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Genetic Mapping and Physiological Consequences of *metE* Mutations of *Bacillus subtilis*

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Three *metE* mutations of *Bacillus subtilis*, which cause cells to have a 25- to 200-fold decrease in L-methionine *S*-adenosyltransferase (EC 2.5.1.6) activity, were mapped between *bioB* and *thr*. The corresponding three *metE* mutants contained three- to fourfold less intracellular *S*-adenosylmethionine (SAM) but at least sevenfold more methionine than the *metE*$^+$ strain when grown in synthetic medium. This indicates a strong feedback control of SAM on its synthesis. However, only the *metE2* strain, with the lowest SAM concentration, grew at a slightly lower rate than the parent, which showed that an intracellular concentration of about 25 μM SAM was critical for growth at the normal rate. Neither DNA methylation (measured by bacteriophage φ105 restriction) nor sporulation was affected at this low SAM concentration. Addition of methionine to the growth medium caused an increase in the pool of SAM in some but not all *metE* mutants. Coaddition of adenine did not change this result. However, the extent of sporulation (induced by mycophenolic acid) was decreased 50-fold in all mutants by the addition of methionine and adenine. Therefore, the combination of methionine and adenine suppresses sporulation regardless of whether it causes an increase in the level of SAM.

In previous work from this laboratory, exposure of a *Bacillus subtilis relA1* strain (strain 61885; for detailed genotypes, see Table 1) to ethionine (optimal concentration, 1 mM) greatly (10^4 fold) increased the sporulation frequency (in the presence of glucose, which normally represses sporulation) and caused a decrease in L-methionine *S*-adenosyltransferase (SAM synthetase; EC 2.5.1.6) activity (20). Cells of a "*metE1*" mutant (strain 62258), isolated by resistance to the methionine (Met) analog ethionine at 10 mM, had greatly decreased *S*-adenosylmethionine (SAM) synthetase activity and sporulated spontaneously (in the presence of glucose) at about a 10-fold-higher frequency than its parent (strain 61885) (20). The increased sporulation frequency apparently depended on the *metE1* mutation, because a transformant (strain 62302), isolated by transformation of a different *relA1* strain (strain 62262) with DNA of the "*metE1*" mutant (strain 62258) and plating on 10 mM ethionine, also showed elevated spontaneous sporulation (20). These results were interpreted as indicating that the decrease of SAM synthetase activity was responsible for the increased spontaneous sporulation. Furthermore, addition of SAM or of the SAM precursors Met and adenosine, greatly decreased this sporulation, suggesting that increased SAM levels in the cells may suppress sporulation (20).

It was later realized that the original parent (strain 61885) already contained a mutation (called *ethA1*) which caused resistance to intermediate (2 mM) ethionine concentrations and was required for the continual sporulation caused by ethionine. The sporulation resulted from the production of *S*-adenosylethionine rather than from the decrease of intracellular SAM levels and did not simply depend on the ratio of *S*-adenosylethionine to SAM (1). Thus, the ethionine-mediated decrease in SAM synthetase levels could no longer explain the elevated sporulation of the (renamed) *ethA1* strain (strain 61185), and it was no longer clear whether the increased spontaneous sporulation of the (renamed) *ethA1 metE1* strain (strain 62258) could be attributed to the *metE1* mutation alone or whether the *ethA1* mutation also had to be present. To examine the effects of the *metE* mutations alone, we isolated two additional *metE* mutations (*metE2* and *metE3*), mapped all three *metE* mutations, and inserted them into cells that contained no *ethA1* mutation. Using these *metE* mutants and their parents, we examined the effects of *metE* on growth, on spontaneous as well as mycophenolate-induced sporulation, and on the suppression of sporulation by Met and adenine.

