

Properties of *metK* Mutants of *Escherichia coli* K-12

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Received for publication 26 March 1973

Some of the properties of three *metK* mutants of *Escherichia coli* K-12 have been examined. All three strains have lower than normal levels of SAM (S-adenosyl-L-methionine) synthetase and elevated levels of cystathionine synthetase and cystathionase. One strain (RG73) appears to have an unstable SAM synthetase, suggesting that it carries a structural gene mutation. The two strains (RG62 and RG109) which have the lowest levels of SAM synthetase when grown on minimal medium have appreciably higher levels of enzyme when grown on complete medium. Growth on defined media supplemented with leucine or methionine causes a several-fold increase in the specific activity of SAM synthetase with associated decreases in cystathionine synthetase and cystathionase, but the changes are not as large as those seen in cells grown on LB broth. The SAM pools of strains RG62 and RG109 are markedly lower than normal while that of strain RG73 is slightly below normal. The methionine pools of all three strains are elevated several-fold. The *metK* strains are able to synthesize cyclopropane fatty acids, but the rate of their formation is slowed. Modification and restriction of phage 21 appears to be normal, suggesting that these strains are able to methylate DNA.

The methionine biosynthetic pathway of enterobacteriaceae is highly branched with both divergent and convergent sections. Although the structural genes are scattered on the chromosome, they appear to be subject to a common regulatory system (29). Mutations at two loci lead to overproduction of the methionine biosynthetic enzymes. The properties of *metJ* mutants are consistent with the interpretation that the locus codes for an aporepressor (3, 30), but the function of the *metK* locus has not been clearly established. *metK* strains were first isolated in *Salmonella typhimurium* as methionine analog resistant mutants (13). The *metK* locus lies clockwise from *serA* on the conventional map of the *Salmonella* chromosome. The *metK* gene product of *Salmonella* appears to be a protein as judged by the dominance of *metK*⁺ to *metK*⁻ (3) and by the isolation of amber suppressible *metK* mutants (18). We have isolated a series of mutants of *Escherichia coli* that have elevated levels of the methionine biosynthetic enzymes and lower than normal levels of

SAM (S-adenosyl-L-methionine) synthetase (adenosine 5'-triphosphate:L-methionine S-adenosyltransferase, EC 2.5.1.6) (10). All the mutants of this type which have been examined lie in the same region as the *metK* locus of *Salmonella* (15; Greene, Hunter, and Su, unpublished data). We believe that our *metK* mutants involve the structural gene for SAM synthetase, but the *metK* mutants of *Salmonella* that have been examined do not appear to have an altered SAM synthetase (23, 29). The *metK* region has not been mapped in detail because neither *metK*⁺ nor *metK*⁻ can be adequately selected on plates, and there are no good selective markers in the immediate vicinity, so the possibility remains that the region contains more than one locus affecting the control of methionine synthesis.

Because SAM is the precursor for many cellular constituents such as spermidine, methylated bases of nucleic acids, and cyclopropane fatty acids, it would be expected that a mutation causing an extreme deficiency of SAM would be lethal. Although some of our mutants have markedly reduced levels of SAM synthetase (a few percent of normal), they all grow on

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minimal medium with a slightly prolonged division time. Thus, we investigated these strains to estimate the extent of the deficiency of SAM and its metabolites.

MATERIALS AND METHODS

Materials. Glutathione, disodium adenosine 5'-triphosphate (ATP) and L-leucine (methionine and isoleucine-free) were obtained from the Sigma Chemical Co., L-methionine from Schwarz/Mann, L-cystathionine and pyridoxal phosphate from Calbiochem, NADH from P-L Biochemicals Inc., 5,5'-dithio-bis (2-nitrobenzoic acid) from the Aldrich Chemical Co., BRIJ-58 from the Pierce Chemical Co., beef heart lactic dehydrogenase and beef pancreatic deoxyribonuclease from the Worthington Biochemical Corp., egg white lysozyme from the Boehringer Mannheim Corp., and ATP-8-¹⁴C and H₂³⁵SO₄ (carrier-free) from New England Nuclear Corp. O-succinyl homoserine was synthesized by the method of Flavin and Slaughter (7). Unlabeled SAM was prepared by the method of Schlenk et al. (24) and chromatographically purified by a method similar to that of Shapiro and Ehninger (27). All other chemicals were from standard commercial sources.

Since methionine contamination of leucine could influence the results, the leucine used in these experiments was analyzed for methionine. A solution of leucine was oxidized with performic acid according to the method of Moore (19). After removal of the reagent, the oxidized amino acid was dissolved in citrate buffer with a pH of 2.2 and a 1,000-fold excess was analyzed for methionine sulfone with a Beckman amino acid analyzer. Several small peaks of approximately equal size were seen in the acidic region of the chromatogram. Although none of them corresponded exactly to methionine sulfone in the standard chromatogram, the peak nearest its position was measured and was found to contain slightly less than 10⁻⁴ mol of amino acid per mol of leucine.

Media. The standard minimal medium is that of Davis and Mingioli (5) with the omission of sodium citrate, and containing 0.5% dextrose as an energy source. LB broth containing tryptone (Difco) (10 g/liter), yeast extract (Difco) (5 g/liter), and sodium chloride (10 g/liter) was prepared as described by Scott (26). MF (methionine-free) medium is the basic minimal dextrose medium supplemented with several vitamins, adenosine, uridine, and all the protein amino acids except methionine. Except for supplementation with arginine (200 µg/ml) and omission of methionine, this medium is the same as the AF medium of Novick and Maas (20).

