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An *ethA* Mutation in *Bacillus subtilis* 168 Permits Induction of Sporulation by Ethionine and Increases DNA Modification of Bacteriophage φ105

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In contrast to *Escherichia coli* and *Salmonella typhimurium*, *Bacillus subtilis* could convert ethionine to *S*-adenosylethionine (SAE), as can *Saccharomyces cerevisiae*. This conversion was essential for growth inhibition by ethionine because *metE* mutants which were deficient in *S*-adenosylmethionine synthetase activity, were resistant to 10 mM ethionine and converted only a small amount of ethionine to SAE. Another mutation (*ethA1*) produced partial resistance to ethionine (2 mM) and enabled continual sporulation in glucose medium containing 4 mM DL-ethionine. This sporulation induction probably resulted from the effect of SAE, since it was abolished by the addition of a *metE1* mutation. The induction of sporulation was not simply controlled by the ratio of SAE to *S*-adenosylmethionine, but apparently depended on another effect of the *ethA1* mutation, which could be demonstrated by comparing the restriction of clear plaque mutants of bacteriophage φ105 grown in an *ethA1* strain with the restriction of those grown in the standard strain. The phages grown in the *ethA1* strain showed increased protection against *Bsu*R restriction. We propose that SAE induces sporulation through the inhibition of a key methylation reaction.

Vegetative cells of *Bacillus subtilis* differentiate into heat-resistant endospores when the composition of the growth medium becomes insufficient to sustain rapid growth. Numerous conditions which cause massive sporulation have been described (10, 11, 25, 29); investigations of nucleotide changes reveal that a sudden drop in the intracellular concentration of GTP and GDP occurs at the onset of sporulation induced by each of these conditions. Furthermore, the use of both specific inhibitors and mutations affecting only the guanine pathway has shown that this decrease is sufficient to induce massive sporulation (11, 24).

Ochi et al. (28) found that the addition of methionine analogs (either ethionine or selenomethionine) to cultures of *B. subtilis* also induces sporulation. In these experiments, they used a *relA* strain to avoid induction of sporulation by the stringent response.

This paper investigates the effect of ethionine, the more effective of the two analogs, in more detail. Ethionine, the *S*-ethyl analog of methionine, is incorporated into proteins in *Escherichia coli*, *B. subtilis*, and other organisms (1, 5, 6, 13, 22, 40). The eucaryotic *S*-adenosylmethionine (SAM) synthetases (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) use ethionine as a substrate in the synthesis of *S*-adenosylethionine (SAE) (6, 12, 16, 30, 33). But ethionine is not a substrate for the SAM synthetases of *E. coli* or *Salmonella typhimurium* (7, 17, 23). In vitro, SAE inhibits SAM-dependent DNA modification (4). We show here that *B. subtilis* converted ethionine to SAE and that it was SAE which was responsible for the inhibition of growth and the induction of sporulation.

The strain that was susceptible to induction of sporulation by ethionine was resistant to 2 mM DL-ethionine. This partial resistance did not result from an alteration in ethionine transport or in SAM synthetase activity (27). Therefore, we reasoned that the mutant cells (those having an *ethA1* mutation) might have altered activity of an essential modification enzyme which would ensure methylation in the presence of SAE. We show here that phage φ105c30 was indeed more resistant to *Bsu*R restriction when it was grown in cells of an *ethA1* strain than when it was grown in those of an isogenic *ethA1*$^+$ strain.

(Part of this work has been presented previously [R. Allen, H. Wabiko, and E. Freese, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I136, p. 162.].)

MATERIALS AND METHODS

Growth and sporulation. Bacteria were grown in a 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 hydrochloride, 20 mM glutamate (adjusted to pH 7.0 with NaOH), 2% (wt/vol) D-glucose, and 100 μg of L-tryptophan per ml.

For sporulation experiments, cells stored in 25% (wt/vol) glycerol at −70°C were streaked onto plates containing tryptose blood agar base (3.3% [wt/vol]; Difco Laboratories, Detroit, Mich.), incubated overnight at 37°C, and then restreaked onto tryptose blood agar base and incubated overnight at room temperature. Cells were suspended in synthetic medium, and suspensions were diluted into flasks containing 1/10 volume of synthetic medium to a final optical density at 600 nm (OD_{600}) of 0.001. Cultures were shaken (150 strokes per min) at 37°C. When the culture had reached an OD_{600} of 0.5 to 1.0, the culture was diluted to an OD_{600} of 0.001, incubated as before to an OD_{600} of approximately 1.0, diluted to an OD_{600} of 0.001, and incubated to an OD_{600} of

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TABLE 1. *B. subtilis* 168 and phage strains used

