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Novel *Escherichia coli* K-12 Mutants Impaired in *S*-Adenosylmethionine Synthesis

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S-Adenosylmethionine (AdoMet) plays a myriad of roles in cellular metabolism. One of the many roles of AdoMet in *Escherichia coli* and *Salmonella typhimurium* is as a corepressor of genes encoding enzymes of methionine biosynthesis. To investigate the metabolic effects of large reductions in intracellular AdoMet concentrations in growing cells, we constructed and examined mutants of *E. coli* which are conditionally defective in AdoMet synthesis. Temperature-sensitive mutants in *metK*, the structural gene for the *S*-adenosylmethionine synthetase (AdoMet synthetase) expressed in minimal medium, were constructed by in vitro mutagenesis of a plasmid-borne copy of *metK*. By homologous recombination, the chromosomal copy was replaced with the mutated *metK* gene. Both heat- and cold-sensitive mutants were examined. At the nonpermissive temperature, two such mutants had 200-fold-reduced intracellular AdoMet levels and required either methionine or vitamin B_{12} for growth. In the presence of methionine or vitamin B_{12}, the mutants grew at normal rates even though the AdoMet levels remained 0.5% of wild type. A third mutant when placed at nonpermissive temperature had <0.2% of the normal AdoMet level and did not grow on minimal medium even in the presence of methionine or vitamin B_{12}. All of these mutants grew normally on yeast-extract-based medium in which an alternate form of *S*-adenosylmethionine synthetase was expressed.

S-Adenosylmethionine (AdoMet) has numerous roles in metabolism, including acting as a methyl group donor in many reactions, as a propylamine donor in the biosynthesis of the polyamines spermine and spermidine, and in a noncovalent role as a corepressor of the methionine biosynthetic regulon in *Escherichia coli* and *Salmonella typhimurium* (5, 8, 12, 28, 29, 31, 32). A difficulty in studying the behavior of AdoMet-dependent processes in enteric bacteria is the impermeability of the cells to AdoMet and the lack of mutant strains which can be grown with substantially altered AdoMet levels (2, 13, 14, 16, 23). All reported mutants deficient in AdoMet synthetase (ATP:L-methionine *S*-adenosyltransferase) have mapped to *metK*, which encodes the AdoMet synthetase expressed in minimal medium. The reported mutants have maximally fourfold-reduced intracellular AdoMet levels, apparently as a result of production of partially active AdoMet synthetases (9–11, 16–18, 27). These *metK* mutants were isolated as strains resistant to methionine analogs added to the growth medium. Although the *metK* mutants that have so far been isolated are derepressed for production of the enzymes of methionine biosynthesis, it is surprising that the change in total intracellular AdoMet concentration is relatively small. We therefore set out to construct temperature-dependent null mutants of *metK* to verify and reevaluate the roles of AdoMet and AdoMet synthetase in various cellular processes. Through use of in vitro mutagenesis of the cloned *metK* gene and gene replacement methods, we have prepared and characterized new *metK* mutants. These mutants have dramatic temperature-dependent reductions in both intracellular AdoMet synthetase and AdoMet levels and display novel growth behavior.

MATERIALS AND METHODS

The medium components for Vogel-Bonner minimal medium (33) and LB rich medium (20) were purchased from Sigma Chemical Co. and Difco Laboratories, respectively. When present, amino acids were added at 0.01% (wt/vol) and vitamin B_{12} was added at 0.0002%. Biochemicals were purchased from Sigma Chemical Co. Commercial AdoMet was purified before use as described previously (7). Molecular biology reagents were obtained from New England BioLabs or Bethesda Research Laboratories. Other reagents were obtained from commercial sources.

Strains and plasmids. The strains and plasmids used and their sources are listed in Table 1.

Molecular biology methods. Recombinant DNA methods used protocols recommended by the suppliers of the enzymes and other standard procedures (20).

Construction of a *metK*-containing plasmid for mutagenesis. The plasmid used for mutagenesis and the subsequent gene replacement steps, pKA13Kan (Fig. 1), was derived in two steps from the previously described pKA12 (3). pKA12 is a pBR322 derivative which contains *metK*, the *speA* gene which is upstream from *metK*, and ~600 base pairs of DNA downstream from *metK*. pKA13 was obtained by deletion of a 2-kilobase-pair *Eco*RI fragment of pKA12, which removed part of the pBR322 vector and DNA sequences downstream from the *speA* gene. A selectable marker closely linked to *metK* was required for the gene replacement steps. Therefore, a kanamycin resistance cartridge from plasmid pUC4K (25) was inserted into a *Bam*HI site 80 base pairs upstream from the start of the *metK* coding region (21). Restriction maps verified the orientation of the kanamycin cartridge shown in Fig. 1. The insertion of the kanamycin resistance cartridge had no significant effect on level of AdoMet synthetase activity present in strains carrying pKA13Kan versus pKA13.

