTA-3′ (HindIII site underlined) and treated with SalI and HindIII. The amplified fragments were mixed, ligated into plasmid pMAN997, and digested using EcoRI and HindIII. The resultant plasmid, pMAN*thrBC*, was used to obtain the *thrBC*-deficient mutant from W3110 by homologous recombination. The Thr-auxotrophic strain selected was designated WΔ*thrBC*.

A 1-kb fragment containing the region of the *metB* gene was then amplified using primers 5′-GGGCATGCCCAGGGAACTTCATCACATG-3′ (SphI site underlined) and 5′-GGGAATTCTCATGGTTGCGGCGTGAGAG-3′ (EcoRI site underlined) and digested using SphI and EcoRI. The *metJ* gene region was amplified using primers 5′-GGAAGCTTGCGTGAGATGGGGATTAACC-3′ (HindIII site underlined) and 5′-GGGAATTCTACTGCTAGCTGCTCTTGC G-3′ (EcoRI site underlined) and restricted using HindIII and EcoRI. Both of

TABLE 1. List of bacterial strains and plasmids

Strain or plasmid	Genotype or gene	Source or reference
Strains		
W3110	Wild type	Our collection
WΔ*thrBC*	W3110 *thrBC*	This study
WΔ*thrBC*Δ*metJ*	W3110 *thrBC metJ*	This study
WΔ*thrBC*Δ*metJmetK2*	W3110 *thrBC metJ metK2*	This study
WΔ*thrBC*Δ*metJmetK24*	W3110 *thrBC metJ metK24*	This study
WΔ*thrBC*Δ*metJmetK32*	W3110 *thrBC metJ metK32*	This study
Plasmids		
pMW118	Plasmid vector, Amp^r	Nippon Gene
pMWP*thrmetA*	Wild-type *metA* gene under the control of P_{thr}	This study
pMWP*thrmetA4*	*metA4* gene under the control of P_{thr}	This study
pMWP*thrmetA5*	*metA5* gene under the control of P_{thr}	This study
pMWP*thrmetA9*	*metA9* gene under the control of P_{thr}	This study
pMWP*thrmetA4+9*	*metA* gene with combined mutation of *metA4* and *metA9* under the control of P_{thr}	This study
pMWP*thrmetA5+9*	*metA* gene with combined mutation of *metA4* and *metA9* under the control of P_{thr}	This study
pMWP*thrmetA4+5+9*	*metA* gene with combined mutation of *metA4*, *metA5*, and *metA9* under the control of P_{thr}	This study