MATERIALS AND METHODS

Chemicals and filtration equipment. DL-Ethionine was purchased from Sigma Chemical Co., St. Louis, Mo.; L-[*ethyl*-1-^{14}C]ethionine was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif.; L-[*methyl*-^{14}C]methionine was purchased from New England Nuclear Corp., Boston, Mass.; antibiotic medium 3 and tryptose blood agar base (TBAB) were purchased from Difco Laboratories, Detroit, Mich.; type HA nitrocellulose filters (pore size, 0.45 μm; diameter, 25 mm) were purchased from Millipore Corp., Bedford, Mass.; type BA 85 filters (pore size, 0.45 μm; diameter, 100 mm) were purchased from Schleicher & Schuell, Inc., Keene, N.H.; the Nalge 115-ml filter unit (pore size, 0.45 μm; diameter, 50 mm) was purchased from Nalge Co., Rochester, N.Y.; and inulin-[^{14}C]carboxylic acid was purchased from Amersham Corp., Arlington Heights, Ill. DNase I was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Bacterial strains and media. The strains of *B. subtilis* used are listed in Table 1. TBAB plates contained 33 g of tryptose blood agar base per liter. The mineral salts medium (M) of Anagnostopoulos and Spizizen (2) was supplemented with

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TABLE 1. *B. subtilis* strains

Strain	Relevant genotype	Source or reference
61710	*aldA1 aroG932 leuA8 trpC2*	4 (QB936)
61711	*hisA1 thr-5 trpC2*	4 (QB 917)
61852	*lys relA1 trpC2*	26
61885	*ethA1 ilvB1 kauA1 relA1*	21
61950	*metC7 relA1 trpC2*	21
62258	*ethA1 ilvBϕ1 kauA1 relA1 metE1*	20
62280	*argA2 aroG932 bioB141 sacA321*	BGSC[a], 1A92
62281	*argA15 hsrM hsmM leuA8*	14 (RM125)
62293	*ethA1 ilvBϕ1 kauA1 metE2 relA1*	61885, spontaneous
62294	*ethA1 ilvBϕ1 kauA1 metE3 relA1*	61885, spontaneous
62356	*aldA1 leuA8 trpC2*	Td[b] of 61720 by 61885
62358	*aldA1 relA1 trpC2*	Td of 62356 by 61852
62360	*aldA1 metE1 relA1 trpC2*	Td of 62484 by 61852
62391	*leuA8 metB5 purB6*	14 (101)
62396	*leuA8 metB5 hsrM*	14 (1012)
62435	*aldA1 aroG932 relA1 trpC2*	Td of 61710 by 61852
62439	*aldA1 metE2 relA1 trpC2*	Td of 62435 by 62293
62441	*aldA1 metE3 relA1 trpC2*	Td of 62435 by 62294
62484	*aldA1 leuA8 metE1 trpC2*	Td of 61710 by 62258

[a] Obtained from the *Bacillus* Genetic Stock Center, The Ohio State University, Columbus.
[b] Td: PBS1-mediated transduction of the first strain by the second one.

0.5% (wt/vol) glucose and 0.4 mM L-glutamate (MGG medium). S7G medium consisted of 10 mM ammonium sulfate, 5 mM potassium phosphate (pH 7.0), 100 mM morpholinopropanesulfonate (MOPS; adjusted to pH 7.0 with KOH), 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_2$, 1 μM $ZnCl_2$, 2 μM thiamine, 20 mM L-glutamate (adjusted to pH 7.0 with NaOH), and 2% (wt/vol) glucose. Supplements required by the auxotrophs were added at 50 μg/ml for amino acids, 10 ng/ml for D-biotin, and 100 μg/ml for adenine (S7G plus supplements medium). The rich TB medium was described by Shibata and Ando (24). MM medium contained (per liter) 10 g of tryptose (Difco), 5 g of yeast extract, 9.2 g of NaCl, 5 mmol of $CaCl_2$, and 0.1 mmol of $MnCl_2$. PM buffer consisted of 0.1 M potassium phosphate (pH 6.5) and 1 mM $MgCl_2$.

Transduction. Donor strains were grown in 10 ml of 1.75% (wt/vol) antibiotic medium 3 at 37°C. When the optical density at 600 nm (OD_{600}) reached 1.0, 5 ml of bacteriophage PBS1 lysate (1×10^3 to 3×10^3 transducing particles per ml of antibiotic medium 3) was added. After 30 min at 37°C without shaking, chloramphenicol was added (final concentration, 5 μg/ml), and the cultures were incubated overnight at 37°C without shaking. The lysates were treated with 10 μg of DNase I per ml for 30 min at 37°C. Cell debris were removed by filtration through a membrane filter (Nalge 115-ml filter unit), and the filtrate was stored at 4°C. Recipient strains were grown in 10 ml of antibiotic medium 3 to an OD_{600} of 1. PBS1 lysate (5 ml) prepared from donor strains as above was added, and the mixture was incubated for 30 min at 37°C without shaking. The infected cells were collected by centrifugation (4,000 × *g* for 10 min), washed twice with 10 ml of PM buffer, and plated onto selective plates.