Bacteria and phage. The wild-type *E. coli* K-12 was obtained from Samson Gross of Duke University, *E. coli* C was obtained from the American Type Cultural Collection (ATCC 13706), and phage 21 was a gift of Phillip Harriman of Duke University. Spontaneous ethionine-resistant mutants were isolated by spreading 0.1 ml of an overnight culture of wild-type *E. coli* K-12 onto an ethionine-containing minimal or leucine-supplemented plate, from which colonies were picked and purified by two successive single-colony isolates on the same medium. Strains RG62

(*metK* 84) (formerly strain D7) and RG73 (*metK* 85) (formerly strain E4) were picked from DL-ethionine (2 mM), plates and strain RG109 (*metK* 86) was picked from an L-ethionine (0.05 M) L-leucine (0.2 mM) plate. The higher ethionine concentrations (0.02–0.05 M) in the presence of L-leucine (5 mM) or L-leucine, L-aspartic acid and L-alanine (0.2 mM each) give better yields of *metK* mutants. The *metK* strains were selected from the groups of ethionine-resistant mutants on the basis of their low SAM synthetase activities.

Growth of cells and enzyme assays. Cultures were grown at 37°C in a rotary shaking incubator, using shake flasks (Bellco) filled less than half full to assure adequate aeration. Cells were harvested by rapid chilling in ice and by centrifugation during late log phase (usually 3–4.5 mg of cells/ml [wet weight]). The packed cells were washed with 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer with a pH of 7.6 and stored in a freezer until used.

For enzyme assays, cells were suspended in 0.02 M Tris-hydrochloride 0.001 M ethylenediaminetetraacetic acid (EDTA), with a pH of 7.6. Sonic extracts were prepared by a treatment of 2-ml portions of cell suspension with a Branson S-125 Sonifier equipped with a microtip at a power setting of two (output ~2.8 amperes) for 2 min. The disrupted cell suspensions were centrifuged at 18,000 rpm for 20 min in a Sorvall RC-2B centrifuge. The cell debris was discarded and the protein contents and enzyme activities of the supernatant solutions were measured. Toluene-treated cells were prepared by addition of a 0.05-volume of toluene to the cell suspensions and incubation was at 37°C for 10 min. Cell lysates were prepared by a modification of the method of Godson and Sinsheimer (8), in which sucrose was omitted from the lysozyme, Tris, EDTA incubation mixtures, and both incubations with lysozyme and with deoxyribonuclease (DNase) MgCl₂ and BRIJ 58 were done at room temperature instead of at 0°C.

SAM synthetase and cystathionase were assayed as previously described (12) regardless of whether toluene-treated cells or cell extracts were used. Cystathionine synthetase was assayed as previously described (12) when toluene-treated cells were used, but when the enzyme activity was measured in cell extracts the procedure was slightly modified to remove the precipitated protein from the acidified incubation mixtures before neutralization with potassium carbonate.

For protein determinations, samples of cell extract were mixed with equal volumes of 6% perchloric acid and centrifuged. The precipitated protein was redissolved in 0.1 M NaOH and its concentration was determined by treatment with a biuret reagent. Measurement of the absorbance was at 310 nm (31). A solution of crystalline bovine serum albumin standardized by its absorbance at 279 nm was used to prepare standards for the biuret procedure.

Measurement of SAM and methionine pools. Several one-step chromatographic procedures were tested for rapid measurement of SAM pools, but none of them adequately resolved SAM from unidentified

interfering compounds. Thus the multistep procedure described below was used. In addition to isolating and measuring SAM the results were verified by measuring the 5'-thiomethyl adenosine produced by thermal decomposition of SAM. The SAM and methionine pools were determined by growing cells for many generations in medium with ^{35}S -sulfate and by measuring the radioactivity in the separated compounds. The medium used in these experiments was a modified minimal medium in which the magnesium and ammonium sulfates were replaced by equimolar amounts of the chloride salts, and which was made 0.2 mM in ^{35}S -sulfate with a specific activity of about 20 $\mu\text{Ci}/\mu\text{mol}$. In preliminary short-term experiments the modified minimal medium apparently supported growth of each strain at the same rate obtained in minimal medium, but small differences in growth rate may not have been detected.