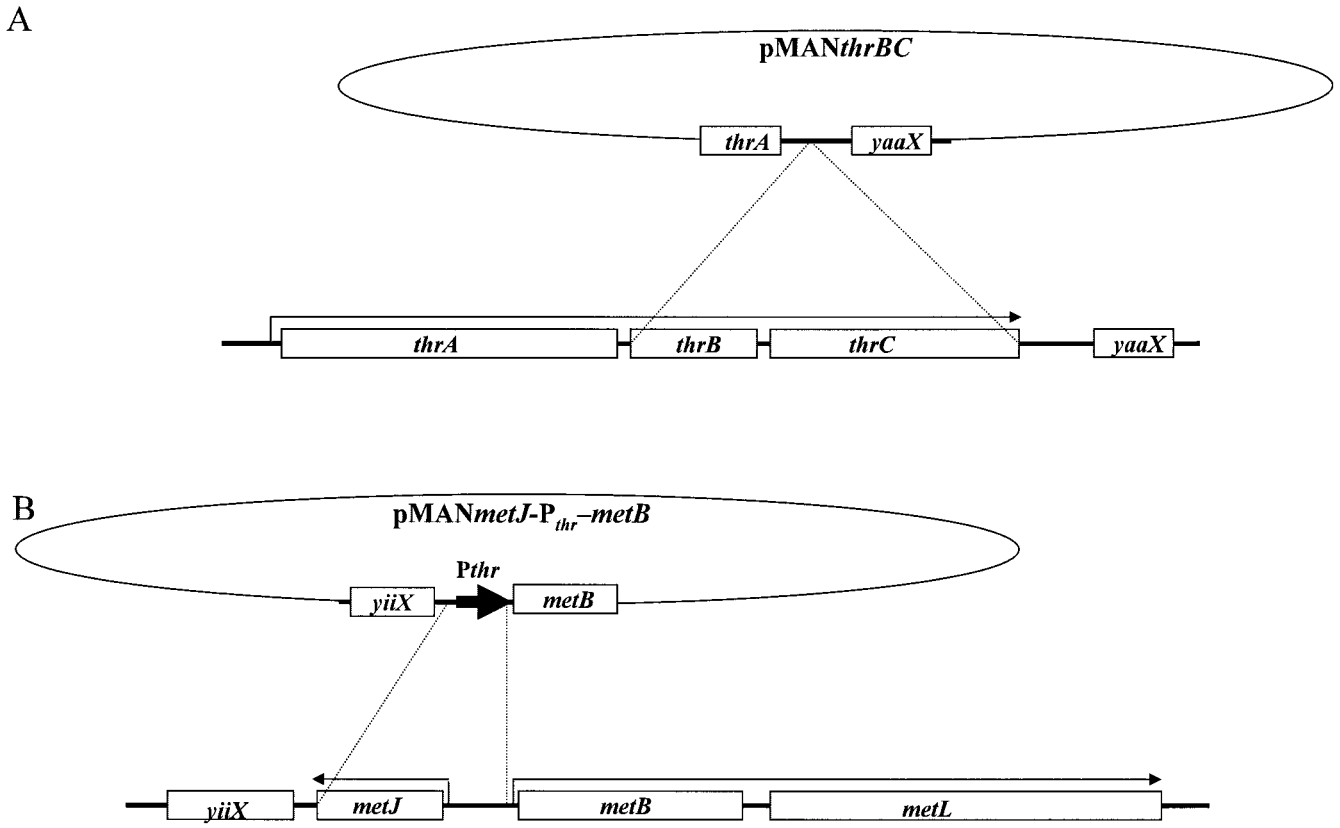

FIG. 2. Schematic representation of recombination strategies. (A) Construction of *thrBC* disrupted strain. The lower part depicts the structure of plasmid pMAN*thrBC*. The upper part depicts the gene organization of the wild-type *thr* operon (*thrABC*) on the chromosome. The dotted lines show the points of homologous recombination. (B) Construction of the strain with *metJ* disruption and *thr* operon promoter (P_{thr}) insertion into the *metBL* operon. The lower part depicts the structure of plasmid pMAN*metJ*-P_{thr}-*metB*. The upper part depicts the gene organization of the wild-type *metJ* and *metBL* operon on chromosome. P_{thr} is indicated by a boldface arrow. The dotted lines show the points of recombination.

the 75-bp strands of the *thr* operon promoter (P_{thr}) region, 5′-GGAAGCTTAA AATTTTATTGACTTAGGTCACTAAATACTTTAACCAATATAGGCATA GCGCACAGACGCATGCCC-3′ (HindIII and SphI sites underlined, respectively), were synthesized, annealed, and digested using HindIII and SphI.

Three fragments and plasmid pHSG298 digested with EcoRI were ligated. Plasmid pHSG*metJ*-P_{thr}-*metB*, into which the three fragments were inserted, was then selected and sequenced for confirmation. The *metJ*-P_{thr}-*metB* fragments that were excised from pHSG*metJ*-P_{thr}-*metB* were inserted into pMAN997, and the resultant plasmid was designated pMAN*metJ*-P_{thr}-*metB*. Using this plasmid, a *metJ*-deficient strain and a strain with the promoter replaced were derived from WΔ*thrBC* and W3110 and designated WΔ*thrBC*Δ*metJ* and WΔ*metJ*, 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′-GGAAGCTTAAGCAGAGATGCAGAGTG CG-3′ and 5′-GGAAGCTTGGTGCGGTATAAGAGGCCAC-3′ (HindIII sites underlined). Mutations in the *metK* structural gene were identified by DNA sequencing using the following six internal sequencing primers: 5′-CAACAGT TTGAGCTAACC-3′, 5′-GCGGTTTTTTTGCCGGATGC-3′, 5′-TCGGCTAC GCAACTAATG-3′, 5′-GAGAATGCACCGCCACCG-3′, 5′-TGGCGCGTCA CGGTGGCG-3′ and 5′-GCACGTCGGTTTCATTAG-3′. To introduce the mutation into the host strains, fragments amplified from the *metK* mutants were introduced into plasmid pSTV28 at the HindIII site, transferred to pMAN997, and then subjected to homologous recombination.

