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Suppression of Temperature-Sensitive Sporulation of a *Bacillus subtilis* Elongation Factor G Mutant by RNA Polymerase Mutations

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A class of rifampin-resistant (*rfm*) mutations of *Bacillus subtilis* suppresses the temperature-sensitive sporulation of a fusidic acid-resistant mutant, FUS426, which has an altered elongation factor G. The *rfm* mutation suppressed only the sporulation defect caused by the elongation factor G mutation, but could not suppress other types of induced sporulation defects. Genetic and biochemical analyses showed that the sporulation suppression by the *rfm* mutation was caused by a single mutation in RNA polymerase. After the early sporulation phase, the apparent rate of RNA synthesis of FUS426, measured by [^{3}H]uracil or [^{3}H]uridine incorporation into RNA, became lower than that of the wild-type strain, and this decrease was reversed by the *rfm* mutation. However, when the total rate of RNA synthesis of FUS426 was calculated by measuring the specific activity of [^{3}H]UTP and [^{3}H]CTP, it was higher than that of the *rfm* mutant, RIF122FUS426. The possible mechanism of the functional interaction between elongation factor G and RNA polymerase during sporulation is discussed.

Sporulating cells and spores of *Bacillus subtilis* are biochemically and morphologically different from vegetative cells. These differences are controlled, at least in part, at the transcriptional level, because sporulation-specific mRNA's are detected in the sporulating cells (4, 30). In addition, structural and functional alterations of the initiation factors (3) and ribosomes (5-9, 18, 24, 33) have been observed, and the importance of translational control during sporulation has been suggested.

Recently, a functional relation between the translational apparatus and the transcriptional system has been reported. For example, Kaplan et al. (13) showed that the ribosomes have an important role in the stringent control of RNA synthesis. Pongs and Ulbrich (22) reported that *Escherichia coli* fMet-tRNA$_f^{Met}$ and RNA polymerase formed a 1:1 complex and that the binding of the initiator tRNA stimulated the transcription of λp*lac* DNA. Chakrabarti and Gorini (2) found that a class of temperature-insensitive *rfm* mutations became temperature sensitive in the presence of a specific temperature-insensitive *rpsL* mutation, which suggested an interaction between ribosomes and RNA polymerase. Elongation factor G (EF-G) mutants with an altered stable RNA synthesis character were also isolated from *B. subtilis* (15) and *E. coli* (34).

These facts suggest a possibility that the translational apparatus may regulate the sporulation at the transcriptional level by interacting functionally with RNA polymerase. Kobayashi et al. (16) isolated a fusidic acid-resistant, temperature-sensitive sporulation mutant of *B. subtilis* (FUS426), whose sporulation is blocked at an early stage at the nonpermissive temperature, and showed that the fusidic acid resistance and the temperature-sensitive sporulation were caused by a single mutation in EF-G. To examine the possibility that EF-G may regulate sporulation at the transcriptional level, we have isolated rifampin-resistant mutants from strain FUS426 and found that the temperature-sensitive sporulation character of strain FUS426 was suppressed by a class of *rfm* mutations. Analysis of these mutants suggests that the sporulation is controlled through the functional interaction between EF-G and RNA polymerase.

(Preliminary results of this work were presented at the Seventh International Spore Conference, Madison, Wis., October 1977 [17].)

MATERIALS AND METHODS

Bacterial strains. The characters of the strains used in the present paper are described in Table 1.

Isolation of mutants. The rifampin-resistant mutants were isolated spontaneously from FUS426. Cells were transferred from a single colony to a test tube

TABLE 1. *List of B. subtilis strains*[a]

Strain	Genetic marker	Sporulation phenotype	Source, derivation (reference)
168	*thy trp*	Wild type	Institute of Applied Microbiology, University of Tokyo
FUS426	*thy trp fus426*	Temperature sensitive	Spontaneous from 168 (16)
FUS429	*thy trp fus429*	Conditional	Spontaneous from 168 (16)
RIF110FUS426	*thy trp fus426 rfm110*	Wild type	Spontaneous from FUS426
RIF117FUS426	*thy trp fus426 rfm117*	Wild type	Spontaneous from FUS426
RIF122FUS426	*thy trp fus426 rfm122*	Wild type	Spontaneous from FUS426
RIF122FUS429	*thy trp fus429 rfm122*	Wild type	Transformation of *rfm122* from RIF122 to FUS429
Spo080	*thy trp spo0*	Asporogenous	Spontaneous from 168 (21)
RIF117	*thy trp rfm117*	Wild type	Transformation of *rfm117* from RIF117FUS426 to 168
RIF122	*thy trp rfm122*	Wild type	Transformation of *rfm122* from RIF122FUS426 to 168
RIF122Spo080	*thy trp rfm122 spo0*	Asporogenous	Transduction of *rfm122* from RIF122 to Spo080
CEL[b]	*cusA ery leu*	Wild type	Institute of Applied Microbiology, University of Tokyo

[a] *fus*, Fusidic acid resistance (10 μg/ml); *rfm*, rifampin resistance (5 μg/ml); *spo0*, sporulation is blocked at stage 0.