Strains	Genotype	Origin and other designations[a]	Source[b] (reference)
B. subtilis			
60001	*trpC2*		
61885	*ilvB kauA relA ethA1*		
62381	*trpC2 ethA1*	61885 → 60001 (resistance to 2 mM ethionine)	
62360	*trpC2 aldA1 metE1 relA1*		
62378	*trpC2 metE1*	62360 → 60001 (resistance to 5 mM ethionine)	
62496	*trpC2 metE1 ethA1*	62360 → 62381 (resistance to 10 mM ethionine)	
62258	*ΔilvB1 kauA1 relA1 ethA1 metE1*		
62412	*sup-3 metB5 thr-5 leuA8* (SPβ)$^{-c}$	CU1050	S. Zahler (31)
62423	*sup-3 metB5 thr-5 leuA* (SPβ)$^+$	1L4, CU1050(SPβ)$^+$	BGSC[d]
62426	*ΔilvB1 kauA1 citK1* (SPβ *c*1) (SPβ *c2 d citK1*)	CU1227	S. Zahler (31)
62396	*leuA8 metB5 hsrM*	1012	S. Ikawa (19)
62395	*leuA8 metB5 hsrM hsrR*$^+$	ISR11; (*B. subtilis* R → 1012)	S. Ikawa (19)
62410	*trpC2 metB10 xin-1* (SPβ)$^-$	YB886	R. Yasbin (37)
62523	*trpC2 met*$^+$ *xin-1* (SPβ)$^-$, *TF-1*[e]	61885 → 62410	
62524	*trpC2 met*$^+$ *xin-1* (SPβ)$^-$, *TF-4*[e]	61885 → 62410	
62525	*trpC2 met*$^+$ *xin-1* (SPβ)$^-$, *TF-5*[e]	61885 → 62410	
62526	*trpC2 met*$^+$ *xin-1* (SPβ)$^-$ *ethA1, TF-1*[e]	61885 → 62410	
62527	*trpC2 met*$^+$ *xin-1* (SPβ)$^+$	SPβ lysogen of 62523	
62528	*trpC2 met*$^+$ *xin-1* (SPβ)$^+$	SPβ lysogen of 62524	
62530	*trpC2 met*$^+$ *xin-1 ethA1* (SPβ)$^+$	SPβ lysogen of 62526	
Phage			
φ105 *c*30		1P8, clear plaque mutant of temperate phage φ105	BGSC
φ105DI:2c		1P14, clear plaque deletion mutant of phage φ105	BGSC
φdoc39		Clear plaque deletion mutant of phage ρ14	BGSC (21)
SPO2 *c*12		Clear plaque mutant of temperate phage SPO2	R. Yasbin
SPβ *c*1		Clear plaque mutant of temperate phage SPβ. Induction with mitomycin C of strain 62426 and subsequent propagation of a clear plaque	S. Zahler (31)

[a] Arrow, Strain construction by DNA transformation.
[b] Source was the authors' laboratory unless otherwise specified.
[c] (SPβ)$^-$ denotes the absence of prophage SPβ as revealed by sensitivity to phage SPβ *c*1, a clear plaque phage mutant.
[d] Bacillus Genetic Stock Center, Ohio State University, Columbus.
[e] TF-1, TF-4, and TF-5 are different isolates from a single DNA-mediated transformation.

0.5. Prolonged exponential growth was necessary to reduce the background spore titer. Samples (5 ml) were transferred to 125-ml flasks containing DL-ethionine and were shaken for 13 h. Spore titers were determined by heating portions of the cultures at 75°C for 20 min, followed by dilution in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$ and spread plating on tryptose blood agar base plates.

Bacterial strains. Bacterial strains (all derived from *B. subtilis* 168) are listed in Table 1. The origin of the ethionine resistance mutation in strain 61885 remains unknown.

MIC determination. Cells were grown in synthetic medium at 37°C to an OD_{600} of 0.5. The culture was diluted 1:1,000 in synthetic medium, and 0.1-ml samples were added to 0.9-ml portions of synthetic medium with different concentrations of DL-ethionine. The MIC was defined as that concentration of DL-ethionine which prevented 10^4 cells from growing to visible turbidity within 24 h at 37°C.

SAM and SAE measurements. Formic acid extracts of cells grown in synthetic medium were prepared by the method of Ochi et al. (28). Vacuum-evaporated extracts were dissolved in 100 μl of glass-distilled water, and 90 μl of the solution was applied to a Partisil PXS 10/25 SCX column (Whatman, Inc., Clifton, N.J.) which had been equilibrated with 0.1 M KH_2PO_4 (adjusted to pH 4.0 with H_3PO_4). The column was part of an ALTEX model 420 high-pressure liquid chromatography (HPLC) system with a Schoeffel Instruments Monochromator model GM 770 UV detector set at 254 nm and connected to a Spectra-Physics (San Jose, Calif.) SP4100 computing integrator. SAM and SAE were eluted at a flow rate of 1.5 ml/min by a gradient prepared from a low-ionic-strength buffer (0.01 M KH_2PO_4, adjusted to pH 4.0 with H_3PO_4) and a high-ionic-strength buffer (1.0 M KH_2PO_4, adjusted to pH 4.0 with H_3PO_4). The percentage of buffer with high ionic strength was 0 for the first 10 min, and then it linearly increased to 10% over 15-min, where it remained for 40 min. SAM and SAE were quantitated by comparison with standards. The amount of SAM or SAE per cell was expressed in picomoles per OD_{600}.