In vitro mutagenesis. Plasmid pKA13Kan (6 μg) was mutagenized by treatment with 1 M hydroxylamine–1 mM EDTA, pH 6.0, for 45 min at 0°C, followed by incubation for 30 min at 70°C (15). Subsequently, the hydroxylamine was removed by five cycles of concentration and dilution into 1 ml of 30 mM $CaCl_2$, using an Amicon Centricon concentra-

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TABLE 1. *E. coli* strains and plasmids used in this work

Strain or plasmid	Genotype	Source or reference
Strain		
JC7623	F⁻ *arg his leu pro thr thi strA recB21 recC22 sbcB15*	B. Bachmann (19)
DM22	*pro his metG metK serA* Δ(*speC glc*) *leu rpsL thi hsd sup*	G. D. Markham (3)
GW5180	Δ(*lac pro*) *proAB* Z M15 *lacI thi strA endA sbcB15 hsdR4 supE recA938*::*cat* F′ *traD36*	G. Walker (34)
DM50	DM22 *recA938*::*cat*	Transduction (DM22 × GW5180)
DM101	F⁻ *arg his leu pro thr thi strA recB21 recC22 sbcB15 metK501*	This work [gene replacement using linear DNA of pKA13Kan(*metK501*)]
DM102	F⁻ *arg his leu pro thr thi strA recB21 recC22 sbcB15 metK502*	This work [gene replacement using linear DNA of pKA13Kan(*metK502*)]
DM103	F⁻ *arg his leu pro thr thi strA recB21 recC22 sbcB15 metK503*	This work [gene replacement using linear DNA of pKA13Kan(*metK503*)]
Plasmid		
pKA12	*bla speA metK*	S. M. Boyle et al. (3)
pKA13	*bla speA metK*	This work
pKA13Kan	*bla speA kan metK*	This work
pKA13Kan(*metK501*)	*bla speA kan metK501*	This work (hydroxylamine mutagenesis of pKA13Kan)
pKA13Kan(*metK502*)	*bla speA kan metK502*	This work (hydroxylamine mutagenesis of pKA13Kan)
pKA13Kan(*metK503*)	*bla speA kan metK503*	This work (hydroxylamine mutagenesis of pKA13Kan)

tor; the mutagenized plasmid was finally placed in a volume of 0.2 ml of 30 mM $CaCl_2$.

Construction of chromosomal mutations. The mutations from each plasmid were transferred into the *E. coli* chromosome as described by Winans et al. (34). DNA containing the *metK* region without an origin of replication was obtained by an *Eco*RI-*Bal*I double digest of the plasmids (Fig. 1). The *metK*-containing DNA was isolated from agarose gels (1) and transformed into strain JC7623, a *recBC sbcB* strain which is readily transformed with linear DNA. In the absence of an origin of replication on the transforming DNA, all drug-resistant transformants have replaced the chromosomal DNA with the transforming DNA carrying the selectable marker. Transformants were plated on kanamycin-containing medium at the permissive temperature.

Mapping of chromosomal mutations. P1 *vir* lysates of the strains bearing each of the three chromosomal mutations were prepared, and transductions were carried out as described by Miller (26).

Determination of AdoMet pools. Intracellular AdoMet levels were determined by ion-exchange high-performance liquid chromatography with UV detection at 254 nm. A Whatman SCX column (0.46 by 25 cm) was attached to a Beckman 320 high-performance liquid chromatography system. The elution buffer contained 33.5 ml of concentrated NH_4OH per liter adjusted to pH 4.0 with formic acid. Isocratic elution at 1.5 ml/min was used because this gave rapid (~15-min total time) chromatography suitable for quantitation of AdoMet levels. AdoMet eluted as a well-resolved peak at 8.2 ml. AdoMet levels were quantified by comparison of peak heights or areas (with equivalent results) to standard samples. Standards showed elution positions (in milliliters) for adenosine (4.0), *S*-adenosylhomocysteine (4.3), 5′-methylthioadenosine (6.0), and adenine (5.7).

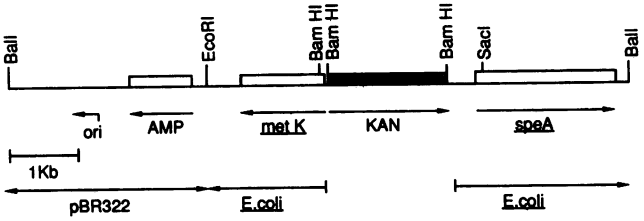

FIG. 1. Restriction map and gene organization in plasmid pKA13Kan. KAN and AMP represent the genes (*kan* and *bla*) that encode proteins that confer resistance to the antibiotics kanamycin and ampicillin, respectively. The origin of replication is represented as Ori, and the direction of transcription of the genes on the plasmid is shown. The gene encoding kanamycin resistance (*kan*) is inserted into pKA13 at the *Bam*HI site upstream of the first codon of the *metK* gene. The originless *Eco*RI-*Bal*I linear DNA fragment that contains *speA*, *metK*, and the gene for kanamycin resistance was used to transform *E. coli* JC7623 to replace the *speA-metK* region on the chromosome. Kb, Kilobase.

Preparation of cell extracts for intracellular AdoMet pool determinations. Cells were harvested by centrifugation of cultures of known density. The cell pellet was extracted with 0.1 volume of cold 10% trichloroacetic acid. After centrifugation, the trichloroacetic acid was removed by three extractions with an equal volume of water-saturated diethyl ether. Volumes between 25 and 250 μl were injected onto the high-performance liquid chromatographic column without alteration of peak width or retention time; chromatograms were obtained in duplicate.