[b] Used as a recipient in the transformation experiments.

containing 5 ml of modified NB medium (16) and grown at 37°C for 6 h. The culture was centrifuged, and the cells (about 5×10^8 cells) were plated on a Schaeffer sporulation plate (27) containing 5 μg of rifampin per ml and incubated at 45°C for 4 days; then the sporulation ability of the *rfm* mutants appearing was determined by the color of the colonies (the asporogenous colonies were translucent, whereas the colonies of spore-forming strains were brown). As a rule, only one brown colony was picked up from one plate.

Growth and sporulation. Cells were grown in Schaeffer sporulation medium supplemented with 10 μg of thymine per ml. To obtain synchronous sporulation, cells were transferred two times to fresh medium at an early stationary phase. Heat-resistant spores were counted by plating the cells after heating the cell suspensions for 10 min at 80°C.

Genetic analysis. Transforming DNA was prepared by the phenol extraction method (26). Transformation was carried out by the method of Wilson and Bott (35). *B. subtilis* Marburg strain CEL (*cysA ery leu*) was used as a recipient. Cys^+ transformants were selected on minimal medium plates supplemented with leucine (50 μg/ml), and unselected markers and sporulation ability were determined on Schaeffer sporulation plates containing rifampin (5 μg/ml) and/or fusidic acid (10 μg/ml). Fus^r transformants were selected on Schaeffer sporulation plates containing fusidic acid, and unselected markers were determined on appropriate plates.

Preparation and analysis of RNA polymerase. RNA polymerase was prepared by the rapid micromethod of Gross et al. (10) with the following modifications. A 150-ml culture of late-log-phase cells was cooled on ice and centrifuged, and the cells were washed once with washing buffer (19) containing 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 1 M KCl, 0.01 M ethylenediaminetetraacetic acid, and 300 μg of phenylmethylsulfonylfluoride per ml. Cells were treated as described by Gross et al. (10), except that lysozyme treatment was done at 37°C for 10 min and freeze-thawing was repeated three times to lyse cells completely. To prevent proteolytic action during the isolation procedure, 300 μg of phenylmethylsulfonylfluoride per ml was added to all solutions.

The reaction mixture for the assay of RNA polymerase activity contained, in 0.25 ml: 40 mM Tris-hydrochloride (pH 7.9); 2.5 mM $MnCl_2$; 0.4 mM KH_2PO_4; 4 mM β-mercaptoethanol; 150 mM KCl; 125 μg of bovine serum albumin; 0.2 mM ATP; 0.05 mM UTP; 0.6 μCi of [^{3}H]UTP (25.2 Ci/mmol); 6 μg of polydeoxyadenylic acid-polydeoxythymidylic acid; and 15 to 20 μg of the enzyme (step iv extracts [10]). Reaction mixtures were incubated at 37°C for 10 min. The reaction was stopped by the addition of 4 ml of cold 5% trichloroacetic acid. The precipitates were collected on GF/C Whatman glass fiber filters, washed with 5% trichloroacetic acid, and dried. The radioactivity was counted in a liquid scintillation counter. Step iv extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in Tris-glycine buffer (29).

RNA pulse-labeling. Samples of cells (1 ml) were exposed to [^{3}H]uracil or [^{3}H]uridine (1 μCi/ml) plus 2 μg of cold uracil or uridine for 2 min at 42°C. The labeling was stopped by the addition of 2 ml of cold 10% trichloroacetic acid containing 200 μg of uracil or uridine per ml. Zero time backgrounds were subtracted.