Bacteriophage growth and assay. Phage stocks were obtained by propagating phage originating from a single plaque.

Bacterial strains were grown in M medium with 0.1% glucose plus calcium, magnesium, and manganese (39). Cultures were infected with phage (multiplicity of infection, 0.1) at an OD_{600} of 0.25 to 0.5 (1×10^8 to 2×10^8 cells per ml). Culture lysis was complete within 3 to 4 h. Phage titers were obtained on M plates. Lysates were filtered through membrane filters (Millipore Corp.; pore diameter, 0.2 μm) and used in that state for subsequent experiments. The presence of the SPβ prophage was determined by cross-streaking cells from a single colony across a smear of SPβ *c1* (5×10^8 phage per ml) on M plates. SPβ lysogens were isolated by purifying clones from the center of a turbid plaque of phage SPβ, a lysate of which was originally obtained by mitomycin C induction of *B. subtilis* 62423 (36).

DNA-mediated transformation. DNA-mediated transformation was largely based on a previously published procedure (38). At DNA saturation, transformation occurred in 0.5 to 2% of the bacterial culture.

Reagents. *S*-Adenosyl-L-methionine (iodide salt grade I; 85 to 90% purity), *S*-adenosyl-L-ethionine (iodide salt; 85 to 90% purity), and DL-ethionine were purchased from Sigma Chemical Co., St. Louis, Mo. L-[ethyl-1-^{14}C]Ethionine (specific activity, 9.8 mCi/mmole) was purchased from ICN Pharmaceuticals Inc., Irvine, Calif. KH_2PO_4, an analytical reagent, was purchased from Mallinckrodt, Inc., St. Louis, Mo. All other chemicals were of reagent grade. Glass-distilled water was used in the preparation of solutions for HPLC. Deionized water was used for all other procedures.

RESULTS

Ethionine induction of sporulation. The effect of different concentrations of DL-ethionine on sporulation was measured in the standard strain (60001) and in several ethionine-resistant mutants 13 h after ethionine addition (Fig. 1). Only the *ethA1*-containing mutants (strains 62381 and 61885) sporulated, with an optimal concentration of 2 mM DL-ethionine. No sporulation was observed at any ethionine concentration with the standard strain (60001), a *metT* strain lacking ethionine transport (27), and a *metE1* strain (62378) that produces only 3% of the normal SAM synthetase activity (27). The *ethA1* mutant (62381) is biochemically and genetically distinct from other ethionine-resistant mutants because it has the normal ethionine transport (*metT*$^+$) and the normal SAM synthetase activity (*metE*$^+$) and because *ethA1* maps at a different location than *metE1* and *metT* (Wabiko, et al., unpublished data; Allen and Freese, unpublished data).

To determine the time dependence of the spore increase under conditions of an essentially constant cell titer, a culture of the *ethA1* strain (62381) was exposed to ethionine when the OD_{600} was 0.25 and then was twofold diluted in an ethionine-containing medium whenever the OD_{600} reached 0.5 (2×10^8 cells per ml). The spore titer increased later and much more slowly than that of a culture maintained in a similar fashion in which sporulation was induced by the addition of decoyinine (an inhibitor of GMP synthetase) (Fig. 2) (9). The increase lasted for at least 20 h after the addition of ethionine.

Evidence that SAE was responsible for the induction of sporulation. To demonstrate that *B. subtilis* could convert ethionine into SAE, exponentially (OD_{600}, 0.5) growing cells of the standard strain (60001) were exposed to 2 mM DL-ethionine for 2 h and then extracted by using formic acid. When analyzed by HPLC on a negatively charged column, the extract produced a large peak coeluting with standard SAE (Fig. 3). When the culture contained L-[^{14}C]ethionine,