Preparation of cell extracts for enzyme assays. Cells were grown to stationary phase or as described in the figure legends and concentrated by centrifugation to 4×10^9 to 20×10^9 cells per ml. Cells were suspended in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)/KOH–50 mM KCl–0.1% 2-mercaptoethanol (pH 8.0) buffer and broken by sonication, using the microtip of a Branson sonicator (at 100 W for 6 min at 50% duty cycle). After removal of debris by centrifugation (10 min at 10,000 × *g*), aliquots were removed for AdoMet synthetase assays or for protein determination. Protein was determined by the method of Bradford (4), using a kit from Bio-Rad; bovine serum albumin (BSA) was used as the standard.

Enzyme assays. AdoMet synthetase assays monitored the incorporation of [$^{14}CH_3$]methionine (New England Nuclear) into AdoMet as described previously (22), except that the methionine concentration was 0.2 mM and the specific activity was 51.8 mCi/mmol. Whatman P81 ion-exchange

filters were used to retain the AdoMet formed. Assays were conducted at 20 ± 2°C unless otherwise noted. The rate of AdoMet formation was determined by regression analysis of the data obtained by quenching aliquots of the reaction after various incubation times.

Antibody production. AdoMet synthetase was purified from an overproducing clone harboring the *metK*-containing plasmid pKA8 (3), by the method of Markham et al. (22). Antibodies were raised in guinea pigs by using purified *S*-adenosylmethionine synthetase (*metK* product) as the antigen. Preimmune sera were obtained from each animal prior to immunization. AdoMet synthetase, 0.15 mg in complete Freund adjuvant, was injected intradermally. After 6 weeks, the animals were injected with 0.15 mg of enzyme in incomplete Freund adjuvant. This was followed after 3 weeks by another injection of 0.1 mg of enzyme in incomplete Freund adjuvant. Antisera were obtained 2 weeks after the third injection. After removal of erythrocytes, the sera were stored in aliquots at −70°C. Antisera were used without further fractionation.

Immunodiffusion analysis. Ouchterlony double-diffusion experiments were carried out as described before (30), except that the 1% agarose contained 50 mM HEPES/KOH–50 mM KCl (pH 8.0) instead of buffered saline.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by McGrogan et al. (24).

Immunoblotting. Immunologically cross-reactive protein bands were detected on immunoblots with the Vectastain ABC peroxidase kit obtained from Vector Laboratories. The kit was used as described by the supplier, with the following modifications. The SDS-polyacrylamide gel was laid against nitrocellulose (BA-85, 0.45 μm; Schleicher & Schuell) and electrophoresed in a Bio-Rad transblot apparatus for 4 h at 150 mA, using a buffer of 25 mM Tris, 193 mM glycine, and 10% methanol. The nitrocellulose filter was then soaked in 10 ml of BSA buffer (3% BSA, 0.9% NaCl, 10 mM Tris hydrochloride, pH 7.4) overnight at 4°C. To this solution ~10 μl of anti-AdoMet synthetase antibody was added, and the filter was incubated on a rocking platform for ~2 h at 25°C. Following six washes with distilled water (total time, ~1 min), ~100 μl of biotinylated anti-guinea pig immunoglobulin G (1.5 mg/ml) in BSA buffer was added, and the solution was allowed to mix for 1 h at 25°C. The filter was rinsed with distilled water as described above and then soaked in a mixture containing ~100 μl each of solution A and solution B (vector ABC kit) in 5 ml of BSA buffer for 1 h at 25°C. Once again, the filter was washed with distilled water as described before, and 10 μl of peroxidase substrate (0.6 mg of 4-chloro-1-naphthol per ml in methanol) and 10 μl of 30% H_2O_2 were added to the filter in 10 ml of BSA buffer.

RESULTS

Construction and characterization of plasmid-borne temperature-sensitive *metK* mutants. The strategy for obtaining plasmids with *metK* mutations relied on the use of a *metG metK* strain. A typical *metG* mutant (which has a mutation in methionyl-tRNA synthetase that results in a high K_m for methionine) has a higher than normal requirement for methionine for growth and therefore is a methionine auxotroph. However, the chromosomal *metK* mutation causes derepression of the genes for the methionine biosynthetic enzymes, resulting in overproduction of methionine. Thus, a *metG metK* double mutant such as strain DM50 is a methionine prototroph (6). However, strain DM50 becomes a methionine auxotroph when it is transformed with a plasmid which provides a *metK* gene that is expressed even at the level of a single chromosomal equivalent copy.

TABLE 2. AdoMet synthetase activities of plasmid-borne *metK* mutants[a]

Plasmid	Growth temp (°C)	AdoMet synthetase activity (nmol/min per mg of protein)
metK$^+$	30	0.95
metK$^+$	42	0.94
pKA13Kan	30	14.5
pKA13Kan	42	16.2
pKA13Kan(*metK501*)	30	1.2
pKA13Kan(*metK501*)	42	10.6
pKA13Kan(*metK502*)	30	13.1
pKA13Kan(*metK502*)	42	1.2
pKA13Kan(*metK503*)	30	10.7
pKA13Kan(*metK503*)	42	1.0

[a] Plasmids were harbored in strain JC7623. Cells were grown to stationary phase in Vogel-Bonnet medium containing appropriate amino acids. When plasmids were present, 50 μg of kanamycin per ml was included. The uncertainty in specific activity is estimated to be ±10%.