Tubes containing 5 ml of ^{35}S -minimal medium were inoculated with 0.05-ml volumes of LB broth cultures of the various strains and incubated at 37 C for about 8 h. Flasks containing 25 ml of ^{35}S -minimal medium were inoculated with 0.5-ml volumes of the tube cultures and incubated overnight. In the morning, duplicate or triplicate sets of flasks each containing 45 ml of ^{35}S -minimal medium were inoculated with 7.5 mg of cells (1–6 ml of inoculum depending on the extent of growth of the particular culture) and incubated. Growth was followed by measurement of absorbancy at 550 nm. Cultures were harvested when they reached a density of about 1.5 mg of cells per ml. The density of each culture was measured, a 30-ml sample was taken for SAM determination and a 5-ml sample for methionine determination. The samples were centrifuged for 10 min at room temperature, and the supernatant culture fluid was carefully decanted. The samples were harvested at room temperature to avoid the loss of amino acids that occurs when *E. coli* cells are cooled below 8.5 C (22). The cells were not washed and were handled as rapidly as possible to minimize the time between their separation from growth medium and the addition of acid. The methionine content of the residual medium is too low to seriously influence the results (10). For the SAM determination each cell pellet was suspended in 2 ml of 6% perchloric acid containing a known amount of cold SAM (usually 0.4 μmol), whereas the cells for the methionine determination were suspended in 1 ml of 1 N HCl containing 0.5 μmol of unlabeled methionine. Both suspensions were stored in the cold room overnight, after which they were centrifuged, the extracts were recovered, and the extracted cells were discarded. Advantage was taken of the unusual acid stability of the adenine riboside bond of SAM (25) by heating the perchloric acid extracts for SAM measurement in a boiling-water bath for 5 min to destroy potentially interfering compounds such as S-adenosylhomocysteine. The heated extracts were then poured through columns of Dowex 50 X-2 (H) 200 to 400 mesh (0.6 by 4 cm). Each column was then washed with three 10-ml portions of 1 N HCl and the absorbed SAM was eluted with 10 ml of 3 N HCl. The 3 N HCl eluates were evaporated to dryness with a lyophilizer and the dried samples were dissolved in 3 ml of wa-

ter. The redissolved samples were then applied to columns (0.6 by 8 cm) of Dowex 50 X-8 minus 400 mesh (the resin was freshly washed with 12 N HCl and water to remove ultraviolet absorbing material which bleeds from the resin). The columns were then eluted with 200-ml 0 to 6 N HCl gradients. The SAM-containing fractions, which emerge slightly after the middle of the gradient, were located by measuring the absorbancy of the fractions at 260 nm. The fractions containing most of the SAM were combined and evaporated to dryness with a lyophilizer. The dried fractions were dissolved in 4 ml of water and 2-ml volumes were placed into pairs of 12-ml conical centrifuge tubes. The solutions were taken to dryness again, and one sample from each pair was dissolved in 0.1 ml of water for direct SAM isolation while the other was dissolved in 0.1 ml of 0.05 M ammonium formate, pH 4, for conversion of the SAM to 5'-thiomethyladenosine. The ammonium formate solutions were heated in a boiling-water bath for 30 min, and the samples were placed in a lyophilizer overnight to remove the water and ammonium formate. The lyophilized samples were dissolved in 0.1 ml of water for further processing. Portions (0.05 ml) of both types of samples were applied to sheets of Whatman 3 MM paper and subjected to electrophoresis in 8% formic acid for 30 min (SAM) or 60 min (5'-thiomethyladenosine) at 2,000 V (GME high-voltage electrophorator model D). The SAM and 5'-thiomethyladenosine spots were located by observation with ultraviolet light. Squares of paper containing the spots and blank squares of equal area from regions of the paper where no sample was applied were cut into small pieces and soaked overnight in 0.01 N HCl. The paper fragments were removed by centrifugation and the absorbancy at 260 nm of the sample and blank eluates were measured. Portions (0.5 ml) of the eluates and 0.5-ml volumes of a 1:100 dilution of ^{35}S -minimal medium (1 nmol of sulfur) were placed in counting vials containing 3.5 ml of water and 17 ml of 2:1 toluene Triton X-100 scintillation mix (21). The samples were counted at 1 C in a Packard TriCarb model 3375 liquid scintillation spectrometer. The SAM pools were then calculated from the weight of cells taken in the original sample, the quantity of ^{35}S and the fraction of the added SAM recovered in the eluates of the electrophoresis strips.

Samples (0.75 ml) of the extracts for methionine analysis were evaporated to dryness on a lyophilizer, and the residues were dissolved in 75 μl of water. Twenty- μl iter volumes of these solutions were applied to sheets of Whatman no. 1 filter paper and chromatographed with a solvent containing 80 parts of *n*-butanol, 20 parts of acetic acid, and 20 parts of water. The chromatograms were cut into strips and passed through a Packard Model 7201 radiochromatogram scanner. Each strip showed a resolved peak corresponding to methionine. The areas of the paper corresponding to the peaks were cut into 1-cm sections, and the small paper strips were placed in counting vials containing 5 ml of toluene scintillant [4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter]. Standards were prepared by applying ^{35}S -minimal medium (10

μliters) to the same sized strips of Whatman no. 1 filter paper. The vials were counted at 1 C in a Packard TriCarb Model 3375 liquid scintillation counter. After counting, the strips were recovered from the vials and treated with ninhydrin. The ninhydrin reactivity of the carrier methionine corresponded to the radioactivity of the strips. The methionine content of the cells was calculated from the ^{35}S content of the methionine spots and from the corresponding cell weight.