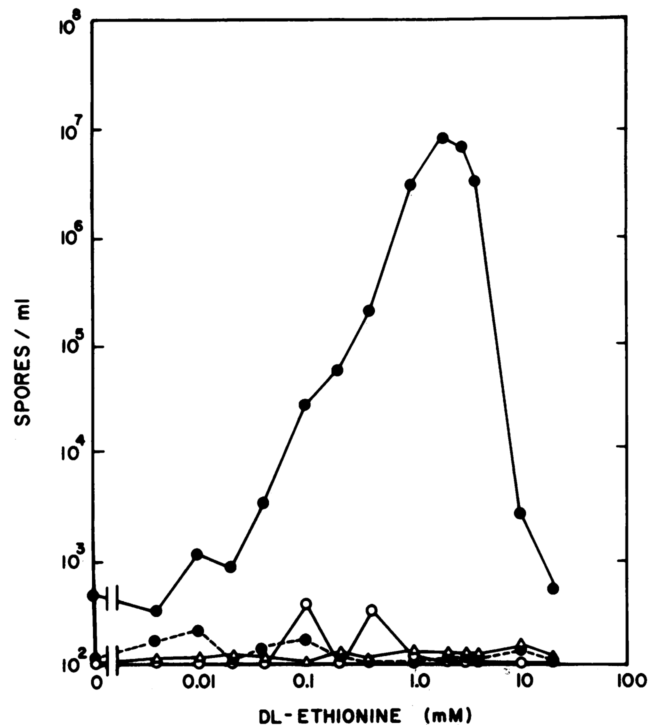

FIG. 1. Induction of sporulation by ethionine in strains 60001 (○——○), 62381 (●——●), 62378 (△——△), and 62496 (●-----●). DL-Ethionine was added to cells at an OD_{600} of 0.5. Samples were collected 13 h later for determination of viable and spore titers.

the extract contained some ethionine, which eluted with in 7 min, and material with the retention time (43 min) of SAE, which eluted later as a large radioactive peak (Fig. 3). Three additional small peaks of radiolabeled material, the largest of which, eluting after SAE, did not show any UV absorption at 254 nm, remained unidentified. Under these conditions, only a small amount of SAM (5% of the amount in control cells without ethionine; Table 2) was produced by the standard strain; this indicated that ethionine (or SAE) inhibited the pathway to methionine or competed with methionine for SAM synthetase.

The *metE1* mutant (62378), which can grow in the presence of 5 mM ethionine and which has only 1.2% of the normal SAM synthetase activity (27), still produced 42% of the amount of SAM found in the standard strain; apparently SAM or methionine synthesis is normally feedback controlled by SAM (3). In the presence of ethionine, this mutant produced only 6% of the amount of SAE made by the standard strain (Table 2). This demonstrated that SAE was made by SAM synthetase.

In the presence of ethionine, the *ethA1* mutant (62381) produced much more SAM than did the standard strain (Table 2), which showed that the *ethA1* mutation interfered with the inhibition of SAM synthesis caused by ethionine (or by SAE). Since the *ethA1* mutation does not affect SAM synthetase activity (27), the mutation apparently caused a decreased feedback control of methionine synthesis by SAM (and by SAE). To examine whether SAE was responsible for the sporulation caused by ethionine, we transferred the *metE1* mutation into an *ethA1* background. In the resultant *ethA1 metE1* double mutant (strain 62496), the addition of ethionine at any concentration no longer caused sporulation (Fig. 1), although the strain sporulated normally in nutrient

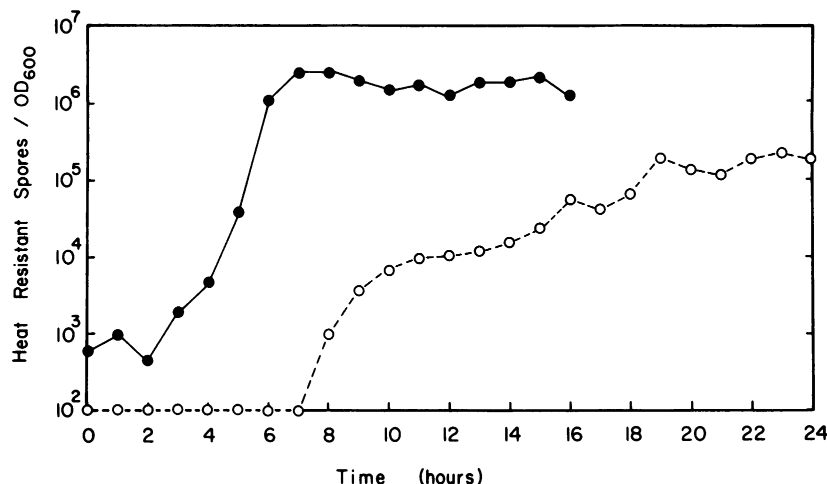

FIG. 2. Effect on sporulation of decoyinine or ethionine added to cells maintained at constant turbidity. Cells were grown in synthetic medium (1 mM isoleucine and 2 mM valine were added to strain 61885; 100 μg of tryptophan per ml was added to strain 62381). When the OD_{600} reached 0.5, decoyinine (400 μg/ml) was added to the culture of strain 61885 (*ilvB1 kauA relA1 ethA1*) (●), or DL-ethionine (4 mM) was added to the culture of strain 62381 (*ethA1 trpC2*) (○). The cultures were maintained at an OD_{600} between 0.25 and 0.5 by periodic dilution with the same supplemented media.

sporulation medium or after decoyinine addition (data not shown). Similarly to the *metE1* mutant, the double mutant was deficient in SAM synthetase activity and produced little SAE (Table 2). Therefore, sporulation caused by ethionine resulted from the effect of SAE rather than from ethionine itself.

