A Decrease in *S*-Adenosylmethionine Synthetase Activity Increases the Probability of Spontaneous Sporulation

KOZO OCHI AND ERNST FREESE*

Laboratory of Molecular Biology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

Received 16 April 1982/Accepted 30 June 1982

Starting with a relaxed (*relA*) strain, mutants with reduced activity of adenosine triphosphate:L-methionine *S*-adenosyl transferase (EC 2.5.1.6; SAM synthetase) were isolated in *Bacillus subtilis*. One such mutant (gene symbol *metE1*) had only 3% of the normal SAM synthetase activity but grew almost as well as the parent strain. Another mutant was isolated (gene symbol *spdC1*) as being able to sporulate continually at a high frequency; it had one-half the normal SAM synthetase activity at 33°C. Both mutants continually and spontaneously entered spore development at a higher frequency than the parent strain in a medium containing excess glucose, ammonium ions, and phosphate. Sporulation was prevented by a high concentration of SAM (1 mM or more) or by the combination of adenosine and methionine (0.5 mM or more each), both of which are precursors of SAM. In contrast to this continual increase in the spore titer, addition of decoyinine, an inhibitor of GMP synthetase, rapidly initiated massive sporulation. Various amino acid analogs also induced sporulation in the *relA* strain, the methionine analogs ethionine and selenomethionine being most effective.

Like many other microorganisms, cells of *Bacillus subtilis* leave their growth cycle and enter differentiation when nutrients get scarce. Many of these deprivation conditions result in the transient deficiency of one or more amino acids and the ensuing "stringent response" (3) which, by causing a decrease in guanine nucleotides, initiates sporulation (2, 8, 9). The partial deprivation of even a single amino acid can thus initiate sporulation, as has been shown for several auxotrophs including a $\Delta ilvB1$ *kauA1* mutant (indicating a deletion of the genetic area around *ilv*) whose supply of isoleucine was controlled by the extracellular concentration of oxomethylvalerate (12). Relaxed (*relA*) mutants, unable to produce the stringent response to amino acid deprivation, do not sporulate under these conditions, but their sporulation can be induced by partial deprivation of guanine nucleotides, which can be produced, e.g., by addition of decoyinine, a specific inhibitor of GMP synthetase (8, 12). Because amino acid deprivation does not induce their sporulation, *rel* mutants are ideal for analyzing whether any amino acid analog, used at an appropriate intermediate inhibitory concentration, can increase the sporulation frequency independent of the stringent response. In such experiments, we found two methionine analogs (ethionine and selenomethionine) to be most effective. Because some of the ethionine-resistant mutants isolated in other or-

ganisms have a reduced activity of methionine *S*-adenosyl transferase (SAM synthetase; adenosine triphosphate:L-methionine *S*-adenosyl transferase, EC 2.5.1.6), we isolated such mutants in *B. subtilis* to determine whether the concentration of SAM or its metabolites is somehow involved in the control of sporulation. This paper shows that some of these mutants enter spore development continually and spontaneously at a higher rate than does the parent strain.

MATERIALS AND METHODS

Growth and sporulation conditions. The bacteria were grown in synthetic medium consisting of 10 mM ammonium sulfate–5 mM potassium phosphate (pH 7.0)–100 mM morpholinopropane sulfonate (adjusted to pH 7.0 with KOH)–2 mM $MgCl_2$ –0.7 mM $CaCl_2$ –50 μM $MnCl_2$ –5 μM $FeCl_3$ –1 μM $ZnCl_2$ –2 μM thiamine–55 mM D-glucose–20 mM L-glutamate (adjusted to pH 7.0 with KOH). In most experiments, this medium was supplemented with 0.5 mM each isoleucine and valine (Ilv medium), and in some experiments this medium contained 110 rather than 55 mM glucose (Ilv medium with 2% glucose). Nutrient sporulation medium contained 0.8% (wt/vol) nutrient broth (Difco Laboratories, Detroit, Mich.)–1 mM $MgCl_2$ –0.7 mM $CaCl_2$ –50 μM $MnCl_2$ –1 μM $FeCl_3$ –10 mM potassium phosphate buffer (pH 7.0)–5 mM potassium acetate (pH 7.0) and supplements as required by auxotrophs.

Cells from cultures (in 25% glycerol), kept frozen at $-20^\circ C$ for short-term and at $-70^\circ C$ for long-term

storage, were streaked onto a plate containing tryptose blood agar base (TBAB; 3.3%, wt/vol; Difco) and incubated for 6 to 8 h at 37°C. The cells were suspended in synthetic medium, and the absorbance at 600 nm (OD_{600}) of the appropriately diluted suspension was measured. Each suspension was diluted, to give an OD_{600} of 0.001 to 0.01, into flasks containing 1/10 volume of synthetic medium and supplements of required compounds (0.5 mM L-isoleucine, 0.5 mM L-valine, 0.25 mM L-tryptophan, or 0.25 mM L-methionine). These precultures were shaken for 8 to 14 h at the indicated temperature (usually 37°C). When the OD_{600} reached the desired value (0.1 or 0.5), 10-ml aliquots of each culture were distributed into flasks containing various amounts of the compounds to be tested, and the flasks were vigorously shaken at the indicated temperature (usually 33 or 37°C). At indicated times, each culture was diluted in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$, and the dilutions were plated on TBAB plates to determine the viable cell titer. The spore titer was measured by heating the diluted cultures for 20 min at 75°C and then plating.

Bacterial strains and preparation of mutants. The strains used in this study were (see also reference 12) the stringent strains 60015 (*metC7 trpC2*) and 61886 (*ΔilvB1 kauA1*) and the relaxed strains 61852 (*lys relA1 trpC2*) and 61885 (*ΔilvB1 kauA1 relA1*). All other strains were derived from these as described below.