Measurement of fatty acid composition. Cells were harvested from 100-ml volumes of culture by centrifugation. The cells were suspended in a few ml of 0.02 M Tris buffer, pH 7.6, transferred to a 15-ml centrifuge tube, and resedimented. The moist cell pellet was suspended in 2 ml of a chloroform methanol (2:1) and magnetically stirred for 5 h (9). The suspension was then centrifuged and the insoluble pellet was discarded. The supernatant solution was evaporated to dryness under a stream of air and residual traces of water were removed by addition of a drop each of benzene and ethanol. Re-evaporation yielded a clear oil. The oily residue was dissolved in diethyl ether and transferred to a screw-capped tube. The ether was evaporated under a stream of air and the residue was dissolved in 1 ml of 2% H_2SO_4 in methanol. The tubes were closed and the fatty acids were converted to their methyl esters by heating to 70 C for 12 h (28). One milliliter of 0.05 M NaCl was added to each tube and the suspensions were extracted three times with 1-ml volumes of ether. The ether extracts were evaporated to dryness, dissolved in 1-ml volumes of chloroform, and back-extracted one time with 0.05 M NaCl. Portions of the chloroform extracts were analyzed on a Varian Aerograph 1200 using diethylene glycol succinate at 185 C. The relative amounts of the different fatty acid methyl esters were calculated from the height and the retention time of each peak (1).

Modification and restriction. The pour plate technique (6) was used for growth and titer of stocks of phage 21. Medium for plates contained 0.88% agar and that for pour tubes contained 0.5% agar. Complete medium was supplemented with MgCl_2 (3×10^{-3} M) added sterily after autoclaving, minimal medium was as described above. Plates were freshly prepared for each experiment. Phage stocks were prepared by adding 5×10^5 to 5×10^6 plaque-forming units of phage (in 0.1 ml) to pour tubes containing 2 ml of appropriate medium and 0.2 ml of a freshly grown culture of bacteria at 40 C. The contents of the tubes were well mixed and poured onto plates containing the same medium as in the pour tubes. After the agar layers had solidified, the plates were incubated overnight at 37 C. Phage were harvested by layering the plates with 2 ml of the same medium as in the plates and allowing them to stand for 5 h at room temperature. The liquid layers were recovered with sterile Pasteur pipettes, centrifuged to remove bits of agar, and filtered through sterile membrane filters (0.45 μm pore size; Millipore Corp.). Each phage stock was grown two times successively on the same strain to reduce the fraction of inappropriately modified phage which might be carried over. Phage

stocks were titered on the same strain and under the same conditions that had been used in their preparation except that a series of dilutions of phage suspensions were used to obtain countable plaques. After the stocks had been prepared and titered, their plating efficiencies on the different strains were determined by using sufficient phage to give 50 to 200 plaques per plate.

RESULTS

The SAM synthetase deficiencies, which were used as one of the criteria for selection of the mutants, are evident in either toluene-treated cells or sonic extracts of cells as shown in Table 1. The SAM synthetase activities of toluene-treated cells of the wild type, strains RG62 and RG109, are about the same as those of sonic extracts prepared from the same cell suspensions. Strain RG73 behaves differently in that the SAM synthetase activity of toluene-treated cells is much higher than that of a sonic extract. Similar results are obtained when sonically disrupted cell suspensions are assayed without removal of insoluble debris and when cells are broken by treatment with EDTA, lysozyme, BRIJ 58, and DNase (8). These results suggest that strain RG73 makes an altered enzyme which is too unstable to survive extraction. If so, the mutation is located in the structural gene for SAM synthetase. The instability of the enzyme from strain RG73 shows the advantage of using toluene-treated cells to reveal the existence of an enzyme which may function in the cell but which is lost during preparation of cell-free extracts.

Previous results (10) have shown that the cystathionase and cystathionine synthetase activities of *metK* strains can be partially repressed by growth on media containing methionine. To further investigate the repressibility of these strains, the enzyme activities of cells grown on several different media were measured (Table 2). The SAM synthetase activities of toluene-treated cells and sonic extracts pre-

TABLE 1. SAM synthetase activities of toluene-treated cells and sonic extracts

Strain	Pertinent genotype	SAM synthetase activity (nmol per min per incubation mixture)	
		Toluene-treated cells	Sonic extracts
Wild type	<i>metK</i> ⁺	4.2	3.9
RG62	<i>metK</i> 84	0.08	0.09
RG73	<i>metK</i> 85	0.33	0.04
RG109	<i>metK</i> 86	0.12	0.09

TABLE 2. *Effects of growth medium on enzyme activities*

Strain	Pertinent genotype	Medium	Cystathionine synthetase ^a	Cystathionase ^a	SAM synthetase ^a	SAM synthetase (nmol per min per incubation mixture)	
						Toluene-treated cells	Sonic extract
Wild type	<i>metK</i> ⁺	Minimal	4.3	20.3	7.7	5.1	5.7
		Min + 0.01 M L-met	0.8	9.7	2.2	1.1	1.5
		MF medium	7.3	31.1	11.3	10.0	10.2
		MF + 0.01 M L-met	0.6	4.5	2.4	1.4	2.3
		LB broth	0.7	3.1	2.7	1.2	2.1
RG73	<i>metK</i> 85	Minimal	25.5	50.4		0.72	0.16
		Min + 0.01 M L-met	5.5	26.6		0.30	0.04
		MF medium	21.3	58.9		0.47	0.15
		MF + 0.01 M L-met	6.1	23.4		0.31	0.08
		LB broth	5.8	18.1		0.55	0.09
RG62	<i>metK</i> 84	Minimal	94.7	82.2	0.18	0.10	0.16
		Min + 0.01 M L-met	31.0	60.2	0.39	0.20	0.34
		MF medium	25.6	61.8	0.99	0.40	0.94
		MF + 0.01 M L-met	10.5	37.7	0.87	0.36	0.78
		LB broth	4.7	17.2	1.35	0.48	1.13
RG109	<i>metK</i> 86	Minimal	68.7	80.0	0.20	0.06	0.15
		Min + 0.01 M L-met	39.2	63.3	0.49	0.23	0.35
		MF medium	27.3	66.4	0.92	0.32	0.74
		MF + 0.01 M L-met	10.9	40.8	0.90	0.45	0.80
		LB broth	4.5	20.7	1.62	0.46	1.38