Plasmid pKA13Kan (Fig. 1), which contains the *metK* and *speA* genes with a kanamycin resistance cartridge inserted in the intergenic region 80 base pairs upstream from the beginning of the *metK* coding region (see Materials and Methods), was mutagenized with hydroxylamine, and the treated plasmid was transformed into strain DM50 (*metK metG*). Transformants were selected at 30 and 42°C in the presence of methionine.

These transformants were checked for methionine requirement at both temperatures. Kanamycin-resistant transformants which were Met$^+$ at 30 and 42°C contained plasmids which did not produce a fully active AdoMet synthetase at the temperature used. Based on the levels of AdoMet synthetase present in DM50, the equivalent of at most ~10% of the enzyme activity from a single copy of wild-type *metK*$^+$ would allow transformants to grow in the absence of methionine, corresponding to a very low activity for the enzyme produced from *metK* on a multicopy plasmid. Several hundred transformants were tested for temperature sensitivity of the Met$^+$ phenotype. Three temperature-sensitive transformants were chosen for further study; these were denoted as pKA13Kan(*metK501*), pKA13Kan(*metK502*), and pKA13Kan(*metK503*). Plasmids pKA13Kan(*metK502*) and pKA13Kan(*metK503*) allowed growth in the absence of methionine at 42°C, but not at 30°C, while plasmid pKA13Kan(*metK501*) allowed methionine-independent growth at 30 but not 42°C. Plasmids were isolated from these strains and were retransformed into DM50; the phenotypes of the resulting strains were the same as the original isolates, indicating that the temperature sensitivity was associated with the plasmid.

To characterize the mutations, the plasmids were transformed into strain JC7623. AdoMet synthetase levels were determined for strain JC7623 lacking plasmid, containing the unmutagenized plasmid pKA13Kan, and those containing the three plasmids with putative *metK* mutations grown in the presence of methionine at 30 and 42°C (Table 2). Strain JC7623 was chosen since it was also used in studies of these mutations when present on the chromosome. At the nonpermissive temperature, the AdoMet synthetase activities in the extracts of the strains containing the plasmid-borne mutant *metK* were indistinguishable from JC7623 lacking a *metK* plasmid and were ~6% of the level of JC7623(pKA13Kan). At permissive temperatures, the AdoMet synthetase activi-

ties were similar to those of JC7623 bearing unmutagenized pKA13Kan. Analogous results were obtained when the enzyme activities were measured in extracts of strain DM50 containing these plasmids. These results confirmed that the methionine-dependent growth phenotype observed in strain DM50 containing the plasmids is associated with mutations affecting *metK*. To investigate the nature of the mutations further, extracts prepared from cells grown at each temperature were incubated for 30 min at both growth temperatures before assay. Preincubation at 42°C of the extract prepared from JC7623(pKA13Kan[*metK503*]) grown at 30°C reduced the enzyme activity to that of cells grown at 42°C, indicating a reduction in protein stability of the mutant (data not shown). In contrast, the enzyme activity present in extracts of strains carrying plasmids pKA13Kan(*metK501*) and pKA13Kan(*metK502*) was unaltered by preincubation at either 30 or 42°C, suggesting a temperature-dependent deficiency of protein quantity.

To examine a possible temperature-dependent alteration of in vivo AdoMet synthetase protein levels, extracts were electrophoresed on SDS-PAGE gels; after transfer of proteins to nitrocellulose, reactivity to anti-AdoMet synthetase antibodies was examined. At permissive temperature, AdoMet synthetase protein levels produced by all three mutant plasmids were similar to those of the parent plasmid, while much less protein was present at the nonpermissive temperature (data not shown). Immunoblotting results confirm the decreased protein quantity in strains carrying pKA13Kan(*metK501*) and pKA13Kan(*metK502*) at the nonpermissive temperature. The reduced level at 42°C for the unstable *metK* protein in strains carrying pKA13Kan (*metK503*) is consistent with the failure of nonfunctional AdoMet synthetases produced from other mutant *metK* genes to accumulate to appreciable levels, apparently due to susceptibility to proteolysis in vivo (unpublished results).

Replacement of a chromosomal MetK with the temperature-sensitive mutants. The *metK* mutants described above were used to replace the chromosomal *metK*$^+$ of strain JC7623 as described by Winans et al. (34). An *Eco*RI-*Bal*I restriction double digest of the mutant pKA13Kan-derived plasmids was performed, and the fragment containing *metK* was purified by agarose gel electrophoresis. The purified DNA was used to transform strain JC7623. Transformants were selected on medium containing kanamycin at the permissive temperature for the particular *metK* allele. Approximately 50 transformants were obtained in each case, and characterization of 25 individual isolates of each allele indicated that each behaved identically. When the kanamycin-resistant transformants were tested for growth at nonpermissive temperature, it was found that in no case did the transformants grow on the minimal medium which had previously supported growth at the permissive temperature. When the medium was supplemented with either methionine or vitamin B_{12}, both *metK501* and *metK502* mutants grew normally at nonpermissive temperature, whereas no growth of *metK503* was observed at 42°C. Replacing methionine with homocysteine did not allow growth of *metK501* or *metK502* at the nonpermissive temperature. One isolate of each type was selected for further characterization.