Strongly ethionine-resistant mutants 62258 (*ΔilvB1 kauA1 metE1 relA1*) and 62290 (*ΔilvB1 kauA1 metT1 relA1*) were obtained after 3-day culture of strain 61885 in Ilv medium containing 5 to 10 mM L-ethionine. Suitable dilutions were plated on TBAB plates, and the colonies were checked for resistance to ethionine on plates of Ilv medium containing 10 mM L-ethionine. Several isolates (e.g., 62258 or 62290) produced normal colonies after 1 day, whereas the parent (61885) produced tiny colonies only after 4 days. Both strains 62258 and 62290 sporulated normally in nutrient sporulation medium (spore titer, about 10^8 /ml 24 h after the OD_{600} was 0.1).

The spontaneously sporulating (*spd*) strain 62252 (*ΔilvB1 kauA1 relA1 spdC1*) was derived from strain 61885 after 3 days of culture in synthetic medium containing 5 mM ethionine and compounds required by the auxotrophs. Once every day the culture was heat treated at 75°C for 20 min to select for spores. After 3 days, the heat-treated cells were plated on TBAB plates, and cells of individual colonies were again cultured in ethionine-containing medium as above. After three repeats of this procedure, cells of colonies on TBAB plates were grown in Ilv medium (no ethionine) at 37°C and checked for their ability to sporulate 10 h after the OD_{600} was 0.5. About half of 150 isolates tested showed various increased spontaneous sporulation efficiencies (10^5 to 10^8 spores per ml), whereas the parent (61885) produced only 10^3 spores per ml (all measured 10 h after the OD_{600} was 0.5). One of these mutants, strain 62252, was used here because it sporulated particularly efficiently, and its cells showed no morphological difference from the parent under the microscope (many of the other *spd* mutants exhibited various morphological changes, such as growth in chains or absence of motility or both).

Assay of SAM synthetase. SAM synthetase activity

was assayed by using ^{14}C -labeled methionine and a modification of the method by Markham et al. (11), which had been used for *Escherichia coli* (6). To prepare a crude cell extract, cells were grown in 200 ml of Ilv medium from a low inoculum ($OD_{600} = 0.01$) at 33, 37, or 43°C. When the OD_{600} reached 2.0, the culture was added to a beaker containing ice, chloramphenicol (100-μg/ml final concentration, to stop protein synthesis), and phenylmethylsulfonyl fluoride (0.1 mM final concentration, to inhibit serine proteases). After centrifugation at $12,000 \times g$ for 5 min at 3°C, the pellet was suspended in 50 ml of cold potassium acetate buffer (0.1 M, pH 6.0, by KOH) containing 10 mM $MgCl_2$ -0.1 mM phenylmethylsulfonyl fluoride. The cells were again spun down and then suspended in 50 ml of cold 0.1 M potassium carbonate (pH 10 by HCl) buffer containing 10 mM $MgCl_2$ -1 M KCl-0.1 mM phenylmethylsulfonyl fluoride; this treatment removes extracellular protease (7). After centrifugation ($12,000 \times g$ for 5 min), the pellet (about 1 g, wet weight) was suspended in 1 ml of Tris (0.1 M, pH 8.1, by HCl; Tris-chloride buffer) containing 10 mM $MgCl_2$ -100 mM KCl-1 mM dithiothreitol-20% (vol/vol) glycerol-0.1 mM phenylmethylsulfonyl fluoride. This suspension was passed through a French pressure cell (2,400 lb/in²), and cell debris was removed by centrifugation ($35,000 \times g$ for 20 min at 3°C). The supernatant fluid ("enzyme preparation") contained about 20 mg of protein per ml and was immediately used for enzyme assay. Protein was measured by the method of Lowry et al. (10). The reaction mixture for the SAM synthetase assay (total, 50 μl in an 8-mm-diameter tube) contained (final concentrations): 10 mM ATP, 1 mM (methyl- ^{14}C)methionine (0.02 μCi), 10 mM KCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 200 mM Tris-chloride buffer (pH 8.1). The reaction (run in triplicate) was started by addition of the enzyme preparation (25 μl) at 37°C. The reaction was terminated after 45 min by addition of 0.1 ml of 50 mM EDTA (adjusted to pH 8.0 with NaOH) followed by cooling in an ice bath. The mixture was placed (by a Pasteur pipette) onto a 3-cm square of Whatman P-81 phosphocellulose paper. The papers from several mixtures were spread on a Buchner funnel (8.5-cm diameter) that had been covered with filter paper (Whatman no. 1) and washed with 0.5 liter of distilled water. The filter squares were placed in vials with 10 ml of Aquasol counting fluid (New England Nuclear Corp., Boston, Mass.), and radioactivity was measured in a scintillation counter. The recovery of SAM after the wash with distilled water was 70% as determined with S-adenosyl-L-[2- 3H]methionine. Therefore, the measured amounts of SAM were multiplied by 1.4.

The amount of SAM produced in this assay mixture increased up to 60 min, and then the rate of increase gradually decreased; about 10,000 cpm of radioactivity was obtained after 45 min of incubation with SAM synthetase from the parent strain (61885), whereas the background radioactivity (without enzyme) was 700 to 1,000 cpm and did not change throughout the incubation time. The counts increased linearly with the amount of enzyme preparation added. The optimal assay temperature was 37 to 40°C. Less than half that activity was observed at 27 or 43°C. At 43°C, 70% activity was lost when the enzyme preparation was incubated in the reaction mixture (containing KCl, $MgCl_2$, and ATP, etc.) without methionine for 15 min

before assay at 37°C; apparently the enzyme is unstable at 43°C. The SAM synthetase of the *spd* strain (62252) was similarly sensitive (55% activity was lost after 15 min at 43°C). The SAM synthetase activity also decreased (>90%) if it was precipitated by ammonium sulfate precipitation (0 to 80%) and subsequently dialyzed against 10 mM Tris-chloride buffer (pH 8.0) containing 200 mM KCl–2 mM mercaptoethanol. The enzyme preparation also lost activity (>90%) after storage in a freezer (–20°C) overnight. The SAM synthetase of *B. subtilis*, similar to that of *E. coli* (5, 19), is controlled by feedback repression because the specific activity of the enzyme decreased 95% when cells were cultured (from OD₆₀₀ = 0.1 to 2.0) in Ilv medium containing 0.5 mM SAM.