Isolation of *metA* mutants and construction of *metA* expression plasmids. 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 5 ml of Davis-Mingioli minimal medium 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 of α-MM on minimal medium plates again.

The *metA* fragment was amplified using primers 5′-GGGCATGCTGTAGTG AGGTAATCAGGTT-3′ (SphI site underlined) and 5′-GGGTCGACTTAATC CAGCGTTGGATTCA-3′ (SalI site underlined) and the W3110 genome 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′-TGTCGCTG GGCGGTACA-3′ and 5′-AGAGAGTTTTTCGGTGCG-3′. The wild and mutated *metA* fragments were digested with SphI and SalI, the P_{thr} fragments were digested with HindIII and SphI, and plasmid pMW118 treated with HindIII and SalI was mixed and ligated. The resultant plasmid, which contained the *metA* gene under the control of P_{thr}, was selected and used to produce the *metA* expression plasmid (designated pMWP*thrmetA* for wild-type *metA*). In order to combine the *metA* mutations, 5′-phosphorylated primers containing the *metA* mutation points, 5′-CCAGACGCACAAGAAGTTGTC-3′ for *metA9* and 5′-T AGATCGTATAGCGTGTCTCTGGTAGAC-3′ for the *metA4* mutation plus the *metA5* mutation, were used for site-directed mutagenesis.

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 (Sigma-Aldrich), 0.2 mM L-homoserine, and 2 nM DL-[U-^{14}C]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 acetone, 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 [^{14}C]homoserine to [^{14}C]*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 WΔ*thrBC*.

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 P_{thr} simultaneously using the temperature-sensitive origin of replication plasmid (Fig. 2B). We obtained a *metJ*-deficient mutant from WΔ*thrBC* and mutants WΔ*thrBC*Δ*metJ* and WΔ*metJ* 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 WΔ*metJ* strain (126 mmol/min/mg protein) than in the W3110 strain (0.3 mmol/min/mg protein). Furthermore, the MetB activity of WΔ*metJ* 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 *metK* mutations, as mapped using P1 transduction (1). Using norleucine to isolate *metK* mutants, we obtained 12 resistant strains from W3110. The *metK* region of each strain was amplified using PCR, and the nucleotide sequences were determined. Mutations in the structural *metK* gene were observed in 3 of the 12 norleucine-resistant strains; these mutations were designated *metK2*, *metK24*, and *metK32*. The first two strains both had a nucleotide substitution that led to an amino acid substitution (*metK2* and *metK24*), whereas the third strain had a nucleotide deletion that caused a frameshift in the translated polypeptide (*metK32*) (Table 2). These mutations were introduced into the WΔ*thrBC*Δ*metJ* strain using the plasmid pMAN997 vector, and the resultant strains containing *metK* mutations were designated WΔ*thrBC*Δ*metJmetK2*, WΔ*thrBC*Δ*metJmetK24*, and WΔ*thrBC*Δ*metJmetK32*. In order to test the effects of these mutations on Met excretion, the wild-type *metA* expression plasmid pMWP*thrmetA* was constructed (as described above). This plasmid was introduced into W3110, WΔ*thrBC*, WΔ*thrBC*Δ*metJ*, WΔ*thrBC*Δ*metJmetK2*, WΔ*thrBC*Δ*metJmetK24*, and WΔ*thrBC*Δ*metJmetK32*, and the Met excretion levels were determined by cultivating the strains in MS medium (Table 3). The results showed that the *metJ* deletion had a significant effect on Met excretion in Thr-auxotrophic mutants. Furthermore, of the *metK* mutations, the *metK24* mutation clearly had the greatest impact on Met production.