^aEnzyme activities expressed in nanomoles per minute per milligram of protein.

pared from the same cell suspensions are shown in the two right-hand columns. As seen before, the activities of toluene-treated RG73 cells are higher than those of sonic extracts. Enzyme activities of sonic extracts of cells of the other strains range from the same as those of toluene-treated cells to as much as threefold higher. The discrepancies between toluene-treated cells and sonic extracts is most evident in cells grown on rich medium. The reason for these discrepancies is unknown but possible causes include differences in the susceptibility of the cell membranes to toluene treatment or the presence of an inhibitor of SAM synthetase which is not released from toluene-treated cells. A similar phenomenon has been observed in yeast, in which an inducible arginase inhibits ornithine transcarbamylase when both are present in high concentration, as in cells rendered permeable by treatment with nystatin, but the inhibition is relieved when both proteins are diluted by disruption of the cells (17).

The enzyme levels of wild-type cells respond to the various growth media in the expected manner (Table 2), all three enzymes are repressed in cells grown on complete medium

(LB) or on synthetic media containing methionine, and are derepressed in cells grown on synthetic medium containing all the protein amino acids except methionine (MF). As previously reported (10) the *metK* mutants grown on minimal medium have higher levels of cystathionine synthetase and cystathionase and lower levels of SAM synthetase than their wild-type parent. Strain RG73 shows less derepression of cystathionine synthetase and cystathionase than strains RG62 or RG109, possibly resulting from a larger intracellular pool of corepressor (SAM or its metabolites) which is consistent with its intermediate level of SAM synthetase. Growth of strain RG73 on media containing 0.01 M L-methionine or on LB broth results in a partial repression of cystathionine synthetase and cystathionase. These cells also show a slight repression of SAM synthetase, although as discussed above there is some question whether these results are due to differences in amounts of enzyme or to an idiosyncrasy of the assay using toluene-treated cells. Cells of strain RG73 grown on medium supplemented with all the amino acids except methionine do not show significantly higher enzyme activities

than those grown on minimal medium, suggesting that these growth conditions do not further decrease the concentration of corepressor. Strains RG62 and RG109 respond somewhat differently to the various growth media than the wild type or strain RG73. Although supplementation of minimal medium with 0.01 M L-methionine causes a partial repression of cystathionine synthetase and cystathionase, it causes a slight increase (about twofold) in SAM synthetase. Cells of these strains show about a fivefold increase in SAM synthetase activity when grown on medium supplemented with all the amino acids except methionine, but cystathionine synthetase and cystathionase, instead of being depressed, are repressed to the same extent as in cells grown on medium containing 0.01 M L-methionine. The repression of these enzymes may be a consequence of the increased SAM synthetase activity resulting in a higher concentration of corepressor even though the medium lacks methionine. Supplementation of the amino acid medium with 0.01 M L-methionine has no further effect on the SAM synthetase activity, but causes greater repression of cystathionine synthetase and cystathionase. Cells grown on LB broth have even higher specific activities of SAM synthetase and correspondingly lower levels of cystathionine synthetase and cystathionase.

Although the increase in specific activity of SAM synthetase seen in strains RG62 or RG109 cells grown on methionine is small, the effect is reproducible and appears to be real. To further investigate this phenomenon, the time course of changes in enzyme activity was followed in parallel cultures of strain RG62 with and without methionine. As shown in Fig. 1, addition of methionine to the growth medium causes a slow steady increase in SAM synthetase activity such that, after 4 h, cells grown in the presence of methionine have about three times more enzyme activity than those grown in its absence. The cystathionine synthetase and cystathionase activities of the methionine-grown cells decrease slowly during the growth period, in contrast to cells grown on minimal medium in which the activities of these enzymes show slight increases.

During work on the genetics of *metK* mutants it was observed that although strains RG62 and RG109 will grow on minimal medium (either liquid or solid) when the inoculum is relatively large, no colonies are obtained when a few hundred cells are spread on minimal plates. If the cells are spread on LB broth plates or plates supplemented with vitamin-free casein hydrolysate (1 mg/ml) colonies are obtained in 24 h.