Assay of nucleotide pool and methionine. To determine the intracellular concentrations of GTP, guanosine-5'-diphosphate-3'-diphosphate (ppGpp), and guanosine-5'-triphosphate-3'-diphosphate (pppGpp), cells were extracted with 1 M formic acid, and the nucleotide concentrations in the extracts were measured by high-performance liquid chromatography as described previously (12). To determine the intracellular methionine concentration, we grew cells in 100 ml of Ilv medium at 37°C to OD₆₀₀ = 0.5. Then we rapidly collected the cells on a membrane filter (0.45-μm pore size, 10-cm diameter; Millipore Corp., Bedford, Mass.), washed them with 20 ml of 5 mM potassium phosphate buffer (pH 6.5) containing 2 mM MgCl₂ (whole operation taking <20 s), and immediately placed the filter into a 30-ml centrifuge tube containing 2 ml of 0.3 M HClO₄ plus 1 mM EDTA. After 30 min of extraction, the tube was centrifuged (5,000 × g, 10 min), the supernatant was neutralized with 0.5 M K₂CO₃, and the new precipitate was again removed by centrifugation. The supernatant was adjusted to pH 2.2 by 0.5 M ice-cold HCl, and a suitable volume of it was applied to an amino acid analyzer. The methionine concentration was expressed as nanomoles per AM₆₀₀. (One AM₆₀₀ unit is the amount of cells which would produce an OD₆₀₀ of 1 if suspended in 1 ml. For cells grown in synthetic medium to an OD₆₀₀ of 1, 1 AM₆₀₀ unit corresponds to approximately 0.5 mg, dry weight.)

Reagents. SAM (chloride salt; purity, 85 to 90%), L-ethionine (purity, 95%), and seleno-DL-methionine were purchased from Sigma Chemical Co., St. Louis, Mo. Decoyinine was a gift from G. W. Whitfield of The Upjohn Co., Kalamazoo, Mich. Fusidic acid was kindly given to us by P. Fortnagel, University of Hamburg, Hamburg, West Germany. L-[methyl¹⁴C]methionine and S-adenosyl-L-[2-³H]methionine were purchased from New England Nuclear, and ICN Co., Irvine, Calif., respectively. All other chemicals were from commercial sources and were analytical grade.

RESULTS

Spontaneous sporulation enhanced by amino acid analogs. To determine the effect of amino acid analogs on sporulation, we grew the relaxed strain 61885 (*ΔilvB1 kauA1 relA1*) of *B. subtilis* in Ilv medium to OD₆₀₀ = 0.5 and transferred aliquots of the culture to flasks containing different amounts of the amino acid analogs (Fig. 1).

FIG. 1. Increase of sporulation caused by amino acid analogs. The relaxed strain 61885 was inoculated into Ilv medium at a low OD₆₀₀ (0.001), and the culture was shaken at 37°C. When the OD₆₀₀ reached 0.5, 10-ml aliquots were placed into 125-ml flasks containing various amounts of an amino acid analog (dissolved in water). After 13 h of further incubation, the titers of heat-resistant (75°C for 20 min) spores were determined. Symbols: (a) L-ethionine (●), seleno-DL-methionine (○), α-methyl-DL-methionine (▲), N-acetyl-DL-methionine (Δ), DL-norleucine (□); (b) DL-7-azatryptophan (●), m-fluoro-DL-tyrosine (○), p-fluoro-DL-phenylalanine (▲), DL-β-3-thienyl-α-alanine (Δ).

Although cells in which sporulation has been induced usually sporulate, at most, 8 h later, we measured the titer of heat-resistant spores after 13 h to assure that sporulation could be expressed even if its induction were delayed or if the amino acid analog should reduce the rate of spore maturation. Many analogs produced some increase in the spore titer when they were used at concentrations causing partial growth inhibition; the two methionine analogs ethionine and selenomethionine were most effective.

Although we had used a *rel* mutant, we wanted to make sure that the sporulation was not due to a stringent response resulting from the addition of amino acid analogs. We used the fact that fusidate interferes with the stringent response and the sporulation caused by it (9) (Fig. 2) for the *rel*⁺ strain 61886 (*ΔilvB1 kauA1*). Sporulation was initiated by partial isoleucine deprivation caused by cell transfer from Ilv medium to synthetic medium containing 0.5 mM L-valine and the growth-limiting amount of 0.4 mM DL-oxomethylvalerate (12). A 0.15-μg/ml amount of fusidic acid sufficed to prevent this sporulation completely. In contrast, the sporulation caused by the presence of ethionine or selenomethionine in the *rel* mutant was relatively little affected by fusidate concentrations of as much as 0.2

FIG. 2. Effect of fusidic acid on sporulation caused by isoleucine (oxomethylvalerate) deprivation of the *rel*⁺ strain (61886) and by ethionine or selenomethionine addition to cultures of the *rel* strain (61885). Cells were grown at 37°C in Ilv medium from OD₆₀₀ 0.01 to 0.5. Aliquots of the culture of strain 61885 (*rel*) fusidic acid and either 1 mM L-ethionine (●) or 0.05 mM seleno-DL-methionine (○) was added. Cells of strain 61886 (*rel*⁺) were washed on a membrane filter, suspended in synthetic medium containing 0.5 mM L-valine and 0.4 mM DL-oxomethylvalerate and distributed into flasks containing fusidate (■). The heat-resistant spore titer was measured 13 h later.

μg/ml; similar results were obtained for the other amino acid analogs. The small decrease of sporulation, produced by fusidate addition to the cultures of the *rel* strain, presumably resulted from the inhibition of protein synthesis. The absence of a stringent response in the *rel* mutant after addition of the amino acid analogs was also confirmed by measurement of the intracellular nucleotide concentrations: both ppGpp and pppGpp remained at <4 pmol/AM₆₀₀ up to at least 3 h after addition of ethionine (1 mM). GTP decreased only slightly (from the initial 400 pmol/AM₆₀₀ down to 290 pmol/AM₆₀₀ after 3 h); this small decrease does not suffice to initiate sporulation (12). These results demonstrate that the increased sporulation frequency, caused by the addition of amino acid analogs in the *rel* mutant, was not due to the stringent response and the concomitant decrease of GTP.