Selection and scoring of recombinants. Transductants were selected as described by Dedonder et al. (4). The *metE* mutations were tested for their resistance to 4 mM DL-ethionine on MGG plates. The presence of *relA* was detected by continued RNA synthesis in the presence of granaticin (6).

Growth and sporulation measurements. Strains were inoculated from frozen cultures (in 25% [vol/vol] glycerol at −70°C) onto TBAB plates and incubated at 37°C for 8 to 9 h. The cells were suspended in S7G plus supplements and diluted into the same medium at an initial OD_{600} of 0.001 to 0.003. The cultures were shaken at 150 strokes per min in a reciprocal shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C. When the OD_{600} reached 0.7, 5-ml aliquots of each culture were distributed into 125-ml flasks containing different amounts of decoyinine or mycophenolate to induce sporulation. After 10 h of further shaking, the titers of viable cells and heat-resistant spores were measured (20).

Modification of ϕ105. A clear plaque mutant of ϕ105 (ϕ105c30, from the *Bacillus* Genetic Stock Center, Columbus, Ohio) was prepared and assayed on plates by a double-layer method (10, 24). The strains to be tested for modification activity were grown at 37°C in S7G plus supplements to an OD_{600} of 2. About 10^5 PFU of the phage lysate (in 0.1 ml) was mixed with 0.5 ml of these bacteria and 2.5 ml of S7G medium containing 0.7% (wt/vol) agar, and then the mixture was plated onto S7G plates. The plates were incubated overnight at 37°C. S7G liquid medium (1 ml) was added to each plate, the soft-agar layer was collected, and the liquid was extracted by centrifugation (5,000 × *g* for 10 min). The lysate was filtered through a sterile membrane filter (Millipore). This procedure was repeated to obtain phage lysates with a uniform genetic background. The lysate was diluted in TB medium, and 0.1 ml was plated on MM plates together with 0.1 ml of a culture of the restriction-proficient or restriction-deficient strain (strains 62391 and 62396, respectively) grown to stationary phase in liquid MM medium.

Assay of SAM synthetase. Strains were grown at 37°C in 200 ml of S7G plus supplements to an OD_{600} of 2.0. Chloramphenicol (final concentration, 100 μg/ml) and phenylmethylsulfonyl fluoride (final concentration, 0.1 mM) were added; the cells were collected by centrifugation (12,000 × *g* for 5 min at 4°C), washed twice with 25 ml of PM buffer at 4°C, and stored at −70°C. Cell extracts were prepared and the SAM synthetase assay was performed as described (20). The reaction mixture (50 μl) contained 1 mM L-[*methyl*-^{14}C]methionine (0.4 μCi/ml). The background counts per minute obtained without ATP addition were 0.05% of the total input. This value was the same as that obtained when the reaction mixture contained no cell extract. Protein was measured by the method of Lowry et al. (18).

Measurement of SAM and Met pools. Cells were grown in 100 ml of S7G plus supplements at 37°C to an OD_{600} of 1, rapidly collected on a membrane filter (diameter, 10 cm; Schleicher & Schuell), and extracted with 1.5 ml of 0.5 M formic acid, and the formic acid was removed by freeze-drying as described by Ochi et al. (21). The dried material was dissolved in 100 μl of water. When the culture contained Met, cells on the filter were washed twice with 30 ml of prewarmed medium without Met before the filter was placed on formic acid. The amount of SAM was assayed by high-performance liquid chromatography by the method of Freese et al. (9). The amount of Met was determined by a microbiological assay (23) with *B. subtilis* 61950 as the *met* auxotroph. This strain was grown at 37°C in S7G plus 1 mM Met and 0.25 mM tryptophan (Trp). When the OD_{600} reached 1, the cells from 4 ml of culture were collected by centrifugation (4,000 × *g* for 10 min) and washed twice with 4 ml of S7G plus 0.25 mM Trp. The cells were added to 2 ml of S7G plus 0.25 mM Trp to give an OD_{600} of 0.01, 10 to 60 μl of the above extract was added, the mixture was shaken for 18 h at 37°C, and the OD_{600} was then measured. The amount of Met was determined from a standard curve. The relationship between the amount of Met and the final OD_{600}