Determination of the total rate of RNA synthesis. Cells were precultured in the sporulation medium to early stationary phase and harvested by centrifu-

gation. The cells were suspended (final concentration, 10 Klett units) in 4 ml of the fresh sporulation medium containing [^{32}P]phosphate (30 μCi/ml; 17 μCi/μmol) and incubated at 42°C. At $t_{1.5}$ (1.5 h after the end of exponential growth), 1-ml samples of cells were exposed to [^{3}H]uridine (50 μCi/ml; 47.74 Ci/mmol). At 0.5, 1, 1.5, and 2 min after the addition of [^{3}H]uridine, 100-μl samples were taken and extracted with 50 μl of 4 M formic acid. Nucleotides in the supernatant were separated by thin-layer chromatography (36). The incorporation of [^{3}H]uridine into RNA was determined by precipitating 100-μl samples with 10% trichloroacetic acid at indicated times. The rate of RNA synthesis (UMP incorporation into RNA) was calculated according to Segall et al. (28).

Preparation of unlabeled RNA. Unlabeled rRNA was extracted by the phenol method from the ribosomes of late-logarithmic-growth-phase cells that had been treated for 30 min with rifampin (20 μg/ml) before harvesting.

Total unlabeled RNA was prepared as follows. Nucleic acids were prepared according to the method of Summers (31), dialyzed against 0.01 M Tris-hydrochloride (pH 7.5) and 1 mM $MgCl_2$, and then treated with 20 μg of ribonuclease-free deoxyribonuclease per ml at 37°C for 10 min. RNA was extracted with water-saturated phenol three times, precipitated with ethanol two times, and resuspended in 2× SSC (pH 7.4) (1× SSC = 0.15 M NaCl–0.015 M trisodium citrate).

Hybridization-competition experiments. (i) rRNA assay. Samples of cells (1 ml) were labeled for 2 min with [^{3}H]uridine (100 μCi/ml; 47.74 Ci/mmol), and [^{3}H]RNA was prepared by the method of Summers (31). Then [^{3}H]RNA (0.5 μg per assay) was hybridized with denatured *B. subtilis* DNA (100 μg per assay; trapped on nitrocellulose filters) in the presence or absence of excess amounts (75 μg per assay) of unlabeled rRNA for 20 h at 66°C in 0.75 ml of 6× SSC containing 0.05% sodium dodecyl sulfate, and the relative amount of rRNA in the total RNA was estimated according to the method of Kimura (14).

(ii) mRNA assay. Samples (3 ml) of FUS426 and RIF122FUS426 cells were labeled for 2 min with 80 and 40 μCi of [^{3}H]uridine (47.74 Ci/mmol) per ml, respectively. [^{3}H]RNA was prepared by the method of Summers (31), and mRNA was assayed according to the following procedure. Nitrocellulose filters trapping 10 μg of *B. subtilis* DNA prepared by the method of Saito and Miura (26) were incubated at 66°C for 20 h in vials containing [^{3}H]RNA (5 μg), 6× SSC, an appropriate amount of unlabeled RNA, and 0.1% sodium dodecyl sulfate in a total volume of 1 ml. The filters were washed with 2× SSC and incubated at 37°C for 10 min in 2× SSC containing 20 μg of heat-treated pancreatic ribonuclease A per ml. The filters were again washed with 100 ml of 2× SSC and dried, and the radioactivity was counted in a scintillation counter.

RNA synthesis in toluenized cells. A 15-ml culture of $t_{1.5}$ cells was cooled on ice and centrifuged, and the cells were washed once with washing buffer (19) and once with 0.05 M Tris-hydrochloride (pH 7.5) containing 300 μg of phenylmethylsulfonylfluoride per ml. Cells were suspended in 1 ml of 0.05 M Tris-hydrochloride (pH 7.9) containing 1% toluene and 300 μg of phenylmethylsulfonylfluoride per ml, shaken vigorously for 30 s, and incubated at 0°C for 20 min before assaying. The assay of RNA synthesis was performed as described by Rothstein et al. (25).

Chemicals and enzymes. [5,6-^{3}H]uracil (40 Ci/mmol), [5,6-^{3}H]uridine (47.74 Ci/mmol), and [5-^{3}H]UTP (25.2 Ci/mmol) were purchased from New England Nuclear Corp. [^{32}P]phosphate (200 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. ATP, GTP, CTP, and UTP were gifts from Yamasa Soy-sauce Co. Polydeoxyadenylic acid-polydeoxythymidylic acid was obtained from Boehringer/Mannheim GmbH. Deoxyribonuclease A was from Sigma Chemical Co. Rifampin and fusidic acid were gifts from Daiichi Seiyaku and Sankyo Co., respectively.