Because later results showed that a decrease in SAM synthetase was correlated with an in-

creased spontaneous sporulation frequency, we measured SAM synthetase activity in cells exposed to the above amino acid analogs at the concentration producing optimal sporulation (Fig. 1). The specific activity of SAM synthetase in these cells was inversely correlated to the spore titer observed in the presence of the analog (Table 1); cells grown with ethionine and selenomethionine, which produced the highest spore titer, had the lowest SAM synthetase activity, whereas cells grown with the other amino acid analogs had intermediate SAM synthetase activities. Methionine itself (2 mM) also caused a 90% decrease of SAM synthetase activity (not shown) so that at least the pronounced effect of the two methionine analogs could result from enzyme repression rather than from the incorporation of the analogs into SAM synthetase.

At high concentrations (e.g., 10 mM), the SAM synthetase reaction itself was inhibited by ethionine (29%) and selenomethionine (71%) (data not shown), but the effect was negligible at the concentrations at which the compounds produced optimal sporulation. Thus, unless these analogs were accumulated inside the cell to concentrations of about 10 mM, it appears unlikely that the inhibition of SAM synthetase was responsible for the increase in sporulation caused by the methionine analogs.

Isolation and properties of mutants. It appeared likely that the two methionine analogs were most effective in inducing sporulation be-

TABLE 1. Specific activity of SAM synthetase in cells grown in the presence of different amino acid analogs^a

Cells grown with:	Heat-resistant spores per ml ^b	SAM synthetase activity (pmol min ⁻¹ mg of protein ⁻¹) ^c
No addition	8.5×10^2	119
Ethionine (1 mM)	1.2×10^7	7.5
Seleno-DL-methionine (0.05 mM)	4.7×10^6	7.7
DL-Norleucine (1 mM)	2.9×10^4	67
α-Methyl-DL-methionine (0.1 mM)	2.0×10^4	78
7-Azatryptophan (0.6 mM)	4.5×10^4	25
β-3-Thienylalanine (0.03 mM)	4.0×10^4	74

^a Strain 61885 was grown at 37°C in Ilv medium. When the OD₆₀₀ was 0.5, the stated compound was added at the indicated concentration.

^b The titer of heat-resistant spores was measured 13 h later.

^c When the OD₆₀₀ was 2.0, a crude cell extract was prepared and the SAM synthetase activity was assayed as described in the text.

cause they interfered with the synthesis of SAM. Selenomethionine is converted by SAM synthetase into the corresponding SAM analog in *E. coli* (11), yeast (4), and rat liver (15), and ethionine is converted to 5-adenosylethionine in yeast (18), rat liver (15), and inefficiently ($K_m = 3.3$ mM) in *E. coli* (D. G. Markham, C. W. Tabor, and H. Tabor, personal communication). As some of the ethionine-resistant mutants isolated in *E. coli* have an altered SAM synthetase (11), we isolated such mutants in *B. subtilis*.

Three representative mutants are listed in Table 2, which also shows their SAM synthetase activity in comparison with the parent strain 61885. Two of the mutants were isolated as being able to grow well in the presence of 10 mM ethionine. The third mutant was isolated by multiple subculturing of cells in Ilv medium containing 5 mM ethionine and heat killing all vegetative cells once every day (see Materials and Methods); the selected strain (62252 = *spdC1*) sporulated continually in Ilv medium. To what extent the isolation of this *spd* mutation depended on the presence of ethionine is not known; *spd* indicates a mutation conveying a high frequency of continual spontaneous sporulation. This *spd* strain was not significantly more ethionine resistant than the parent. (The genetic map locations of these new mutations will be published elsewhere [K. Ochi, H. Wabiko, and E. Freese, in preparation].)

One of the strongly ethionine-resistant mutants (62258 = *metE1*) had very low SAM synthetase activity at 33 and 37°C (Table 2). We report both activities because we have performed some of the following experiments at 33°C, at which the effect of the *metE* mutation on sporulation was more pronounced. The low SAM synthetase activity of strain 62258 might have been caused by the intracellular accumula-

tion of an inhibitor. This possibility was excluded because an equal mixture of the enzyme preparation from the parent (61885) and the mutant (62258) produced 54% of the SAM synthetase activity obtained with the parent extract alone (Table 2). The other mutant (62290 = *metT1*) had normal SAM synthetase activity; uptake studies of ethionine (5 μ M) and methionine (5 μ M) showed that this mutant was deficient in active ethionine transport (uptake rate in the first 2 min after ethionine addition decreased by >99%) and had reduced methionine transport (uptake rate reduced by 50%). The *metT* mutant has not been further used in this paper. The *spdC* mutant (62252) had (at 33°C) 50% of the normal SAM synthetase activity (Table 2).

To determine the intracellular concentration of methionine, the three mutants and the parent strain were grown in Ilv medium at 33°C to an OD₆₀₀ of 0.5. After rapid collection of cells and their extraction by 0.3 M perchloric acid, the amino acid pool was analyzed on an amino acid analyzer. The *metE* strain (62258) showed a distinctly higher methionine content (0.7 nmol/AM₆₀₀) than the other three strains (61885, 62290, and 62252), for which the concentration of methionine was below the limit of our measurements (<0.3 nmol/AM₆₀₀). Thus, the deficiency in SAM synthetase causes an accumulation of methionine (presumably due to derepression of methionine-synthesizing enzymes).

Sporulation of the *metE* mutant 62258. When the parent strain was grown at 33°C in Ilv medium (containing 55 mM glucose), about 10⁵ heat-resistant spores per ml were obtained 16 h after the OD₆₀₀ had been 0.1, whereas the *metE* mutant (62258) produced a 10-fold-higher spore titer. We found later that these values fluctuated and a fraction of the cells lysed before their spores could mature into heat-resistant particles. This problem was avoided by the use of 2% instead of 1% glucose. In that medium, the titer of spores, measured 16 h after the OD₆₀₀ was 0.1, was higher in both cultures but again differed by a factor of 10 (10⁷/ml for the *metE* strain and 10⁶/ml for its parent). Addition of high SAM concentrations (higher than 0.2 mM) reduced the background level of sporulation in both strains; with 3 mM SAM the spore titer was below 10³/ml. At SAM concentrations up to 100 μ M, the *metE* mutant (62258) produced about the same or slightly more (factor of 2) spores than without SAM.