TABLE 2. Genetic linkage obtained from two-factor transduction crosses

Donor	Recipient	Genotype of transductants		Cotransduction frequency (%)
		Selected (no.)	Unselected (no.)	
metE1 (62258)	*aroG932* (61710)	*aroG*$^+$ (100)	*metE* (51)	51
	thr-5 (61711)	*thr*$^+$ (108)	*metE* (10)	9.3
metE2 (62293)	*aroG932* (61710)	*aroG*$^+$ (52)	*metE* (21)	40
	thr-5 (61711)	*thr*$^+$ (120)	*metE* (12)	10
metE3 (62294)	*aroG932* (61710)	*aroG*$^+$ (71)	*metE* (31)	44
	thr-5 (61711)	*thr*$^+$ (100)	*metE* (8)	8

was linear up to 20 μM Met in the growth tube. The amount of Met was also determined with an amino acid analyzer. The intracellular pool measurements were expressed in picomoles or nanomoles per OD_{600} unit, where one OD_{600} unit is defined as the number of cells which give an OD_{600} of 1 if suspended in 1 ml.

Intracellular water volume. Strain 62358 was grown in S7G plus supplements at 37°C. When the OD_{600} reached 1.3, three 8-ml aliquots were distributed into centrifuge tubes each containing 8 μCi of inulin-[^{14}C]carboxylic acid. The procedure for determination of the intracellular water volume was exactly the same as described by Freese et al. (9). The average volume was 0.535 ± 0.13 μl per OD_{600} unit.

RESULTS

Mapping of *metE* mutations. In addition to the *ethA1 metE1* mutant 62258 described before (20), we isolated from the same parent (strain 61885) two other spontaneous mutants that were resistant to 10 mM DL-ethionine and showed low SAM synthetase activity. The mutations were designated *metE2* (in strain 62293) and *metE3* (in strain 62294). The generalized transducing phages PBS1, grown on these mutants, were used to transduce all "kit" strains constructed for mapping by Dedonder et al. (4). The selected transductants were tested for resistance to 4 mM DL-ethionine to score *metE* recombinants. (Separate tests showed that *ethA1* mutants, *metE* mutants, and *ethA1 metE* mutants were fully resistant to 2, 4, and 10 mM DL-ethionine, respectively.) All three *metE* mutations were linked closely to *aroG932* and loosely to *thr-5* (Table 2). From each cross (with strain 61710), one of the *aroG*$^+$ ethionine-resistant transductants was isolated; they all had low SAM synthetase activity (see below). The order of genes around the *metE1* mutation was determined by a four-factor cross (Table 3).

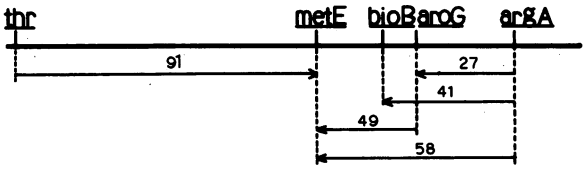

FIG. 1. Portions of the genetic map of *B. subtilis* containing the *metE* mutation. The numbers represent the percentage of recombination, which is given by (1 − cotransduction frequency) × 100. Arrows are drawn from selected to unselected markers.

The order of the genes *bioB-aroG-argA* is known (22) and was confirmed by the results in Table 3. The linkage between *argA* and *bioB* was closer (41% recombination) than that between *argA* and *metE* (58%) (Table 3; Fig. 1), suggesting that the order is *metE-bioB-aroG-argA* or, less likely, *bioB-aroG-argA-metE*. Since *thr-5* was linked to *metE* (91%) (Table 2), but not to *argA* (5), *thr-5* is closer to *metE* than to *argA*, suggesting the order *thr-5-metE-bioB-aroG-argA* (Fig. 1). This order was confirmed by the details of the four-factor cross (Table 3) if we assumed that the minority classes of recombinants were due to four crossover events. The *ethA1* mutation, causing resistance to a maximum of 2 mM ethionine, was not linked to *aroG* and is located elsewhere (data not shown). Because all three *metE* mutations produced the same phenotypic properties and had a similar genetic location, they presumably are located in the same gene.