SAE might have caused sporulation by competing with SAM because the ratio of SAE to SAM was 1.7 in the *ethA1* mutant but was only 0.25 in the *ethA1 metE1* double mutant. To examine this possibility, we attempted to induce sporulation in the standard strain (60001) by 2 mM ethionine in the presence of different concentrations of L- or D-methionine. D-Methionine was used because it could be argued that active transport of L-methionine would not allow control of the intracellular concentration of methionine. Previous experiments have shown that D-methionine can be used for this purpose because it is taken up and converted by a racemase to L-methionine so that the growth rate of a methionine auxotroph depends linearly on the D-methionine concentration in the medium (28). Sporulation was not induced at any concentration of L- or D-methionine (data not shown).

Increased DNA modification in the *ethA1* strain. Because the sporulation effect of ethionine (i.e., of SAE) was observed only in strains containing the *ethA1* mutation and did not seem to depend only on the ratio of SAE to SAM, it appeared likely that the *ethA1* mutation altered one of the many methylation reactions that might be inhibited by SAE. Because the methylation of DNA can be quantitated by determining the restriction of phage, we measured the effect of the *ethA1* mutation on the restriction of phage ϕ105.

A clear plaque mutant of phage ϕ105 (ϕ105 *c30*) was propagated on both the standard strain (60001) and its isogenic *ethA1* derivative (62381). The two lysates were assayed for host-specific restriction by determining titers on a set of isogenic strains carrying individually the *Bsu*B, *Bsu*C, *Bsu*E, *Bsu*F, or *Bsu*R restriction-modification systems (15, 18, 19). The titers were compared to those obtained on the isogenic strain devoid of the corresponding restric-

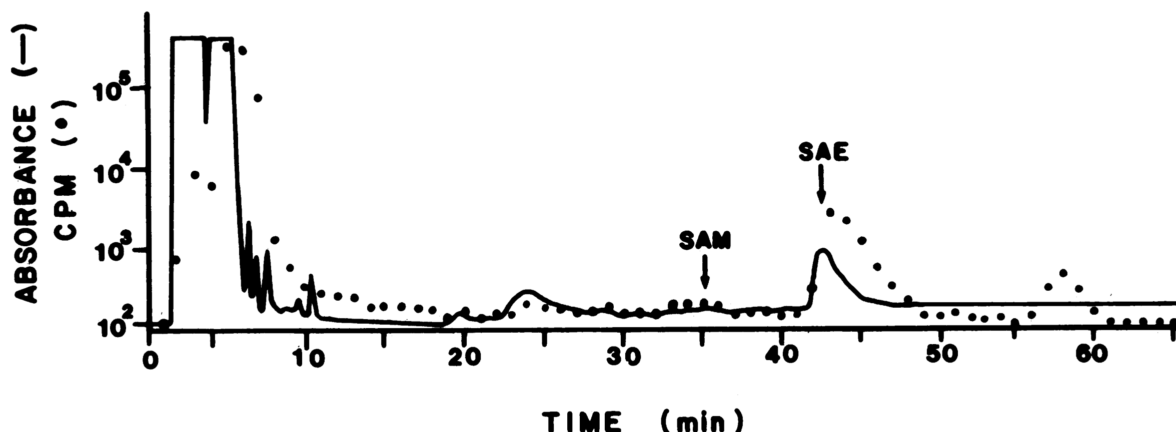

FIG. 3. Conversion of L-[^{14}C]ethionine to SAE by *B. subtilis*. Cells of *B. subtilis* 168 *trpC2* (60001) were grown at 37°C in synthetic medium to an OD_{600} of 0.5. A total of 25 ml was transferred to a 300-ml flask containing 10 μCi L-[^{14}C]ethionine and sufficient DL-ethionine to give a final concentration of 2 mM. The culture was further incubated for 2 h; next, the cells were collected on a membrane filter and were extracted with formic acid. After lyophilization, the formic acid extract was analyzed by HPLC. Eluate fractions were collected into scintillation vials at 1-min intervals with an LKB 2111 Multirac fraction collector. ——, Absorbance; ·····, counts per minute.