To verify that the kanamycin resistance and temperature-sensitive growth were linked, and were associated with *metK*, bacteriophage P1 lysates were made on each strain and were used to transduce strain DM22 to kanamycin resistance. For each strain, all tested transductants (125 of 125) required methionine for growth even at the permissive temperature, as expected for a *metK*$^+$ *metG* strain. Furthermore, the transductants displayed 20% (24 of 125) cotransduction of the *serA* mutation to *ser*$^+$, in good agreement with reported cotransduction frequencies of *metK* and *serA* (9). The introduction of mutations into the chromosomal copy of *metK* in strain JC7623 was also indicated by the elimination of temperature sensitivity for growth of mutant strains when they were transformed with a plasmid containing *metK*$^+$ (e.g., pKA13Kan).

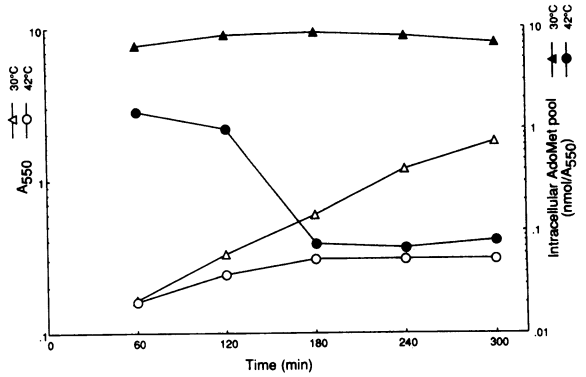

FIG. 2. Growth curve and AdoMet pools in the mutant *metK503* following temperature shift. The cells were grown overnight in limiting glucose (0.01%) in minimal medium containing amino acids and vitamin B_{12} at 30°C. The following day replicate cultures were replenished with glucose (0.2% final concentration) and were allowed to grow at 30 and 42°C for 5 h. Aliquots were withdrawn every hour for determination of AdoMet pools. The viable count determined for the wild type and the mutant strain when cultured at 30°C was 4×10^8 CFU/ml per A_{550} unit.

AdoMet synthetase activities and intracellular AdoMet levels in *metK* mutants. To examine the effects of the temperature-sensitive *metK* mutations on cell growth, each strain was grown to stationary phase at the permissive temperature and then diluted to $\sim 4 \times 10^7$ cells per ml into minimal medium at permissive and nonpermissive temperature. The three mutants behaved similarly, and a typical growth curve for strain *metK503* is shown in Fig. 2. During growth, aliquots were removed for determination of the intracellular AdoMet level (Fig. 2). The intracellular AdoMet levels decreased dramatically at nonpermissive temperature in each case. When growth had stopped, the AdoMet levels had reached 0.5% of wild type for the *metK501* and the *metK502* mutants and <0.2% of wild type for the *metK503* mutant. The temperature shift led to a profound decrease in AdoMet synthetase activity (data not shown). Immunoblots (Fig. 3) showed the disappearance of immunologically reactive protein at the nonpermissive temperature, as expected from the immunoblots with extracts of strains bearing mutant plasmids.

When the medium was supplemented with methionine or vitamin B_{12}, the *metK501* and *metK502* mutants grew indefinitely at the same growth rate as the parent strain at both permissive and nonpermissive temperatures. In the presence of methionine or vitamin B_{12}, the specific activity of the enzyme and the AdoMet levels were similar to that of the wild type at the permissive temperature, but were reduced to 4 and ~0.5% of wild type, respectively, at the nonpermissive temperature (Table 3). The presence of methionine or vitamin B_{12} did not allow *metK503* to grow at the nonpermissive temperature, and AdoMet synthetase activity was undetectable (<1% of wild type) when growth stopped.

The higher AdoMet pool sizes in strains *metK501* and *metK502*, compared with that of a strain carrying *metK503*,

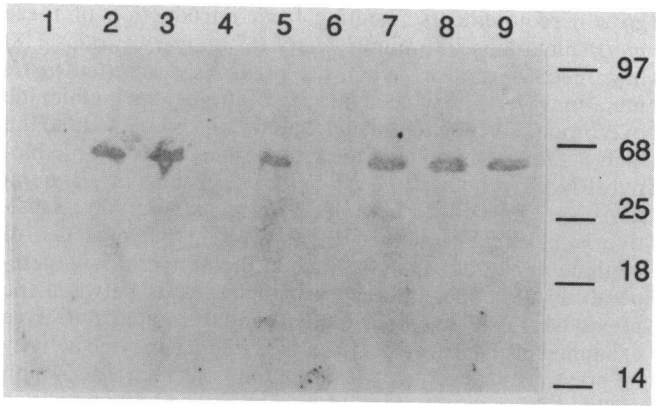

FIG. 3. Immunoblot analysis of SDS-PAGE-separated extracts of wild-type and mutant strains grown in minimal medium at permissive and nonpermissive temperatures. Extracts were prepared from cells cultured as described (see Materials and Methods), while for the mutant *metK503* the cells were cultured as described in the legend to Fig. 2. Equivalent amounts of protein (~100 μg) of each extract and ~0.3 μg of purified AdoMet synthetase (*metK* gene product) were electrophoresed on a 17.5% SDS-polyacrylamide gel. Numbers on the right correspond to the molecular mass in kilodaltons of the prestained protein standards. Lanes 1 to 8 correspond to extracts of mutant and wild-type strains. Incubation temperatures are indicated in parentheses: lane 1, *metK501* (30°C); lane 2, *metK501* (42°C); lane 3, *metK502* (30°C); lane 4, *metK502* (42°C); lane 5, *metK503* (30°C); lane 6, *metK503* (42°C); lane 7, wild type (30°C); lane 8, wild type (42°C); lane 9, purified AdoMet synthetase (*metK* gene product).