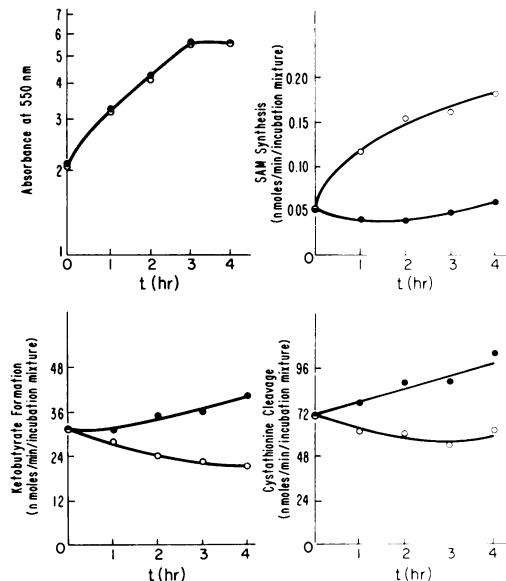


FIG. 1. Cells from an overnight minimal culture of strain RG62 were harvested by centrifugation, suspended in minimal medium (about 1.7 mg of cells per ml), and incubated for 2 h at 37°C to assure that the culture was growing. The cells were again harvested and suspended in fresh minimal medium to give a cell concentration of 3 mg/ml. The suspension was incubated at 37°C without further addition (●) and in the presence of 0.01 M L-methionine (○). Samples of the culture were taken hourly for 4 h. SAM synthetase incubation mixtures contained 12 mg of toluene-treated cells, the cystathionine synthetase assay mixture contained 3 mg of washed toluene-treated cells, and cystathionase assays used lysozyme BRIJ 58 lysates from 1.5 mg of cells.

Investigation of the effects of individual amino acids on the growth of these two strains showed that colonies can be obtained in about 2 days on plates supplemented with low concentrations of leucine, alanine, and aspartic acid (0.2 mM each) or a higher concentration (5 mM) of leucine alone. In view of these results and the stimulation of SAM synthetase activity seen in cells grown on MF medium, the effect of leucine and a mixture of leucine, aspartic acid, and alanine on enzyme activities were measured (Table 3). The concentrations of leucine, alanine, and aspartic acid are the same as in MF medium except for one pair of flasks which has 10 times as much leucine. Cells grown in medium supplemented with leucine alone, at the same concentration as that of MF medium, have a three- to fourfold elevation in the level of SAM synthetase, and have partially repressed levels of cystathionine synthetase and cystathionase. The enzyme levels of these cells are

almost the same as those of MF-grown cells, indicating that the effects of MF medium can largely be attributed to its leucine content. Growth of cells on medium containing 10 times as much leucine leads to a slight increase in SAM synthetase activity and a significant further decrease in cystathionine synthetase activity, with little effect on the level of cystathionase. Addition of alanine and aspartic acid has little or no effect on the enzyme levels of the cells. Since these results could have been influenced by methionine contamination, the leucine used in these experiments was analyzed for methionine. The maximum methionine content of the leucine used in these experiments was 10^{-4} mol per mol. Thus, the maximum methionine content of the leucine-supplemented media was 0.4 μ M, a value lower than that excreted into the medium by *metK* strains of this type (10). These results explain the ability of leucine to stimulate growth of colonies on plates but do not account for the stimulatory effect of alanine and aspartic acid when the leucine concentration is low.

SAM pools of wild type and *metK* strains.

Since it has not been clearly established that cells have an absolute requirement for SAM or its metabolites, it is of interest to measure the SAM pools in strains with low SAM synthetase activities. The SAM and methionine pools of the three *metK* strains and their wild-type parent in cells grown to a density of 1.5 mg/ml in low sulfur minimal medium (0.2 mM sulfate) are given in Table 4. The SAM pools were determined by direct measurement of 35 S-SAM and by measurement of 35 S-thio-methyladenosine, produced by heating SAM at pH 4. The SAM pools shown in Table 4 for strains RG62, RG73, and RG109 are, respectively, 39, 76, and 16% of wild type. In other experiments, SAM pools as low as 10% of normal were seen in strains RG62 and RG109, whereas the smallest SAM pool observed in RG73 was 50% of that of the wild type. Although initial control experiments showed that cells grown for short periods in the low sulfur medium have the same SAM synthetase activity and about the same growth rate as cells grown in minimal medium, later investigations revealed that prolonged incubation of the mutant strains in this medium, used

TABLE 3. Effect of amino acid supplements on enzyme activities^a

Strain	Pertinent genotype	Medium	Cystathionine ^b synthetase	Cystathionase ^b	SAM synthetase ^b
RG62	<i>metK 84</i>	Minimal	82.2	57.9	0.12
		Minimal + leucine	26.3	31.0	0.33
		Minimal + 10 \times leucine	17.7	43.4	0.49
		Minimal + leucine alanine aspartic acid	32.0	35.5	0.27
		MF medium	26.1	35.2	0.45
RG109	<i>metK 86</i>	Minimal	66.1	50.0	0.09
		Minimal + leucine	30.7	40.0	0.38
		Minimal + 10 \times leucine	18.7	39.6	0.44
		Minimal + leucine alanine aspartic acid	28.2	38.1	0.21
		MF medium	26.3	39.6	0.38

^aAmino acid supplements are: leucine, 50 μ g/ml (0.38 mM); 10 \times leucine (Leu), 500 μ g/ml (3.8 mM); alanine, 400 μ g/ml (4.5 mM); aspartic acid, 120 μ g/ml (0.90 mM); MF supplements are described in the text.

^bEnzyme activities are expressed in nanomoles per minute per milligram of protein.