Physiology of *metE* mutants. To determine the physiological consequences of the *metE* mutations, we constructed isogenic *metE*$^+$ and *metE* strains which no longer contained the *ethA1* mutation (*metE* mutations were transferred to strain 61710 by cotransduction with *aroG*$^+$). To avoid the initiation of sporulation by the stringent response (6, 21), we also introduced the relaxed marker (*relA*) into these *metE* strains by cotransduction with *leuA*$^+$ (Table 1). The cell extracts of the three resulting *metE relA* strains had 25- to 210-fold-lower specific activities of SAM synthetase than the isogenic *metE*$^+$ *relA* strain 62358 did (Table 4). The activities of the *metE1* and *metE2* strains (strains 62360 and 62439, respectively) were close to the limit of detection, but, statistically, the *metE2* cells had the lowest SAM synthetase activity.

Despite their low SAM synthetase activity, the *metE* mutants, grown without Met, had only three to four times less intracellular SAM than the *metE*$^+$ strain did (Table 4). The *metE2* strain (strain 62439) contained the lowest SAM concentration. Using the water volume of the cell (see Materials and Methods), we calculated the intracellular concentration of SAM in cells of the *metE*$^+$ strain (strain 62358) to be 80 μM. The exponential growth rates in S7G

TABLE 3. Four-factor transduction cross

Donor	Recipient	Recombinants			
		Selected marker	Class	No.	Crossover events
metE1 (62258)	*aroG932 bioB141 argA2* (62280)	*argA*$^+$	*aroG bioB metE*$^+$	29	2
			aroG bioB$^+$ *metE*$^+$	0	4
			aroG bioB metE	0	4
			aroG bioB$^+$ *metE*	0	4
			aroG$^+$ *bioB metE*$^+$	14	2
			aroG$^+$ *bioB*$^+$ *metE*$^+$	20	2
			aroG$^+$ *bioB metE*	1	4
			aroG$^+$ *bioB*$^+$ *metE*	44	2

TABLE 4. Various properties of *metE* mutants grown in the presence and absence of methionine[a]

Strain	Relevant genotype	SAM synthetase[b] (pmol/min per mg of protein)		SAM pool[c] (pmol/OD_{600} unit)			Met pool (nmol/OD_{600} unit)			Doubling time[d] (min)	
		−Met	+Met (1 mM)[e]	−Met	+Met (1 mM)	+Met (10 mM)	−Met[f]	+Met (1 mM)	+Met (10 mM)	−Met	+Met (1 mM)
62358	*metE*$^+$	195 ± 20	98	43 ± 4.0	207 ± 0.5	224	<0.02 (−)	5.1 ± 0.06	14.7	88	89
62360	*metE1*	2.4 ± 1.3	2.7	14 ± 0.3	203 ± 11	146	0.14 ± 0.01 (0.14)	5.8	14.2	91	88
62439	*metE2*	0.91 ± 0.3	ND	11 ± 3.9	47.1 ± 3.8	49	0.24 ± 0.02 (0.11)	4.3	13.5	100	90
62441	*metE3*	7.7 ± 2.4	ND	15 ± 1.6	11.7 ± 3.2	11	0.17 ± 0.01	4.1	11.3	88	102

[a] Cells were grown in S7G plus 0.25 mM tryptophan with or without Met at 37°C. Means of two to three determinations are presented with their standard deviations.
[b] When the OD_{600} reached 2.0, the crude extracts were prepared and SAM synthetase was assayed.
[c] When the OD_{600} reached 1.0, the cell extracts were prepared with formic acid. The amount of SAM was measured by high-performance liquid chromatography, and Met was determined by bioassay.
[d] The doubling time was measured during exponential growth in the OD_{600} range from 0.5 to 5.00.
[e] Concentration of Met in the growth medium.
[f] The values shown in parentheses were determined by an amino acid analyzer; (−), below the limit of detection.
[g] ND, Not determined.

plus supplements were the same for the *metE*$^+$, *metE1*, and *metE3* strains. Only the *metE2* strain grew slightly more slowly (10% higher doubling time) than the other strains (Table 4). Its intracellular SAM concentration was 11 pmol/OD_{600} unit (Table 4), or about 20 μM. Even on rich medium, such as on a TBAB plate, the *metE2* strain grew slowly, producing smaller colonies than those of the other strains. Addition of spermine or spermidine, whose production requires decarboxylated SAM, did not restore the growth of the *metE2* strain (data not shown).