TABLE 2. *metK* mutations found in norleucine-resistant strains

Mutation	Change in nucleotide	Change in amino acid(s)
metK2	^{907}A → C	^{303}Ile → Leu
metK24	^{554}T → A	^{185}Val → Glu
metK32	^{1132}C deletion	^{378}Arg-Asp-Ala-Ala-Gly-Leu-Lys-stop → ^{378}Ala-Met-Leu-Pro-Val-stop

Desensitization of MetA. MetA is the first unique enzyme in the Met biosynthetic pathway, which catalyzes the succinylation 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 IS*2* transposition (6) with a duplication of five nucleotides (^{886}ATCTC) 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 ^{298}Pro onward was altered to ^{298}Arg-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 DL-[^{14}C]homoserine as a

TABLE 3. Met excretion levels of wild-type strains with the *metA* gene introduced[a]

Strain	Plasmid	Optical density at 600 nm	Excreted Met concn (g/liter)
W3110	pMW118	21.3 ± 1.7	ND[b]
W3110	pMWP*thrmetA*	23.1 ± 1.0	ND
WΔ*thrBC*	pMWP*thrmetA*	9.5 ± 0.8	0.008 ± 0.001
WΔ*thrBC*Δ*metJ*	pMWP*thrmetA*	7.9 ± 0.1	0.022 ± 0.004
WΔ*thrBC*Δ*metJmetK2*	pMWP*thrmetA*	6.9 ± 0.2	0.014 ± 0.002
WΔ*thrBC*Δ*metJmetK24*	pMWP*thrmetA*	6.9 ± 0.6	0.141 ± 0.004
WΔ*thrBC*Δ*metJmetK32*	pMWP*thrmetA*	6.5 ± 0.1	0.023 ± 0.002

[a] Each value is the mean ± standard deviation of the mean for two replicate cultures.
[b] ND, not detected.

TABLE 4. *metA* mutations in α-MM-resistant strains

Mutation	Change in nucleotide	Change in amino acid
metA4	^{887}T → G	^{296}Ile → Ser
metA5	^{893}C → T	^{298}Pro → Leu
metA9	^{79}C → T	^{27}Arg → Cys
metA7	IS insertion	^{298}Pro-Tyr-Asp-Leu-Arg-His-Met-Asn-Pro-Thr-Leu-Asp-stop → ^{298}Arg-Leu-Ala-Pro-stop
metA8	IS insertion	^{298}Pro-Tyr-Asp-Leu-Arg-His-Met-Asn-Pro-Thr-Leu-Asp-stop → ^{298}Arg-Leu-Ala-Pro-stop

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 *metA7* extract as a result of the amino acid modification caused by the IS*2* 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: pMWP*thrmetA4*, pMWP*thrmetA5*, and pMWP*thrmetA9* harbored single mutations; pMWP*thrmetA4+9* and pMWP*thrmetA5+9* harbored double mutations; and pMWP*thrmetA4+5+9* harbored all three mutations. These plasmids were introduced into host strain WΔ*thrBC*Δ*metJmetK32*, 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.

TABLE 5. Inhibition to desensitized MetA derived from α-MM-resistant mutants

Inhibitor	MetA activity (mmol/min/mg protein)				
	W3110	*metA9*	*metA4*	*metA5*	*metA7*
None	22.3 (100)[b]	5.0 (100)	4.5 (100)	4.5 (100)	ND[a]
0.1 mM α-MM	18.6 (83)	4.9 (99)	4.1 (93)	4.6 (102)	ND
1 mM α-MM	7.0 (31)	2.7 (54)	4.6 (103)	4.8 (107)	ND
0.1 mM Met	14.3 (64)	2.5 (51)	4.5 (101)	4.2 (94)	ND
1 mM Met	0.8 (4)	2.2 (44)	4.0 (89)	4.0 (88)	ND
0.1 mM SAM	17.0 (76)	1.1 (22)	4.6 (103)	3.6 (79)	ND
1 mM SAM	3.0 (13)	0.5 (10)	2.6 (58)	3.3 (72)	ND
0.1 mM Met + SAM	0.0 (0)	0.9 (19)	5.6 (125)	2.8 (61)	ND

[a] ND, not detected.
[b] Numbers in parentheses are relative activities (%).