TABLE 2. Intracellular concentrations of SAM and SAE in *B. subtilis*

Strain no.	Relevant genotype		Concn (pmol/OD_{600}) for treatment[a]		
			No ethionine (SAM)	2 mM DL-ethionine	
	ethA1	*metE1*		SAM	SAE
60001	+	+	44.9	2.3	176.0
62381	−	+	44.4	27.8	48.5
62378	+	−	18.7	16.1	10.0
62496	−	−	22.6	27.0	6.7

[a] Cells were grown at 37°C in synthetic medium to an OD_{600} of 0.5. The culture was then split into two parts, with one receiving DL-ethionine followed by further incubation for 2 h. Cells (25-ml samples) were collected on membrane filters (diameter, 10 cm; pore size, 0.45 μm; Schleicher & Schuell, Inc., Keene, N.H.) and extracted with 1.5 ml of 0.5 M ice-cold formic acid. SAM and SAE concentrations were measured by HPLC. Measurements shown above are averages from two independent experiments.

TABLE 4. Comparison of *Bsu*R restriction on phages φ105DI:2c, φdoc39, and SPO2*c*12 previously propagated on *B. subtilis* 168 *ethA1*

Phage and propagation strain	*ethA1* genotype	PFU/ml on strain: 62396 (*hsrR*)	PFU/ml on strain: 62395 (*hsrR*$^+$)	Restriction ratio[a]	Protection ratio[b]
φ105DI:2c					
60001	+	8.9×10^9	6.6×10^5	7.4×10^{-5}	19
62381	−	1.5×10^{10}	2.1×10^7	1.4×10^{-3}	
φdoc39					
60001	+	7.6×10^9	1.9×10^5	2.5×10^{-5}	48
62381	−	1.1×10^{10}	1.3×10^7	1.2×10^{-3}	
SPO2 *c*12					
60001	+	7.3×10^{10}	2.9×10^6	4.0×10^{-5}	2.0
62381	−	5.9×10^{10}	4.8×10^6	8.1×10^{-5}	

[a] For definition, see Table 3, footnote *a*.
[b] For definition, see Table 3, footnote *b*.

tion-modification activity. The ratio of these numbers (the restriction ratio) is an indicator of the levels of specific DNA modification (methylation) of the phage genome by the two hosts in which the phages were grown. Both phage lysates were equally restricted by all of the indicator strains (data not shown) except the *Bsu*R strain. Therefore, all of the following work concerned only restriction by the *Bsu*R restriction system. Phage progeny from the *ethA1* host was less restricted by the *Bsu*R restriction system than phage from the standard host (Table 3). This increased protection against *Bsu*R restriction was also obtained with other hosts carrying the *ethA1* mutation. As expected, when the phage was multiplied in a strain containing the *Bsu*R restriction-modification system, full resistance was observed (Table 3). The effect of the *ethA1* mutation was not limited to this particular phage, since the same phenomenon was observed with another clear plaque mutant of φ105 (φ105DI:2C) and a clear plaque mutant of phage ρ14 (φdoc39) (21), both carrying deletions that reached into the phage immunity region (Table 4). Phage ρ14 has the same immunity, host range, and serological properties as does φ105 (21). The effect was not observed with SPO2 *c*12 (Table 4), a phage similar in genome size and base composition to φ105 but susceptible to M-*Bsu*R modification (32, 34).

The difference in phage modification did not result from differences in any growth property of phage φdoc39 between the two hosts, since one-step growth curves on the *ethA1* strain (62381) and the standard strain (60001) revealed the same degree of phage absorption (more than 98% within 10 min) and similar latent periods (about 25 min), times to completion of lysis (70 min), and burst sizes (305 and 185, respectively). The phage was 55 times less susceptible to *Bsu*R restriction after one cycle of growth in the *ethA1* than after one cycle of growth in the *ethA1*$^+$ strain (data not shown).

The enhanced protection against *Bsu*R restriction was lost after subsequent phage multiplication in the standard strain (60001), consistent with a host-controlled restriction-modification phenomenon (Table 5). No evidence of increased restriction activity in the *ethA1* mutant was evident when the titers of phage grown on the standard strain were determined for this host and the *ethA1* mutant (data not shown).

Most *B. subtilis* 168 strains, including standard strain 60001 (unpublished observations), are lysogenic for phage SPβ (36). This phage codes for a *Bsu*R-specific DNA methyltransferase which is normally expressed upon induction of the prophage (35). Conceivably, the *ethA1* mutation could increase the spontaneous induction of SPβ and thereby increase methylation. To determine whether the increase in DNA modification activity caused by the *ethA1* mutation corresponded to the SPβ-coded enzyme, the *ethA1* mutation was moved, by DNA-mediated transformation at saturating DNA concentrations, from strain 61885 *ilvB kauA relA*

TABLE 3. Protection against *Bsu*R restriction of phage φ105c30 propagated on strains carrying the *ethA1* mutation