Because cells use SAM as a methyl group donor, the decreased SAM pool in *metE* mutants might affect methylation. Therefore, we measured the in vivo effect of DNA methylation via the modification of ϕ105 phage DNA. This phage is susceptible to the restriction modification system of *B. subtilis* Marburg 168 (*hsrM hsmM*) (24), and the *hsmM*-associated methylation reaction requires SAM as a methyl donor (15). The phages, prepared from various strains, were tested for their plating efficiency on restriction-proficient and restriction-deficient strains (strains 62391 and 62396, respectively). The *hsrM*$^+$ strain 62391 restricted the phages grown on the modification-deficient *hsmM* strain 62281 50-fold. However, the phages grown on the *metE*$^+$ strain or on any of the *metE* mutants were not restricted by strain 62391. These results indicate that the decrease of the SAM concentration in the *metE* mutants was not sufficient to decrease the DNA modification activity, at least with respect to *hsmM*.

Effect of Met on the accumulation of SAM. SAM synthetase requires both Met and ATP as substrates. In addition to de novo biosynthesis, ATP can be derived from adenine or adenosine by means of the purine salvage pathways of *B. subtilis* (7). We investigated the accumulation of Met and SAM following the addition of Met, adenosine, or adenine to the medium. Addition of Met increased the Met pool to a similar level in all strains (Table 4).

The SAM pool of the *metE*$^+$ strain increased fivefold in the presence of 1 mM Met (Table 4). This amount of SAM represents saturation, because it increased only little when the Met concentration was increased to 10 mM. Neither adenine nor adenosine addition increased the SAM pool (30 pmol/OD_{600} unit with 1 mM adenine and 23 pmol/OD_{600} unit with 1 mM adenosine). The combination of both 10 mM Met and 1 mM adenine resulted in essentially the same concentration of SAM (241 pmol/OD_{600} unit) as with 10 mM Met alone. The addition of 1 or 10 mM Met to the medium greatly increased the SAM accumulation in the cells of the *metE1* and *metE2* mutants, but not in the cells of the *metE3* mutant (Table 4). Cells of the *metE1* mutant accumulated about the same amount of SAM as those of the *metE*$^+$ strain did; the 40- to 80-fold-lower level of SAM synthetase in this strain apparently sufficed to allow this accumulation. Cells of the *metE2* mutant accumulated about the same amount of SAM as that normally found in cells of the *metE*$^+$ strains without Met; the normal rate of growth was restored. In contrast, for cells of the *metE3* mutant, the SAM pool and the growth rate were slightly decreased (Table 4).

Suppression of sporulation by methionine and adenine. In synthetic medium (S7G plus supplements) the *metE*$^+$ strain, as well as the three *metE* mutants, sporulated at very low frequencies (10^2 to 10^3 spores per ml 10 h after the viable cell titer had reached 2×10^9 to 4×10^9 per ml). Sporulation was induced in exponentially growing cells by the addition of decoyinine (an inhibitor of GMP synthetase) or mycophenolate (an inhibitor of IMP dehydrogenase), both of which decrease the intracellular concentration of guanine nucleotides (8, 17). The four strains sporulated about equally well 10 h after the addition of 400 μM decoyinine (3×10^7 to 8×10^7 spores per ml) (data not shown) or 50 to 200 mM mycophenolate (4.5×10^7 to 6.5×10^7 spores per ml) (shown for *metE*$^+$ and *metE3* strains in Fig. 2). (They also sporulated normally in nutrient sporulation medium.) The sporulation frequency decreased two- to threefold when 1 mM adenine or Met was included in the medium (Fig. 2). In the *metE*$^+$ strain, the minor effect of Met could conceivably result from the fivefold increase in the SAM pool (addition of 150 μM mycophenolate caused only a slight decrease of SAM, from 207 to 150 pmol/OD_{600} unit after 15 min). However, adenine alone did not increase the SAM pool (see above). When 1 mM each adenine and Met were included in the medium, the suppression of sporulation was more pronounced and could not be counteracted by increasing the concentration of mycophenolate (Fig. 2). The possibility that the presence of adenine and Met delayed spore maturation was excluded by the fact that the spore titer did not increase between 10 and 13 h after mycophenolate (150 μM) addition to the *metE*$^+$ strain. The effect of adenine plus Met might have resulted from an increase in the level of SAM, but the results with the *metE3* mutant negated this possibility. The cells of this mutant, grown in the presence of adenine and Met, contained only 29 pmol of SAM per OD_{600} unit and maintained this value for at least 2 h after mycophenolate (150 μM) addition. This value was lower than that of the