The addition of methionine at concentrations of >0.2 mM or of adenosine at concentrations of >0.5 mM produced a significant decrease in the spore titer; this effect was enhanced by the addition of both compounds (Table 3). Up to 5 mM guanosine or putrescine or its metabolites, spermidine and spermine, caused less than a

TABLE 2. SAM synthetase activity of different strains^a

Strain	Relevant genotype	SAM synthetase activity (pmol min ⁻¹ mg of protein ⁻¹)	
		33°C	37°C
61885	Parent	123	128
62258	<i>metE1</i>	2.7	3.2
61885 + 62258	1:1 mixture	ND ^b	69
62290	<i>metT1</i>	118	126
62252	<i>spdC1</i>	65	81

^a Cells were grown in Ilv medium at 33 or 37°C to OD₆₀₀ = 2. They were harvested and extracted by French press treatment, and the supernatant of the centrifuged lysate was assayed for SAM synthetase activity at 37°C for 45 min (see text).

^b ND, Not determined.

TABLE 3. Effect of various compounds on the spontaneous sporulation of strains 62252 and 62258^a

Compound added	Concn (mM)	No. of spores per ml	
		62252 (<i>spdC</i>)	62258 (<i>metE</i>)
None		1.2×10^8	1.3×10^7
Methionine	1	1.7×10^7	5.5×10^4
	10	5.5×10^6	1.0×10^3
Adenosine	1	4.1×10^6	5.0×10^4
	10	1.4×10^6	2.5×10^4
Methionine plus adenosine	1	4.0×10^4	8.8×10^2
	2	6.0×10^5	5.3×10^2
	10	6.0×10^5	6.0×10^2
SAM	1	7.7×10^6	3.0×10^3
	2	3.0×10^5	2.7×10^2
	5	1.0×10^3	6.0×10^1
Homoserine	1	2.2×10^7	2.6×10^7
	10 ^b	2.5×10^6	4.5×10^5

^a Both strains were grown in Ilv medium to OD₆₀₀ = 0.1, strain 62252 at 43°C (to suppress sporulation) and strain 62258 at 33°C. Aliquots (10 ml) were transferred to flasks containing the stated compound, and the cultures were incubated for 16 h at 33°C. For strain 62258 the transfer flasks also contained SAM (final concentration, 10 μM).

^b 10 mM homoserine significantly inhibited growth.

factor 2 change in the sporulation of the *metE* mutant (62258) with the exception of 5 mM spermine, which decreased sporulation by a factor of 5 (not shown).

To establish the importance of the *metE* mutation for the increased sporulation frequency, another *rel* strain (62262; *trpC2 relA1*) was transformed with DNA of strain 62258, and colonies resistant to 10 mM ethionine were selected. Such a transformant (62302) produced at 33°C in Ilv medium containing 2% glucose, 1.6×10^7 spores per ml, whereas the recipient (62262) produced 1.5×10^6 spores per ml.

The spore titer of the *metE* mutant (62258) continually increased throughout growth in Ilv medium at a higher rate than that of the parent (61185) (Fig. 3). If cells of the *metE* mutant were pregrown with more than 1 mM SAM, which inhibited growth somewhat, they grew after SAM removal at the normal rate only after some adaptation time, which increased with the prior SAM concentration; e.g., with 4 mM SAM the (extrapolated) lag time was 1.5 h (not shown). The lag was not prevented by addition of methionine (1 mM). When the cells were pregrown with 1 mM SAM, which inhibited growth only slightly, SAM removal caused a lag of <20 min, but the cells still began to sporulate only after a

5-h delay (Fig. 4); this shows that SAM prevented the initiation of sporulation.

Comparison of sporulation caused by SAM deprivation versus that caused by GTP deprivation. Because cells sporulated when the synthesis of either GMP (and thus GTP) (2) or SAM was reduced, we wanted to see the relationship between the two processes. When the *metE* mutant (62258) or its parent (61185) was grown in Ilv medium and, at OD₆₀₀ = 0.5, exposed to 2 mM decoyinine, the spore titer of both the parent and the mutant increased drastically 6 to 8 h later (Fig. 4). This response was quite distinct from the continual increase in the spore titer without decoyinine. Similar results were obtained when commitment to sporulation was measured (not shown): when 3 mM guanosine or 2 mM SAM was added at different times after decoyinine addition, the final spore frequency (after 12 h) was reduced only when the addition occurred up to 2 h after decoyinine addition; after that time most cells were committed to sporulate. In contrast, when cells were grown (in Ilv medium containing 10 μM SAM) from OD₆₀₀ = 0.1 on and excess SAM (1 mM final concentration) was added at different times later to stop further initiation of sporulation, the

FIG. 3. Effect of preculture with or without SAM on production of heat-resistant spores. The *metE* strain (62258) was grown at 33°C in Ilv medium with or without 1 mM SAM from OD₆₀₀ = 0.01. When the OD₆₀₀ reached 0.1, cells were collected on a membrane filter, washed, and suspended at the same density in Ilv medium without SAM. After different times of shaking at 33°C, 2-ml aliquots were removed to measure the heat-resistant spore titer. Symbols: ●, preculture without SAM; ○, with SAM.

FIG. 4. Time course of increase in spore titer. The parental (61885) and *metE* (62258) strains were grown in Ilv medium at 33°C. When the OD₆₀₀ reached 0.5, 50-ml portions were placed into flasks containing SAM (final concentration, 10 μ M) for 62258 (no SAM for 61885) and decoyinine (2 mM final, closed symbols) or no addition (open symbols). The cultures were shaken, and at the indicated times 2-ml aliquots were removed and assayed for heat-resistant spore titer. Symbols: $\circ$, $\bullet$, 62258; Δ , $\blacktriangle$, 61885.

fraction of committed cells slowly increased for 10 h (this was observed by measuring the spore titer 16 h after the OD₆₀₀ had been 0.1). The same was observed if cells were grown in the presence of excess SAM (1 mM) and transferred to Ilv medium containing 10 μ M SAM to allow initiation of sporulation, and 1 mM SAM was subsequently added at different times to arrest further initiation. Thus, the GTP deficiency caused by decoyinine addition rapidly initiated massive sporulation, whereas the SAM deficiency only increased the frequency at which cells entered the spore development.