TABLE 4. SAM and methionine pools of wild type and *metK* strains^a

Strain	Pertinent genotype	SAM Pools (nmol/g of cells)			Methionine pools ^a (nmol/g of cells)
		Measured as SAM	Measured as TMA	Average	
Wild type	<i>metK</i> ⁺	54.6 \pm 4.2	45.7 \pm 1.5	50.2 \pm 5.6	76.0 \pm 2.9
RG62	<i>metK 84</i>	21.0 \pm 0.4	18.1 \pm 1.6	19.5 \pm 1.9	625 \pm 54
RG73	<i>metK 85</i>	40.5 \pm 1.4	35.6 \pm 5.7	38.1 \pm 4.6	297 \pm 9
RG109	<i>metK 86</i>	8.7 \pm 0.6	7.3 \pm 1.4	8.0 \pm 1.2	712 \pm 117

^aPool sizes are the average of determinations from triplicate cultures \pm the standard deviation.

to equilibrate endogenous sulfur reserves with the radioactive sulfate, tends to select for revertants with higher levels of SAM synthetase. Enrichment of such revertants appears to be more of a problem with strain RG62 than with the other strains. Thus, the SAM pools of the *metK* strains shown in Table 4 may be artifactually elevated. While the growth times used in these experiments may have been excessive, incomplete equilibration of the sulfur pools, which could result from short incubation periods, would give low values. Regardless of this experimental difficulty, it is clear that the SAM pools of strains RG62 and RG109 are significantly lower than those of the wild type or strain RG73, but the pools of all the *metK* strains are appreciably higher than might have been hoped judging from their SAM synthetase activities. The effect of the harvest procedure on the SAM pools has not been evaluated. Lowry et al. (14) report that harvesting of *E. coli* cells by centrifugation and washing in the cold results in depletion of the ATP pool, but they also find that allowing rapidly filtered cells to stand on the filter for 2 min at room temperature has no effect on the ATP pool. If the ATP pools of the cells were depleted, it would be expected that the SAM pools would change; but since the cells were in the presence of growth medium at or above room temperature during harvest, the changes in the pools were probably small.

The methionine pools of the three *metK* strains are substantially higher than that of the wild type, with the pools of strains RG62 and RG109 being about twice as large as that of strain RG73. The enlarged intracellular methionine pools apparently partially overcome the blocks in SAM synthetase and allow synthesis of appreciable amounts of SAM.

SAM metabolites in *metK* cells. One of the major SAM utilizing reactions in *E. coli* is the conversion of unsaturated fatty acids to cyclopropane fatty acids that occurs as the cells enter stationary phase (4). Cultures of wild type and *metK* cells were grown for various time periods on minimal medium, samples were harvested, the cells were washed, and their lipids were extracted with chloroform methanol. The fatty acid esters prepared from these extracts were analyzed by gas chromatography. Results are expressed as the fractional conversion of unsaturated fatty acids to cyclopropane fatty acids, calculated by dividing the sum of the major cyclopropane fatty acids (C17 + C19) by the sum of the major unsaturated and cyclopropane fatty acids (C16:1 + C17 + C18:1 + C19). The *metK* strains are relatively deficient in cyclopropane fatty acids during the earlier

stages of growth when compared to their wild-type parent (Table 5). On prolonged incubation, however, the relative differences between the *metK* and wild-type strains become smaller and the bulk of the double bonds are converted to cyclopropane groups. Thus, whereas the SAM deficiency of the *metK* strains slows cyclopropane fatty acid synthesis, it does not stop it.

Another type of SAM-utilizing reaction of interest is methylation of the various types of nucleic acids. The restriction and modification of phage 21 was used to evaluate DNA methylation since the K modification consists of the methylation of specific adenines of DNA and the K restriction endonuclease requires SAM (16, 11). *E. coli* C, which neither modifies nor restricts (2), was used as a control in these experiments. Initial experiments using complete medium during preparation and assay of phage stocks showed no difference between *metK*⁺ and *metK*⁻ strains. Since the differences in SAM synthetase activities between wild type and mutant strains is much greater in cells grown on minimal medium than in those grown on complete medium, the phage modification and restriction experiments were repeated using minimal medium. To evaluate the ability of relatively SAM-deficient *metK*

TABLE 5. Cyclopropane fatty acids in wild type and *metK* cells

Strain	Pertinent genotype	Age of culture (h)	Absorbance of culture at 550 nm	Fraction of unsaturated fatty acids converted to cyclopropane fatty acids
				16:1+17:18:1+19
Wild type	<i>metK</i> ⁺	7	1.0	0.32
		8	2.0	0.32
		9.3	3.0	0.58
		43		0.95
RG62	<i>metK</i> 84	11.0	1.0	0.03
		15.5	2.0	0.05
		19.5	3.0	0.16
		43		0.53
RG73	<i>metK</i> 85	7.5	1.0	0.13
		8.8	2.0	0.15
		10.5	3.0	0.21
		66		0.91
RG109	<i>metK</i> 86	9.3	1.0	0.05
		12.7	2.0	0.07
		15.5	3.0	0.09
		43		0.72

strains to modify DNA, stocks of phage 21 were prepared by lysis of strains RG62 or RG109 cells growing on minimal plates. The plaque-forming ability of these stocks was then measured by plating them on wild-type K-12, strains RG62, RG109, and *E. coli* C growing on complete plates where all the K strains should be capable of restriction. The highest titer of both stocks is obtained by plating on *E. coli* C, but the titers on the various K strains are only two- to sixfold lower (Table 6). Such small differences are of questionable significance and may relate to other variables such as differences in phage absorption. Regardless of the reason for the small difference, it is clear that the bulk of the specific K modification sites must be appropriately methylated. Table 6(B) shows the plating efficiency of a stock of phage 21 (C) which is not methylated at the K modification sites. Compared to *E. coli* C, this strain plates with an efficiency of about 10^{-5} on all of the K strains. Since the *metK* strains are clearly able to restrict unmodified DNA, they must be able to modify their own DNA or they could not survive.