RESULTS

Isolation of rifampin-resistant mutants from FUS426. The fusidic acid-resistant, temperature-sensitive sporulation mutant (FUS426) is unable to sporulate at 45°C and produces translucent colonies on Schaeffer sporulation plates. A wild-type strain (*B. subtilis* 168 *thy trp*) sporulates at this temperature and produces brown colonies. Therefore, the sporulation ability of the mutants was easily determined by the color of the colonies. Mutants resistant to rifampin (5 μg/ml) were isolated spontaneously from FUS426 at a mutation frequency of 2.8×10^{-8}. These mutants can be divided into two classes according to their sporulation character. In class I mutants the temperature-sensitive sporulation character of FUS426 was suppressed. They produced brown colonies on the sporulation plates at 45°C. In class II mutants the temperature-sensitive sporulation character of FUS426 was not suppressed, and therefore translucent colonies were produced at 45°C. In the following experiments, the sporulation suppression mechanism of the class I mutants was studied using mostly one typical class I mutant RIF122-FUS426.

Suppression of the temperature-sensitive sporulation by an *rfm* mutation. The effect of the class I mutation on the temperature-sensitive sporulation of FUS426 was determined at various temperatures. The strains RIF122 and RIF117 were obtained by transforming *rfm* mutations of RIF122FUS426 and RIF117FUS426 (class I mutants) to the wild-type strain, respectively. RIF122 and RIF117, as well as the wild-type strain, sporulated at higher temperatures (Fig. 1). On the other hand, FUS426 clearly showed temperature-sensitive sporulation. In RIF122FUS426 and RIF117FUS426, temperature-sensitive sporulation was suppressed. At 30°C, however, the sporulation ability of

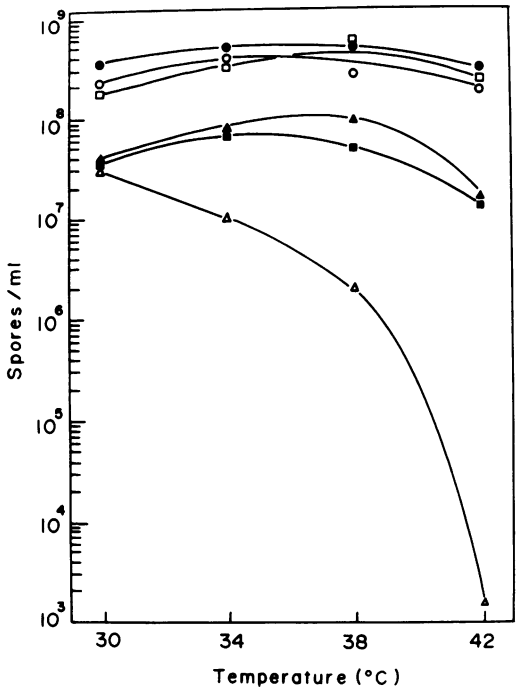

FIG. 1. *Sporulation of the fusidic acid- and/or rifampin-resistant mutants and the wild-type strain at various temperatures. Cells were grown in the sporulation medium at temperatures ranging from 30 to 42°C. At t_{24} the heat-resistant spores were counted. The viable cell number of six strains at t_2 was nearly the same at 42°C (3 × 10^8 to 5 × 10^8 cells per ml). Symbols for strains: (△) FUS426; (■) RIF117-FUS426; (▲) RIF122FUS426; (□) RIF117; (●) RIF122; (○) 168.*

FUS426 was about one-tenth of that of the wild-type strain. This may be due to the slow growth rate of FUS426 (16, 17) caused by the *fus* mutation. The *rfm* mutation cannot suppress this defect of FUS426. These results indicate that EF-G mutation in FUS426 causes two types of alterations, one of which, temperature-sensitive sporulation, is suppressible by the *rfm* mutation, but the other, slow growth rate and reduced sporulation at 30°C, is not.

Specificity of the effect of the *rfm* mutation on sporulation. To know whether the suppressive character of the *rfm* mutation is effective only in the temperature-sensitive sporulation of the FUS426 strain, the effect of *rfm* mutation on other types of sporulation defects was studied. One of the fusidic acid-resistant mutants, FUS429, sporulates very poorly in the presence of fusidic acid (16). The *rfm122* mutation introduced into FUS429 by transformation increased the sporulation ability to a level 50-fold higher than that of strain FUS429 (Table 2). However, the *rfm122* mutation could not suppress other types of sporulation defects. For example, a low concentration of erythromycin (5) or a high concentration of glucose inhibits sporulation without affecting vegetative growth. These inhibitions could not be suppressed by the *rfm122* mutation (Table 2). The *rfm122* mutation could not restore the defective sporulation of strain Spo080. Spo080 is an early blocked asporogenous mutant isolated spontaneously from *B. subtilis* 168 *thy trp*. Morphological and physiological analyses showed that Spo080 belongs to the Spo0a class (21). The introduction by transduction of *rfm122* into the Spo080 strain had no effect on the sporulation defect of Spo080 (Table 2). These results suggest that the *rfm* mutation suppresses the sporulation defect by interacting with the *fus* mutation, and they further suggest a functional interaction between *rfm* and *fus* mutations in sporulation.