Addition of decoyinine (2 mM final concentration) to *metE* mutant cells pregrown in Ilv medium induced sporulation regardless of the SAM concentration in the medium, as long as that was not higher than 0.5 mM (Fig. 5); the spore titer was about a factor of 4 lower if it was measured 10 h after decoyinine addition than if it was measured after 16 h. If the medium contained 1 mM or more SAM, sporulation was distinctly reduced and delayed, as a comparison of the 10- and 16-h curves in Fig. 5 shows. Apparently, SAM prevented the initiation of sporulation not by interfering itself with some enzyme but rather by producing other metabolites (e.g., methylation of some molecule), as the following experiment shows. Cells of the *metE* strain were grown in Ilv medium containing different concentrations of SAM and then transferred to Ilv medium containing no SAM but 2 mM decoyin-

ine. Two hours after decoyinine addition, 3 mM guanosine was added to stop further initiation of sporulation. Increasing SAM concentrations in the pregrowth medium increasingly prevented the initiation of sporulation (Fig. 6). This reduction in the spore titer was not due to a shock which the cells might have experienced when they were transferred from the SAM-containing to the SAM-free medium, because if decoyinine was directly added to the SAM-containing medium, the obtained spore titer was even lower than in the SAM-free medium (Fig. 6).

Sporulation of the *spdC* mutant. The *spdC* mutant (62252) continually entered sporulation at a higher rate than did the *metE* strain or its parent. For example, a culture grown at 33°C in Ilv medium from OD₆₀₀ = 0.1 contained, 16 h later, 1.5×10^8 spores per ml (83% spores per colony-forming unit), whereas a culture of the parent (61885) then contained 2.7×10^4 spores per ml (Table 4). This ability to continually sporulate could be transferred by transformation (congression) to both another *relA* strain (61852) and a *rel*⁺ strain (60015) (Table 4). The sporulation of the *spd* mutant (62252) was suppressed

FIG. 5. Sporulation of the *metE* strain (62258) in the presence of decoyinine and various concentrations of SAM. Strain 62258 was grown in Ilv medium at 33°C to OD₆₀₀ = 0.5. Aliquots of 10 ml were placed into flasks containing various amounts of SAM and either decoyinine (2 mM final concentration) or extra glucose (to give 2% total glucose). The heat-resistant spore titer was measured after incubation at 33°C for 10 and 16 h. Symbols: Ilv medium + SAM + decoyinine after 10 h ($\circ$), after 16 h ($\bullet$); Ilv medium with 2% glucose + SAM (no decoyinine) after 16 h ($\blacktriangle$).

by the addition of SAM or the combination of methionine and adenosine (Table 3). Methionine (or its precursor, homoserine) had only a small effect, whereas adenosine was somewhat more effective. Up to 5 mM polyamines or guanosine did not prevent this sporulation (not shown).

To demonstrate the dramatic difference between the continual sporulation of the *spdC* mutant (62252) and the parent strain (61885), as well as our stringent standard strain (60015), the three strains were grown in Ilv medium (for 62252, the medium initially also contained 4 mM SAM which maintained a low spore titer). When the OD_{600} of each culture was 0.5, the cells were rapidly washed with Ilv medium and suspended at the same OD_{600} in warm Ilv medium (without SAM). These cultures were incubated at 37°C and subsequently diluted twofold whenever the OD_{600} was about 1. In this way the OD_{600} was maintained between 0.5 and 1.0 for 24 h (Fig. 7). The spore titer of the *spdC* mutant (62252) increased between 6 and 9 h after cell transfer and then remained constant at about 10^9 /ml, whereas the spore titers of the other two cultures remained $<10^1$ /ml. The constancy of the spore titer in the *spdC* mutant culture demonstrates also that the sporulation was not caused by the transient shock of cell transfer from the SAM-containing to the SAM-free medium but was a property of the mutant in the SAM-free medium.

The sporulation efficiency of the *spdC* strain (62252) was strongly temperature dependent, optimal (90%) sporulation being observed at 30°C and very little sporulation ($<0.1\%$) at 43°C (Table 5). To determine whether this effect was

TABLE 4. Sporulation of strains containing the *spdC1* mutation and the effect of SAM^a

Strain	Addition	Heat-resistant spores per ml	Viable cells per ml
61885 (<i>relA1</i>)	None	2.7×10^4	6.2×10^8
	2 mM SAM	1.3×10^2	4.9×10^8
62252 (<i>relA1</i>	None	1.5×10^8	1.8×10^8
<i>spdC1</i>)	2 mM SAM	1.7×10^5	2.7×10^8
62259 (<i>relA1</i>	None	1.2×10^8	2.0×10^8
<i>spdC1</i>) ^b			
62263 (<i>rel</i> ⁺	None	1.8×10^8	1.9×10^8
<i>spdC1</i>) ^c			

^a Strains were grown at 33°C in synthetic medium containing 0.5 mM each isoleucine and valine (for 61885 and 62252) or 0.25 mM tryptophan (for 62259 and 62263). When the OD_{600} reached 0.1, each culture was divided into two portions, to one of which 2 mM SAM was added. The viable cell and heat-resistant spore titers were measured 16 h after the transfer.

^b Transformant of 61852 (*trpC2 lys relA1*) by DNA of 62252 obtained by congression for *lys*⁺.

^c Transformant of 60015 (*trpC2 metC7*) by DNA of 62252 obtained by congression for *met*⁺.