Propagation type and strain	Genotype	PFU/ml on strain: 62396 (*hsrR*)	PFU/ml on strain: 62395 (*hsrR*$^+$)	Restriction ratio[a]	Protection ratio[b]
Liquid culture propagation					
60001	*trpC2*	2.7×10^9	9.6×10^4	3.6×10^{-5}	
62381	*trpC2 ethA1*	5.7×10^9	8.6×10^6	1.5×10^{-3}	42
Agar plate propagation					
60001	*trpC2*	2.6×10^9	3.5×10^4	1.3×10^{-5}	
61885	*ilvB1 kauA1 relA1 ethA1*	2.8×10^8	7.3×10^4	2.6×10^{-4}	20
62258	*ilvB1 kauA1 relA1 ethA1 metE1*	3.5×10^8	7.2×10^4	2.1×10^{-4}	16
62395	*leuA8 metB5 hsrM hsrR*$^+$ *hsmR*$^+$	4.9×10^7	6.4×10^7	1.3	

[a] (PFU/ml on strain 62395)/(PFU/ml on strain 62396).
[b] (Restriction ratio for phage propogated on indicated strain)/(restriction ratio for phage similarly propagated on strain 60001).

TABLE 5. Loss of protection against *Bsu*R restriction after propagation of φ105c30 on an *ethA1*$^+$ host

Host bacterial strains for initial and subsequent phage growth	PFU/ml on strain:		Restriction ratio[a]	Protection ratio[b]
	62396 (*hsrR*)	62395 (*hsrR*$^+$)		
60001 (*ethA1*$^+$), 60001 (*ethA1*$^+$)	6.7×10^9	5.3×10^5	7.9×10^{-5}	
60001 (*ethA1*$^+$), 62381 (*ethA1*)	1.5×10^{10}	4.7×10^7	3.1×10^{-3}	39
62381 (*ethA1*), 62381 (*ethA1*)	1.7×10^{10}	3.5×10^7	2.0×10^{-3}	25
62381 (*ethA1*), 60001 (*ethA1*$^+$)	6.7×10^9	1.3×10^6	1.9×10^{-4}	2.4

[a] For definition, see Table 3, footnote *a*.
[b] (Restriction ratio for strain)/(restriction ratio for strain 60001 [*ethA1*$^+$]).

ethA1 into strain 62410 *trpC2 metB10 xin-1* SPβ$^-$. Met$^+$ transformants were tested for tryptophan auxotrophy and for resistance to 2 mM ethionine. A total of 7.5% of Met$^+$ transformants were simultaneously tryptophan independent, and the frequency of Met$^+$ ethionine-resistant transformants was 2.5%. The similarity in congression levels in the two types of transformants suggested that the *ethA1* phenotype resulted from a single mutation. A control experiment with DNA from strain 60001 (*eth*$^+$) showed that, out of 703 Met$^+$ transformants, none were ethionine resistant. Therefore, an upper limit for Met$^+$ transformants carrying a spontaneous mutation to ethionine resistance would be 0.13%, at least 20-fold less than the observed congression frequency of the Met$^+$ and ethionine resistance phenotypes. Examination of the ethionine-resistant transformants revealed that they remained SPβ$^-$ and could be induced to sporulate by ethionine (data not shown).

Phage φdoc39 propagated on a Met$^+$ *ethA1* SPβ$^-$ transformant displayed enhanced protection against *Bsu*R restriction, compared with lysates obtained on three independent Met$^+$ SPβ$^-$ transformants not carrying the *ethA1* mutation (Table 6). When the same strains were lysogenized with phage SPβ, they showed the same degree of protection against *Bsu*R restriction. This indicated that the modification activity modulated by the *ethA1* mutation was not coded for by phage SPβ DNA and that this activity did not require the presence of this phage for its expression. Apparently, only the *ethA1* marker was required to obtain sporulation by ethionine.

DISCUSSION

In the presence of ethionine, *B. subtilis* cells produced a new compound which comigrated (in HPLC) with SAE. Its identity as SAE was confirmed by labeling with [^{14}C]ethionine (see Fig. 3). To our knowledge, this is the first reported evidence of SAE synthesis by bacteria. It has been reported that *E. coli* SAM synthetase cannot use ethionine as a substrate to produce SAE (17, 23), whereas the SAM synthetases from *Saccharomyces cerevisiae* do synthesize SAE (6, 33).

The inhibition of growth and the induction of sporulation, both caused by ethionine, are actually due to the production of SAE, because they are not observed in *metE1* mutants (isolated by their resistance to 10 mM DL-ethionine), which have only 3% of the normal SAM synthetase activity (27) and which have greatly reduced production of SAE. SAM controls methionine synthesis by feedback, as follows from the observation that a SAM synthetase-deficient (*metE1*) mutant produced much more SAM (42% above normal) than was expected from the low SAM synthetase activity (1.2% of normal) of this strain. Apparently SAE exerted the same feedback control because the presence of ethionine greatly reduced SAM production in the standard strain, whereas it had almost no effect in the *metE1* mutant. This feedback effect was reduced in the *ethA1* mutant which, in the presence of ethionine, produced much more SAM than did its parent strain.