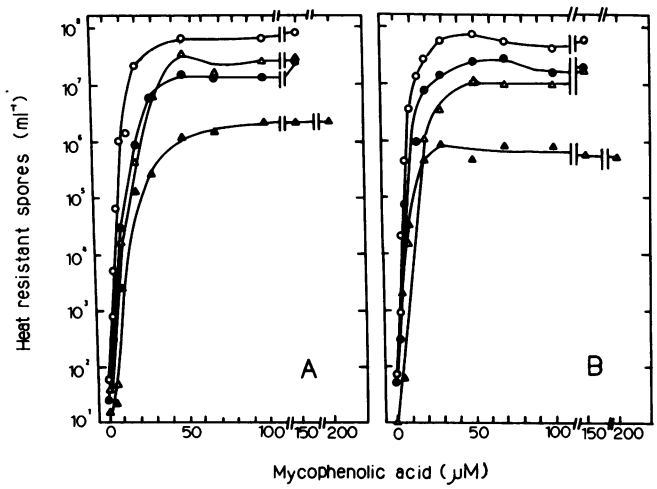

FIG. 2. Effect of adenine and/or methionine on sporulation of wild-type *metE*$^+$ and *metE3* strains. Cells were grown in S7G medium plus 0.25 mM tryptophan with or without Met (1 mM) or adenine (1 mM) at 37°C. When the OD_{600} reached 0.7, the indicated amounts of mycophenolate were added. After an additional 10 h of vigorous shaking, the titers of heat-resistant spores were measured. (A) *metE*$^+$ strain 62358; (B) *metE3* mutant 62441. Symbols: ○, no adenine or Met; ●, plus 1 mM Met; △, plus 1 mM adenine; ▲, plus 1 mM adenine and 1 mM Met.

metE$^+$ strain grown without Met (Table 4), which sporulated well with mycophenolate. The *metE*$^+$ strain was also not suppressed by the accumulation of SAM, because it produced, in the presence of Met plus adenine at high mycophenolate concentrations, a twofold-higher spore titer than the *metE3* mutant did (Fig. 2).

DISCUSSION

Cells of the *metE* mutants, grown in the absence of Met, had 25- to 200-fold-lower SAM synthetase activity than those of the *metE*$^+$ strain, but, surprisingly, only a 3- to 4-fold-lower SAM pool. The SAM synthetase deficiency also caused at least a sevenfold accumulation of Met; this accumulation probably reflects increased Met production rather than decreased Met utilization (two *metE* mutants grew at the same rate as the *metE*$^+$ strain, i.e., they presumably utilized Met at the same rate). In *Escherichia coli*, Met production is feedback inhibited or repressed by SAM (10); this effect is probably responsible for the selection of SAM synthetase-deficient mutants by ethionine (at least in *E. coli*): such mutants produce more methionine, which can compete with the (lethal) incorporation of ethionine into proteins (H. Taber, personal communication). The increased Met pool in the *metE* mutants suggests that SAM feedback inhibits or represses the synthesis of Met in *B. subtilis* also. However, the drastic decrease in SAM synthetase activity in the *metE* mutants did not result in a correspondingly large decrease in the SAM pool, presumably because more Met was available and because the residual SAM synthetase activity functioned at a maximal rate. In the *metE*$^+$, *metE1*, and *metE2* strains, the intracellular Met concentration clearly limited the rate of SAM production, because addition of extracellular Met increased the SAM pool further.