DISCUSSION

Although the slower growth rates of strains RG62 and RG109 (division times 30 to 50% longer than that of their wild-type parent) may be due to a deficiency of SAM or its metabolites, the intracellular concentrations of these compounds are not reduced nearly as much as the activity of SAM synthetase. The lowest SAM pool that we have measured is about 10% of normal and several other measurements showed larger pools. Apparently, accumulation of large methionine pools and the reduced growth rate allow the residual enzyme to synthesize enough SAM to serve the basic needs of the cell. Our results show that the mutant strains can synthesize cyclopropane fatty acids and suggest that they can adequately methylate their DNA. T4 phage grown on *metK E. coli* have the normal amount of 6-methylamino purine (S. Hattman, personal communication). *metK* strains grown on minimal medium have slightly reduced pools of spermidine and slightly increased pools of putrescine (C.-H. Su and S. S. Cohen, in press), but the differences are rather small. The lack of a marked deficiency of SAM and its major metabolites in these strains is disappointing since we are unable to conclude whether or not any of these compounds is absolutely required for growth. The observation that *metK* mutants will not form colonies when a few hundred cells are

TABLE 6. Modification and restriction of phage 21 by wild type and *metK* strains^a

Virus	Titer (plaque-forming units per ml) when plated on:			
	K-12	RG62	RG109	C
A. Modification				
21 (RG62)	3×10^7	4×10^7	3×10^7	16×10^7
21 (RG109)	3×10^7	9×10^7	8×10^7	20×10^7
B. Restriction				
21 (C)	7×10^3	6×10^3	3×10^3	5×10^3

^aTo test modification, stocks of virus were prepared using cells grown on minimal medium and plated on cells grown on complete medium. To test restriction, phage 21 (C) was plated on the various strains growing on minimal medium.

plated on minimal medium suggests that one or more of these metabolites is essential for growth, and that cells in such a low concentration are unable to adequately condition the medium. The formation of colonies on leucine-supplemented plates apparently results from the increased SAM synthetase activities of leucine-grown cells.

Judging from the instability of the SAM synthetase of RG73, the mutation in this strain appears to be in the structural gene for SAM synthetase. The other *metK* strains lie in the same region (Greene, Hunter, and Su, unpublished data), but the resolution of the mapping is insufficient to tell whether they are at the same or adjacent loci. Cells of strains RG62 and RG109 grown on variously supplemented media (leucine, methionine, MF medium, LB broth) have several-fold higher levels of SAM synthetase than cells of the same strains grown on minimal medium. The reason for this behavior is unknown, but it could possibly result from mutation or deletion of a regulatory element which changes control of the cistrons from repressible to slightly inducible by methionine or other metabolites. Alternatively, it is possible that the structural gene in the *metK* region is completely inactivated, and the residual SAM synthetase is a different protein coded by another locus.

The class of mutants used in this work were designated as *metK* because they are located in the same region of the chromosome as the *metK* mutants of *S. typhimurium* (13, 3). Although it appears likely that the structural gene for SAM synthetase is in the *metK* region of *E. coli*, there is no evidence for involvement of SAM synthetase in the *metK* mutants of *Salmonella*. Normal levels of SAM synthetase have been meas-

ured in *metK* mutants of *Salmonella* (23, Gross and Rowbury, unpublished data as cited in reference 29). The SAM synthetase activity measurements of Savin et al. were done under conditions of saturation with substrates (M. Savin, personal communication), so the possibility remains that their *metK* strain had a defective SAM synthetase with relatively normal activity under in vitro assay conditions but with reduced activity in vivo (e.g., elevated K_m for substrates or increased susceptibility to product inhibition). The *metK* strains of *S. typhimurium* were selected as methionine analog resistant mutants whose chromosomal position is clockwise from *serA* while those of *E. coli* were selected as ethionine-resistant mutants having low levels of SAM synthetase with no attempt to isolate other methionine overproducing mutants which might lie nearby. Thus, it is possible that the *metK* region is complex with more than one locus which affects the regulation of methionine synthesis. Regardless of possible complexities of the methionine regulatory system, we believe the results presented here suggest that SAM or its metabolites function in the repression of the methionine biosynthetic enzymes. This hypothesis is based on the observations that the strains with lower SAM synthetase activities show greater derepression of cystathionine synthetase and cystathionase, and that growth conditions which elevate the SAM synthetase activities of these strains lead to partial repression of the other enzymes even though the media contained no added methionine (MF medium or leucine-supplemented minimal medium). We have not been able to directly demonstrate repression by SAM since none of the cell preparations that we have tested are permeable to SAM. The question of the role of SAM either acting alone or in concert with other metabolites will probably only be definitely answered in an in vitro transcription and translation system for the *met* cistrons.

ACKNOWLEDGMENTS

Capable technical assistance was provided by Miss Ellen Mack. This investigation was supported by Public Health Service grant number GM-10317 from the National Institute of General Medical Sciences and by the Veterans Administration Project Number 3300-01.

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