FIG. 6. Effect of preculture in the presence of SAM on the initiation of sporulation by decoyinine. The *metE* strain (62258) was grown at 33°C in Ilv medium to $OD_{600} = 0.1$, at which time 10-ml aliquots were distributed to flasks containing various amounts of SAM. When the OD_{600} reached 0.5, one portion of each flask was added to a flask containing decoyinine (2 mM final concentration) and incubated for 10 h, and the titer of heat-resistant spores was determined (●). The cells of another portion were collected on a membrane filter, washed, and suspended at $OD_{600} = 0.5$ in Ilv medium containing 2 mM decoyinine (but no SAM). After 2 h of incubation, 3 mM guanosine was added to stop further sporulation induction, the culture was incubated for 8 h more, and then the heat-resistant spore titer was determined (○).

correlated with the specific activity of SAM synthetase or the intracellular concentration of GTP, we measured these values at different temperatures (Table 5). The specific activity of SAM synthetase (in strain 62252) decreased threefold when the temperature decreased from 43 to 30°C. In the parent strain (61885), the activity decreased only about 34% in the same temperature range. The concentration of GTP, although lower at the high temperature, showed no difference between the parent and the mutant.

DISCUSSION

Several amino acid analogs increased the sporulation frequency of the *relA* parent (61885)

FIG. 7. Continual sporulation of the *spdC* strain (62252) during long-term culture in Ilv medium. Strain 62252 (●) was grown in Ilv medium at 37°C from $OD_{600} = 0.002$. When the OD_{600} reached 0.02, SAM (4 mM final concentration) was added to prevent initiation of sporulation, and the culture was further incubated to $OD_{600} = 0.5$. The cells were collected on a membrane filter, washed, and suspended in 10 ml of Ilv medium (without SAM) at $OD_{600} = 0.5$, and the culture was incubated at 37°C. Whenever the OD_{600} reached 1.0, a 5-ml portion was transferred to a flask containing 5 ml of fresh Ilv medium (done about every 70 min), and the other portion was used to determine the titer of heat-resistant spores. Strain 61885 (▲) was grown in Ilv medium and strain 60015 (△) was grown in synthetic medium containing 0.25 mM each tryptophan and methionine. When the OD_{600} reached 0.5, 10-ml portions were placed in 125-ml flasks, and incubation and dilution were carried out as above.

if they were present at some partially inhibitory concentration. Cells grown in the presence of these compounds at optimal inducing concentrations had a reduced specific activity of SAM synthetase. This effect could result from the well-known incorporation of these analogs into

proteins, whose activity they can alter (17), or, at least in the case of methionine analogs, from a repression of SAM synthetase. The increased sporulation frequency produced by the analogs could be associated with the alteration of any protein, or it could be due to the decrease of SAM synthetase activity which was correlated with the increase in the spore titer. The most effective analogs, ethionine and selenomethionine, could induce sporulation also because they may be metabolized into SAM analogs or because they repress or inhibit SAM synthetase.

Several of our ethionine-resistant mutants produced only a low SAM synthetase activity. For example, the *metE* mutant (62258) produced only 2 to 3% of the normal activity. Its cells continually entered sporulation at a higher frequency than the parent (61885) (Fig. 4). The increase of methionine in the *metE* mutant and the decrease of SAM synthetase in cells of the parent strain growing in the presence of 0.5 mM SAM or 2 mM methionine indicate that the pathway from homoserine to methionine and SAM is under control of feedback repression, as it is in *E. coli* (5, 19). The increased methionine production could be responsible for the resistance to ethionine, which competes with methionine for activation and conversion to amino-acyl tRNA. We have not yet determined whether *B. subtilis* can convert ethionine to S-adenosylethionine, which might interfere with transmethylation.

At present, we have only little information about the *spdC* mutant (62252), which sporulated continually (Fig. 7) at very high frequency, especially if it grew at 30°C (Table 5). The *spdC1* mutation is located at a different site on the genetic map than the *metE* mutation (Ochi et al., in preparation) and might affect SAM synthetase activity pleiotropically. Although this strain con-

TABLE 5. Effect of temperature on doubling time, sporulation, and SAM synthetase activity

Temp (°C)	Doubling time (min)		Heat-resistant spores per ml ^a		Viable cells per ml ^a		SAM synthetase activity (pmol min ⁻¹ mg of protein ⁻¹) ^b		GTP concn (pmol AM ₆₀₀ ⁻¹) ^c	
	Parent ^d	<i>spdC1</i> ^d	Parent	<i>spdC1</i>	Parent	<i>spdC1</i>	Parent	<i>spdC1</i>	Parent	<i>spdC1</i>
27	105	120	7.3×10^5	9.1×10^7	3.3×10^7	5.5×10^8	ND	ND	ND	ND
30	96	103	9.7×10^5	1.8×10^8	4.3×10^8	2.0×10^8	107	53	ND	ND
33	67	74	2.7×10^4	1.5×10^8	6.2×10^8	1.8×10^8	123	65	425	407
37	47	62	3.3×10^3	2.1×10^7	4.5×10^8	1.8×10^8	128	81	404	400
43	35	35	4.0×10^2	5.0×10^3	2.5×10^8	5.0×10^7	161	154	244	219

^a The titers of viable cells and heat-resistant spores were measured 16 h after each culture had reached an OD_{600} of 0.1.

^b When the OD_{600} reached 2.0, cells were harvested and extracted, and the SAM synthetase activity was assayed as described in Materials and Methods. ND, Not determined.

^c When the OD_{600} reached 0.5, the cells were rapidly collected on a membrane filter and extracted by formic acid, and the nucleotide concentrations were measured by high-performance liquid chromatography (12).

^d Both strains were inoculated from a TBAB plate into Ilv medium at an OD_{600} of 0.01, and the cultures were shaken at the indicated temperature. Parent = strain 61885; *spdC1* = strain 62252.

tained only 50% of the SAM synthetase activity observed in the parent (Table 5) at 30°C, it seems unlikely that this small difference contributes significantly to the highly increased sporulation frequency, especially because the *metE* mutant (62258) has a much lower SAM synthetase activity yet its sporulation is not much increased by the addition of SAM. However, one has to consider that the whole sporulation process involves many different stages, such as the formation of a forespore compartment which may not be accessible to extracellular SAM. This compartment may normally produce its own SAM. Therefore, if SAM is added to a culture of the *metE* strain at one particular concentration, the intracellular concentration of SAM might not have the correct value at all sporulation stages, and, even worse, SAM may not pass at a sufficient rate through the two forespore membranes.