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 *metJ* 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 *metJ* 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 ^{14}C-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) compared with the *metA4* and *metA5* mutations. However, the combination of the *metA9* mutation with *metA4* or *metA5* significantly affected Met excretion. 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 ^{27}Arg 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 well conserved across the bacteria. Similarly, the ^{298}Pro residue that was replaced in the protein encoded by the *metA5* gene, which was located close to the carboxy terminus of the protein, is also conserved across many species. By contrast, the ^{296}Ile 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 ^{298}Pro. 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 WΔ*thrBC* 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 *metJ* 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-

TABLE 6. Met excretion by desentisized metA gene-containing strains[a]

Strain	Plasmid	Optical density at 600 nm	Excreted Met concn (g/liter)
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA*	6.5 ± 0.4	0.023 ± 0.002
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA4*	7.4 ± 0.3	0.158 ± 0.008
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA5*	6.9 ± 0.3	0.108 ± 0.007
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA9*	7.3 ± 0.2	0.131 ± 0.006
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA4+9*	8.1 ± 0.2	0.206 ± 0.021
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA5+9*	8.6 ± 0.7	0.207 ± 0.017
W*ΔthrBCΔmetJmetK32*	pMWP*thrmetA4+5+9*	9.5 ± 0.3	0.236 ± 0.007

[a] Each value is the mean ± standard deviation of the mean for two replicate cultures.

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 metJ* 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 ^{54}Ser by Asn, which accumulated Met in the culture medium. This mutation had an effect similar to the effect of MetJ disruption. Chattopadhyay and coworkers (2, 3) also described enhanced methionine production in Thr analogue- and 5-bromouracil-resistant mutants. However, from the point of view of industrialization, the amounts of Met excretion reported in these studies were far from sufficient.

Many factors must be considered in order to increase the accumulation of Met. The most notable feature in the biosynthesis of this amino acid is the incorporation of sulfur as L-cysteine (Cys); this step is catalyzed by cystathionine γ-synthetase, which is encoded by *metB*. Furthermore, C_1 transfer is involved in the last step of the pathway, which is catalyzed by methionine synthase (encoded by *metE* and *metH*). These steps are both potentially rate limiting in the large-scale production of Met. The C_1 unit provided as methyl-tetrahydrofolate is derived from L-glycine (Gly), and both Cys and Gly are synthesized from L-serine (Ser). Therefore, the balanced synthesis of Cys and Gly from Ser, as well as the synthesis of Ser and oxaloacetate from 3-phosphoglycerate, is essential for the coordinated biosynthesis of Met. Cys production by modified serine acetyltransferase has been reported previously (16). Another factor that might be effective in promoting Met excretion is the Met exporter, and recent studies have revealed the importance of efflux carriers for amino acid excretion (5, 9, 12, 23). A Met exporter might be functional when excess Met is toxic inside the cell. This may explain why there were no negative effects on growth due to Met overproduction under the deregulated conditions for Met biosynthesis in this study (Table 6). Met biosynthesis seems to be regulated tightly, possibly because Met is valuable for cells due to the requirement for energy, including sulfur reduction. We were able to observe Met excretion into the medium only when energy was in excess due to growth restriction by auxotrophy. Therefore, a combination of deregulation of Met biosynthesis, restricted growth, and Met export activity is necessary for excretion of large amounts of Met.

In conclusion, in this study, we deregulated some of the controls of Met biosynthesis and metabolism in the W3110 wild-type strain of *E. coli*. 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*.

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