The induction of sporulation by ethionine was observed only in *ethA1* mutants, in which the inhibitory effect of SAE was reduced, as was shown by a decrease in the ratio of SAE to SAM in the *ethA1* mutant compared with the ratio in the standard strain. One could propose that sporulation induction requires only that this ratio be correct. However, this is not the case, since obtaining a lower ratio simply by adding less ethionine to the standard strain did not enable sporulation. Also, if solely the SAE-to-SAM ratio were important, increasing the SAM concentration by adding the proper amount of L-methionine or (if active transport would be a problem) of D-methionine would be expected to induce sporulation. D-Methionine is taken up and converted to L-methionine sufficiently slowly (by a racemase) so that its intracellular concentration is proportional to the extracellular concentration (28). Nevertheless, 2 mM DL-ethionine did not induce sporulation in the standard strain, no matter how much L- or D-methionine was present. Therefore, the *ethA1* mutation must have had some other cellular effect which was necessary for sporulation induction by ethionine.

Lytic derivatives of phage φ105, grown on a strain carrying the *ethA1* mutation, were less restricted when assayed on a strain carrying the *Bsu*R restriction-modification system than were phages propagated on an isogenic *ethA1*$^+$ strain (Table 3). This enhanced protection against *Bsu*R restriction was lost after subsequent phage multiplication in an *ethA1*$^+$ strain (Table 4). Therefore, the *ethA1* mutation produced an enzyme activity which modified the DNA recognition site for *Bsu*R restriction. Methylation of the internal cytosine of the sequence GGCC suffices to protect DNA from *Bsu*R restriction (14). Phage SPO2 is also subject to *Bsu*R restriction (2).

TABLE 6. Protection against *Bsu*R restriction of phage φdoc39 propagated on an *ethA1* (SPβ$^-$) host

Transformant	Relevant genotype		Restriction ratio[a]	Avg restriction ratio[b]	Protection ratio[c]
	ethA1	SPβ			
62523	+	−	1.6×10^{-5}		
62524	+	−	6.2×10^{-6}	1.1×10^{-5}	
62525	+	−	1.1×10^{-5}		
62526	−	−	1.2×10^{-4}		11
62527	+	+	7.0×10^{-6}		
62528	+	+	2.2×10^{-5}	1.8×10^{-5}	
62529	+	+	1.7×10^{-5}		
62530	−	+	2.1×10^{-4}		12

[a] For definition, see Table 3, footnote *a*.
[b] Average of the three transformants with same genotype.
[c] Protection ratio = (restriction ratio for phage propogated on *ethA1*$^-$ strain)/(average restriction ratio for phage propogated on *ethA1*$^+$ strains with same SPβ status).

However, this virus showed no enhanced protection against *Bsu*R restriction when grown in an *ethA1* strain (Table 3). This suggested that the enzyme activity affected by the *ethA1* mutation modified GGCC sequences as part of a larger recognition sequence that was present in phage φ105 but not in SPO2.

The *ethA1* mutation had several pleiotropic effects. It reduced the feedback control of methionine synthesis by SAM and SAE, caused a relaxed response of RNA synthesis upon amino acid starvation (data not shown), and increased (or enabled) a particular DNA modification (methylation) activity. These effects would be simply explained if the particular DNA modification activity (presumably DNA methylase) affected by the *ethA1* mutation altered certain operator sites, including one controlling methionine synthesis. Such effects have been reported for *E. coli*, in which enhanced repressor binding to a mutant (5-mC) operator (8), decreased activity of the *trpR* promoter after methylation of adenine in the GATC sequence of the −35 region (26), and increased gene transcription due to absence of methylation of a regulatory region (20) have been observed.

Ethionine (i.e., SAE) presumably induced sporulation by competing with SAM for some other methylase, either inhibiting it or (as the substrate) ethylating some molecule (but enzymatic ethylation of DNA has never been observed). In contrast to compounds producing a rapid decrease in GTP, ethionine did not cause massive sporulation of most cells within a short time span but merely increases the frequency with which sporulation occurs in each cell cycle. To accommodate these two modes of sporulation induction, a model has been proposed in which the attachment of a GTP-regulated (or GTP-binding) protein to a particular DNA region represses sporulation (9). SAE might cause a change in the normal pattern of methylation of this regulatory region, thereby decreasing the affinity of the regulatory protein, and could thus increase the probability that the cell will enter sporulation rather than cell division.

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