and the small amounts of enzyme activity in extracts of these mutants suggested that there might be slight AdoMet synthetase activity produced from *metK* in these mutants at nonpermissive temperature. To test this hypothesis, strains containing these mutations were transformed with the pKA13Kan derivatives containing the same mutations: pKA13Kan(*metK501*) was transformed into *metK501*; pKA13Kan(*metK502*), into *metK502*; and pKA13Kan (*met503*), into *metK503*. When the mutant *metK* genes were also present on a multicopy plasmid, temperature-sensitive growth phenotypes were not observed for either the *metK501* or *metK502* mutants, while the temperature-sensitive growth of the *metK503* mutant was unaltered. These

TABLE 3. AdoMet synthetase activities in *metK* mutants in minimal medium[a]

metK allele	Growth temp (°C)	AdoMet synthetase activity (nmol/min per mg of protein)	AdoMet pool size (nmol/10^8 cells)
metK$^+$	30	0.95	1.4
metK$^+$	42	0.94	0.9
metK501	30	0.035	0.008
metK501	42	0.79	1.3
metK502	30	0.84	1.6
metK502	42	0.044	0.008
metK503	30	0.77	1.2
metK503	42	<0.01	<0.003

[a] Cells (JC7623 and mutants derived from it) were grown to stationary phase overnight in Vogel-Bonner medium containing appropriate amino acids and vitamin B_{12}, but limiting (0.01%) glucose. These cultures were made up in glucose concentrations (0.2%), and replicate cultures were incubated at 30 and 42°C. The cells were harvested at late log phase. The *metK503* mutant was harvested when growth stopped. Kanamycin was included in the growth medium of *metK* mutant strains. The uncertainties in enzyme activity and AdoMet pool sizes are estimated to be ±10 and ±20%, respectively.

TABLE 4. AdoMet synthetase activities in *metK* mutants grown in rich medium[a]

metK allele	Growth temp (°C)	AdoMet synthetase activity (nmol/min per mg of protein)	AdoMet pool size (nmol/10^8 cells)
metK$^+$	30	0.44	1.2
metK$^+$	42	0.49	1.0
metK501	30	0.62	0.8
metK501	42	0.41	0.9
metK502	30	0.51	1.6
metK502	42	0.56	1.3
metK503	30	0.46	1.6
metK503	42	0.54	1.3

[a] Cells (JC7623 and mutants derived from it) were grown to stationary phase in LB medium. Kanamycin was included in the growth medium of the mutants. The uncertainties in enzyme activity and AdoMet pool sizes are estimated to be ±10 and ±20%, respectively.

results indicate that the *metK501* and *metK502* mutations are leaky at the nonpermissive temperature, while the *metK503* is not detectably so.

Properties of mutants. As noted above, even with 0.5% of the wild-type intracellular AdoMet level, the *metK501* and *metK502* mutants showed normal growth rates in medium supplemented with either methionine or vitamin B_{12}. Since homocysteine did not replace methionine for growth, a deficiency in methionine synthesis is suggested at its final biosynthetic step, which can occur through either AdoMet- or vitamin B_{12}-dependent enzymes, the *metE* and *metH* gene products, respectively (29).

AdoMet synthetase activities and intracellular AdoMet levels in *metK* mutants grown in rich media. The temperature-sensitive phenotype of the *metK503* mutant was not observed during growth on rich medium (LB medium). Therefore, AdoMet synthetase activities and AdoMet pool sizes were determined for cells grown at 30 and 42°C in LB medium. The AdoMet pools and AdoMet synthetase activities in extracts of the wild-type and all mutant strains were similar at 30 and 42°C growth temperatures (Table 4). Immunoblot analysis (using antiserum against the *metK* gene product) of the wild type and of the mutant extracts prepared from LB-grown cells at either permissive or nonpermissive temperature showed a cross-reactive protein of ~43 kilodaltons (Fig. 4), indistinguishable in migration from the *metK* gene product. These results are consistent with a 43-kilodalton isozyme of AdoMet synthetase being predominantly expressed in rich media.