Because addition of 1 mM spermine or spermidine, compounds whose synthesis requires putrescine and the decarboxylated SAM as precursor (19), did not affect sporulation of the *metE* or the *spdC* mutant, the effect of SAM apparently results from its only other known cellular effect, which is to serve as methyl donor for the methylation of various molecules. The decreased sporulation initiation at high SAM concentrations could be due to the overmethylation of some compound or it could be simply due to an inhibitory effect of SAM itself. The former is more likely because it took cells grown in excess (4 mM) SAM 2 h to adapt to the removal of SAM from the medium and even longer to resume sporulation. Millimolar concentrations of adenosine and methionine alone or together also decreased the extent of sporulation. This could result from their conversion into SAM or from the inhibition of some metabolic process.

SAM is known to be the methyl donor for the methylation of cytosine and adenine bases of DNA, rRNA, and tRNA, of various proteins, and of small molecules which are important only in higher organisms (13, 14, 20). We do not know which of these methylation reactions is important for sporulation. A sudden decrease in the supply of SAM does not immediately initiate sporulation in most cells but merely increases the frequency at which sporulation is spontaneously initiated in each generation. This contrasts with the much more rapid initiation of sporulation caused by GTP deprivation, which one obtains, e.g., by decoyinine addition or indirectly from a partial stringent response (2). Therefore, it appears likely that methylation and GTP deprivation control sporulation by different, but mutually dependent, mechanisms. For example, the extent of methylation of a macromolecule (e.g., a particular DNA region) could determine

the probability with which a second macromolecule (e.g., a repressor), whose tertiary structure is influenced by GTP, can bind to the first. If a critical region of the first macromolecule is methylated, a more extensive GTP decrease might be required to sufficiently reduce the affinity of the second molecule so that the cell switches from division to differentiation. A number of observations in eucaryotic organisms may guide the search for the molecule whose methylation controls the initiation of sporulation (1, 14, 16, 20). Such experiments are under way.

In experiments to be reported elsewhere (E. R. Allen, H. Wabiko, and E. Freese, manuscript in preparation), it was found that the parent strain (61885) used here already contained a genetic constitution which produces a higher ethionine resistance and a higher frequency of continual spontaneous sporulation than that observed for other strains of *B. subtilis*. This property was found to be necessary for the induction of sporulation by ethionine and for the ability of the *metE* marker to produce the highest ethionine resistance and to increase further the frequency of continual sporulation.

LITERATURE CITED

1. Ehrlich, M., and R. Y.-H. Wang. 1981. 5-Methylcytosine in eukaryotic DNA. *Science* 212:1350-1357.
2. Freese, E. 1981. Initiation of bacterial sporulation, p. 1-12. In H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), *Sporulation and germination*. American Society for Microbiology, Washington, D.C.
3. Gallant, J. A. 1979. Stringent control in *E. coli*. *Annu. Rev. Genet.* 13:393-415.
4. Greene, R. C. 1969. Kinetic studies of the mechanism of S-adenosylmethionine synthetase from yeast. *Biochemistry* 6:2255-2265.
5. Greene, R. C., J. S. V. Hunter, and E. H. Coch. 1973. Properties of *metK* mutants of *Escherichia coli* K-12. *J. Bacteriol.* 115:57-67.
6. Hafner, E. W., C. W. Tabor, and H. Tabor. 1977. Isolation of a *metK* mutant with a temperature-sensitive S-adenosylmethionine synthetase. *J. Bacteriol.* 132:832-840.
7. Hageman, J. H., and B. Carlton. 1973. Effects of mutational loss of specific intracellular protease in the sporulation of *Bacillus subtilis*. *J. Bacteriol.* 114:612-617.
8. Lopez, J. M., A. Dromerick, and E. Freese. 1981. Response of guanosine-5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. *J. Bacteriol.* 146:605-613.
9. Lopez, J. M., K. Ochi, and E. Freese. 1981. Initiation of *Bacillus subtilis* sporulation caused by the stringent response, p. 128-133. In H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), *Sporulation and germination*. American Society for Microbiology, Washington, D.C.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
11. Markham, G. D., E. W. Hafner, C. W. Tabor, and H. Tabor. 1980. S-adenosylmethionine synthetase from *Escherichia coli*. *J. Biol. Chem.* 255:9082-9092.
12. Ochi, K., J. C. Kandala, and E. Freese. 1981. Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. *J. Biol. Chem.* 256:6866-6875.
13. Osawa, S., H. Ozeki, H. Uchida, and T. Yura. 1979.

- Genetics and evolution of RNA polymerase, tRNA and ribosomes. Elsevier/North-Holland Biomedical Press, New York.
14. **Pal, K. W., and S. Kim.** 1981. Protein methylation. John Wiley & Sons, Inc., New York.
 15. **Pan, F., and H. Tarver.** 1967. Comparative studies on methionine, selenomethionine and their ethyl analogues as substrates for methionine adenosyltransferase from rat liver. *Arch. Biochem. Biophys.* 119:429-434.
 16. **Razin, A., and A. D. Riggs.** 1980. DNA methylation and gene function. *Science* 210:604-610.
 17. **Richmond, M. H.** 1969. Amino acid analogues, p. 335-343. *In* R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones (ed.), *Data for biochemical research*. Oxford University Press, New York.
 18. **Schlenk, F., C. R. Zydek, D. J. Ehninger, and J. L. Dainko.** 1965. The production of S-adenosyl-L-methionine and S-adenosyl-ethionine by yeast. *Enzymologia* 29:283-298.
 19. **Tabor, C. W., and H. Tabor.** 1976. 1,4-Diamino butane (putrescine), spermidine and spermine. *Annu. Rev. Biochem.* 45:285-306.
 20. **Udin, E., R. T. Borchardt, and C. R. Creveling.** 1979. Transmethylation. Elsevier/North-Holland Biomedical Press, New York.