Resistance to ethionine has also been used to isolate SAM synthetase mutations in *Saccharomyces cerevisiae* (3, 19, 25) and in *E. coli* (10, 11). The *metK* gene of *E. coli* is the structural gene of SAM synthetase (13). Properties of our *metE* mutations resemble those of *metK* mutations (10) in so many respects that *metE* probably is the structural gene for SAM synthetase.

Similar to the *metK* mutants of *E. coli*, the *metE1* and *metE3* mutants of *B. subtilis* grew in synthetic medium at the same rate as the isogenic *metE*$^+$ strain. Only the *metE2* mutant, with the lowest SAM concentration (and SAM synthetase activity), grew at a 10% lower rate. This result shows that 100 times less SAM synthetase activity than is normally found in *metE*$^+$ strains suffices to enable normal growth in Met-free medium. Because addition of Met to a culture of the *metE2* mutant increased the SAM pool and restored growth to the normal rate, the growth deficiency was a direct result of the SAM synthetase deficiency. For optimal growth, the intracellular SAM concentration must be higher than 11 pmol/OD unit (21 μM), while 14 pmol/OD unit (26 μM) is sufficient. It is likely that additional mutations could be found which would produce a complete deficiency of SAM synthetase and require SAM for growth. Surprisingly, the *metE3* mutant responded to Met addition by producing slightly less SAM, as if the small amount of SAM synthetase in this strain was inhibited by excess Met. At this reduced SAM concentration, the growth rate was lower (Table 4), which again shows that an intracellular SAM concentration of about 14 pmol/OD_{600} unit (26 μM) is critical for normal growth.

Neither the *metE* mutants nor the *ethA1* mutants (1) sporulated spontaneously at the high frequency of the *ethA1 metE1* strain 62258 (20). Apparently, the combination of the two mutations is required for this elevated sporulation. Since strain 62302, which had been isolated as a "*metE1*" transformant after transformation with DNA of the *ethA1 metE1* strain 62258 (20), also sporulated spontaneously at a high frequency, we have to conclude that it presumably had acquired the *ethA1* mutation together with the *metA1* mutation. This now seems likely, because the *ethA1* mutation alone causes resistance to 2 mM ethionine and the *metE1* mutation alone causes resistance to 5 mM ethionine, whereas the combination of both mutations causes resistance to 10 mM ethionine; presumably, on the plate containing 10 mM ethionine a large (i.e., rapidly growing) colony had been picked as the transformant.

The *ethA1* mutation causes pleiotropic effects, but the primary function (enzyme) affected by it remains unknown (1). The *metE* mutations did not interfere with sporulation in nutrient sporulation medium or with that induced by decoyinine or mycophenolate in synthetic glucose medium. The combination of adenine and Met, added to the medium containing one of the *metE* strains, decreased the maximal mycophenolate-induced spore titer about 50-fold, each compound alone being less effective. This effect did not result from an increased production of SAM, because it was also observed in the *metE* mutant, which, in the presence of 1 mM Met, produced about as much SAM as the standard strain without Met, and in the *metE3* mutant, in which Met did not increase the production of SAM. Therefore, the combination of Met plus adenine (and to some extent each compound alone) inhibits sporulation by some other mechanism.

In *S. cerevisiae*, SAM is metabolized to adenosine and homocysteine (16); homocysteine can then be methylated (by methyl tetrahydrofolate) to give methionine. In *B. subtilis* a *purH metC* double mutant, which requires adenine (or adenosine) and Met (but cannot use homocysteine), can grow well with SAM instead of adenine and Met (Wabiko,

unpublished results). This shows that SAM can be metabolized (hydrolyzed) directly to Met and adenosine (or adenine). Therefore, it is not clear whether the reported inhibition of sporulation by >1 mM SAM (20) results from intracellular SAM per se or from its degradation products, Met and adenine (or adenosine).

The *metE* mutations did not affect DNA methylation, at least with respect to the modification of phage ϕ105. This is not surprising, because the K_m for one *B. subtilis* DNA methylase, the *Bsu*R enzyme that is specific for CCGG, is 0.7 μM SAM (12), which is much lower than the lowest concentration of SAM observed (when the *metE2* mutant was grown without added Met).

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