Immunodiffusion analysis of extracts of strains grown in rich media. Although the two forms of AdoMet synthetases were indistinguishable on denaturing polyacrylamide gels (Fig. 4), immunodiffusion experiments (Fig. 5 and 6) showed the presence of two distinct forms of immunologically related protein, depending on the medium in which cells had been grown. In extracts of wild-type and mutant strains prepared from cells grown in rich media at either 30 or 42°C, immunoprecipitin bands were observed distinct from those of purified AdoMet synthetase (*metK* gene product) (Fig. 5). In minimal media, both the mutant cultured at permissive temperature and the wild-type cell extracts showed immunoprecipitin bands with identity to the purified *metK* protein (Fig. 6). However, precipitin bands were not observed in extracts of mutants cultured in minimal media at the nonpermissive temperature, as expected from the immunoblotting results (Fig. 3). In no case were both immunoprecipitin bands seen in the same extract. These results present evidence for the presence of another gene that encodes an

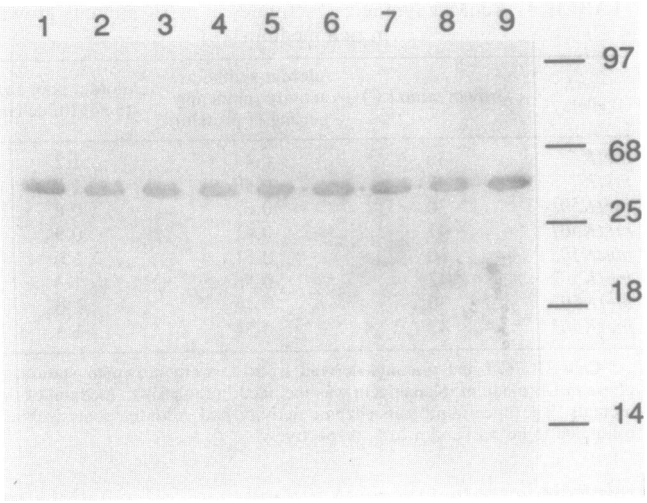

FIG. 4. Immunoblot analysis of SDS-PAGE-separated extracts of wild-type and mutant strains cultured in rich medium (LB) at permissive and nonpermissive temperatures. The cells were grown overnight to stationary phase, and extracts were prepared and electrophoresed as described in the legend to Fig. 3 and immunoblotted (see Materials and Methods). Lanes 1 to 9 are also as described in the legend to Fig. 3.

alternate form of AdoMet synthetase which can be distinguished in its native form from the AdoMet synthetase encoded by the *metK* gene; we denote this gene *metX*.

DISCUSSION

The *metK* mutants constructed in this study confer novel growth phenotypes even in the absence of other mutations in methionine metabolism. Previously obtained *metK* mutants were selected by resistance to methionine analogs, due to the overproduction of methionine. Although none of the previous mutants displayed a distinct phenotype, altered growth requirements resulting from introduction of these *metK* mutations into either *metG* (6) or *metA* strains (18) have been described. When the previously reported *metK* mutations were introduced into *metG* strains, the methionine overproduction due to the *metK* mutation complemented the high K_m for methionine of the mutant *metG* protein, methionyl-tRNA synthetase (6). When these previous *metK* mutations were introduced into *metA* strains, temperature-sensitive *metA metK* double mutants grew in the presence of methionine but not cystathionine at the nonpermissive temperature (18). The differences in phenotypes between the previously described *metK* mutants and the mutants we have examined probably results from the residual enzyme activity of previously described *metK* mutants, which had, minimally, a few percent of wild-type activity and, maximally, a fourfold-reduced intracellular AdoMet level (2, 10, 17, 18). It is the reduction of residual enzyme activity to a much lower level that has enabled us to demonstrate distinct phenotypes in the present set of mutants. The selection we have used is more stringent because of the higher copy number of the plasmid-borne gene, which allows the isolation of *metK* mutants which contain AdoMet synthetase activities at much lower levels than reported previously. It is not clear at present whether the AdoMet synthetase activity found in previous *metK* mutants in minimal media arises from a leaky *metK* mutation or from abnormal expression of the *metX* gene.

Our two mutants with slight (~3%) residual AdoMet synthetase activity, *metK501* and *metK502*, have an ~200-fold reduction in AdoMet pool size at nonpermissive temperatures. The surprising failure of the strains to grow at the nonpermissive temperature in the absence of either methionine or vitamin B_{12}, and their growth at the wild-type rate in the presence of either of these compounds, suggests that the major role of AdoMet is in the final step of methionine biosynthesis, the methylation of homocysteine to yield methionine. The cessation of growth of the *metK503* mutant at the nonpermissive temperature, even in strains that carry the plasmid with the corresponding mutation, is related to the

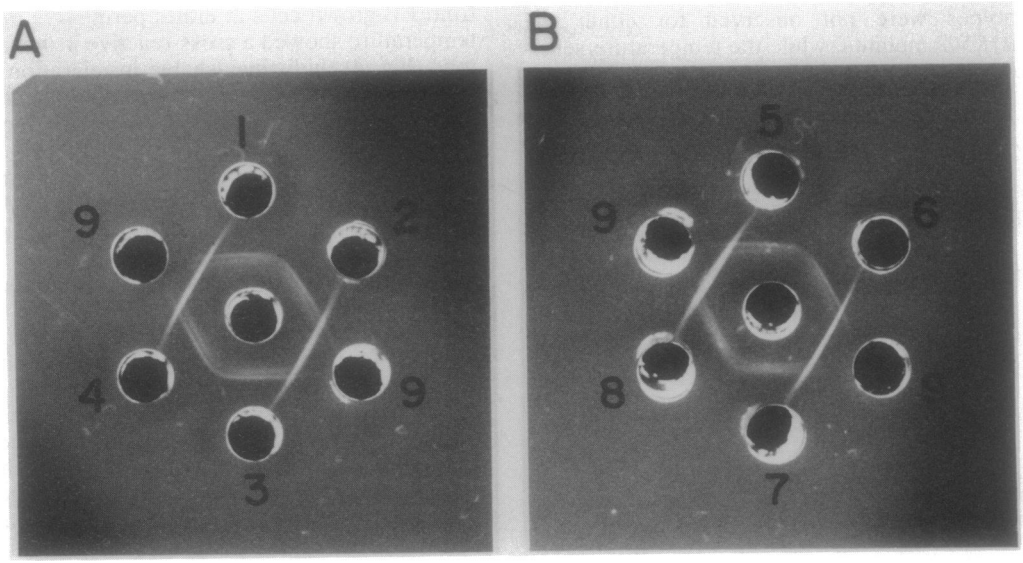

FIG. 5. Immunodiffusion analysis of sonic extracts of wild-type and mutant strains cultured in rich medium (LB) at permissive and nonpermissive temperatures. The cells were grown and the extracts were prepared as described in the legend to Fig. 4. The center well contained 10 μl of antiserum. The sample wells contained 10 μl of sonic extracts of cells or 10 μl of purified *metK* gene product (0.1 mg/ml). The immunodiffusion plates were incubated overnight at 4°C. Numbers 1 to 9 correspond to the lanes described in the legend to Fig. 3.

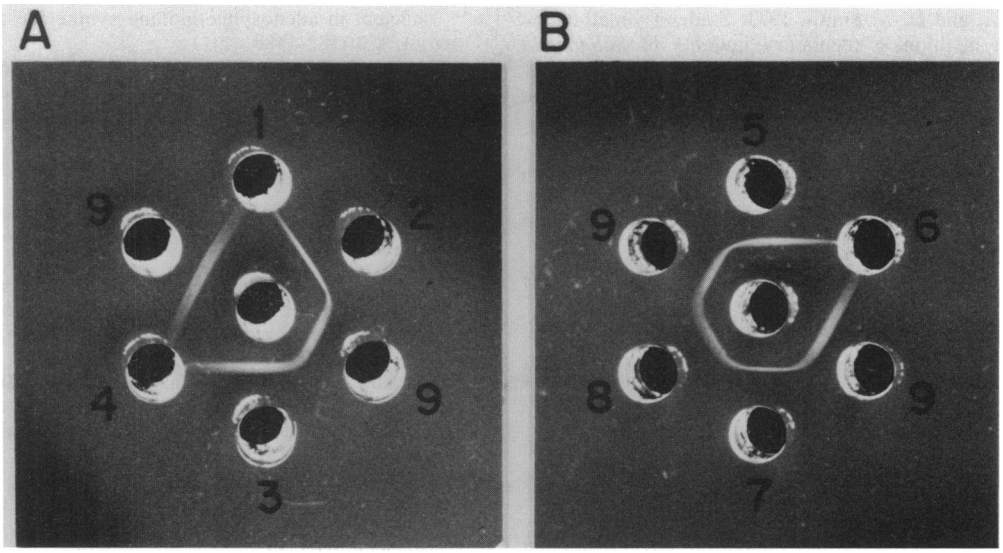

FIG. 6. Immunodiffusion analysis of sonic extracts of wild-type and mutant strains cultured in minimal medium at permissive and nonpermissive temperatures. The cells were grown and the extracts were prepared as described in the legend to Fig. 3. The immunodiffusion was performed as described in the legend to Fig. 5, and numbers 1 to 9 correspond to the lanes described in the legend Fig. 3.

depletion of AdoMet or AdoMet synthetase activity or both. This critical intracellular AdoMet level is <0.2% of wild type. The *metK503* mutant does not grown at the nonpermissive temperature even in the presence of methionine and B_{12}, consistent with a requirement for AdoMet in processes that cannot be satisfied when the AdoMet levels are significantly reduced from those present in *metK501* and *metK502* mutants. This suggested role of AdoMet in other cellular processes is consistent with the observation that the *metK503* mutant strain was not viable after shift of growth temperature from 30 to 42°C in minimal medium. The impermeability of *E. coli* to AdoMet has prevented the demonstration that AdoMet alone is sufficient to restore growth of the *metK503* mutant. It is noteworthy that, of the eluant peaks observed in the cation-exchange high-performance liquid chromatograms, only changes in AdoMet, *S*-adenosylhomocysteine, and 5'-methioadenosine levels were observed when mutants at nonpermissive temperatures were compared with wild-type controls. Therefore, it appears that nucleoside metabolism may not be grossly altered upon AdoMet depletion. The nutritional provision derived from a rich medium by the *metK503* mutant and its role remain elusive. None of the amino acid or vitamin supplements we have tested have substituted for the yeast extract component of Luria broth. However, these new *metK* mutants in minimal media should provide useful tools for the investigation of the levels of AdoMet required for various metabolic pathways as well as the appropriate background for the construction and characterization of mutations in AdoMet synthetase.

Coincidentally, these studies have led to the discovery of another AdoMet synthetase which apparently is normally expressed only in rich media; we have denoted the gene which encodes this isozyme *metX*. The *metX* gene product shares antigenic similarity to the *metK*-encoded AdoMet synthetase. Preliminary DNA hybridization experiments show a second chromosomal restriction fragment that hybridizes to *metK*, and we attribute this to *metX*. Studies directed toward cloning and mapping of the *metX* gene are in progress.

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