Genetic studies. To determine the cause of the *rfm* mutation, three-point transformation crosses were carried out using the *rfm* mutants of strain FUS426 as the donor and the CEL (*cysA ery leu*) strain as the recipient. The *rfm* markers of the three mutant strains tested mapped at almost the same site as the *rfm* marker (RNA polymerase mutation) reported by Harford and Sueoka (12) (Table 3). The *rfm* locus is the structural gene of the β-subunit of RNA polymerase (11, 20). When the sporulation frequency of the recombinants was determined by the color of the colonies on Schaeffer sporulation plates at 45°C and microscopic observation, the sporulation frequency of all of the recombinants (440/440) containing both *rfm* and *fus* markers was much higher than that of the recombinants containing rfm^+ *fus* markers (97/97), but slightly lower than that of the recombinants containing *rfm* fus^+ markers (157/

TABLE 2. *Specificity of the effect of the rfm122 mutation on sporulation*[a]

Strain	Additions	Spores/ml
168		2.4×10^8
FUS429	Fusidic acid, 20 μg/ml	1.7×10^5
RIF122FUS429	Fusidic acid, 20 μg/ml	8.6×10^6
168	Erythromycin, 0.03 μg/ml	2.8×10^5
RIF122	Erythromycin, 0.03 μg/ml	5.6×10^5
168	Glucose, 0.5%	3.2×10^7
RIF122	Glucose, 0.5%	8.7×10^7
Spo080		$<10^2$
RIF122Spo080		$<10^2$

[a] Cells were grown at 37°C in the sporulation medium in the presence or absence of chemicals. Heat-resistant spores were counted at t_{24} by plating the cells after heating the cell suspensions at 80°C for 10 min.

TABLE 3. *Analysis of three-point transformation crosses*

Donor strain	Selected phenotype	Recombinant classes[a]			No. of recombinants	Order and distance of markers[b]
		cysA	*rfm*	*fus*		
RIF110FUS426	CysA$^+$	1	1	1	71	*cysA* *rfm* *fus*
		1	1	0	60	← 0.49 ⟶⟵ 0.27 →
		1	0	1	10	← 0.68 →
		1	0	0	115	
RIF117FUS426	CysA$^+$	1	1	1	66	*cysA* *rfm* *fus*
		1	1	0	33	← 0.48 ⟶⟵ 0.20 →
		1	0	1	9	← 0.64 →
		1	0	0	99	
RIF122FUS426	CysA$^+$	1	1	1	141	*cysA* *rfm* *fus*
		1	1	0	63	← 0.43 ⟶⟵ 0.23 →
		1	0	1	19	← 0.55 →
		1	0	0	136	
	Fus^r	1	1	1	113	*cysA* *rfm* *fus*
		0	1	1	49	← 0.25 ⟶⟵ 0.27 →
		1	0	1	6	← 0.46 →
		0	0	1	53	

[a] The symbols 1 and 0 refer to donor and recipient markers, respectively.
[b] Map distances are defined as 1 minus frequency of cotransfer of the markers.

157). These results suggest that the suppressive character and the rifampin resistance are caused by a single mutation in RNA polymerase or are located very close to each other. Since the *rfm* mutants are isolated spontaneously at a mutation frequency of 2.8×10^{-8}, the probability of a double mutation is very low.

Alteration of RNA polymerase in the RIF122FUS426 strain. To confirm the results obtained by the genetic analysis, we examined the in vitro resistance of the mutant RNA polymerase. The results (Fig. 2) showed that at 0.5 μg of rifampin per ml the activity of FUS426 RNA polymerase was completely inhibited, whereas the RNA polymerase of RIF122FUS426 still retained 56% of the activity of the control. Therefore, the mutant had an altered RNA polymerase.

Linn et al. (20) showed that one rifampin-resistant mutant had an RNA polymerase containing a β-subunit (the second largest polypeptide of RNA polymerase) of altered electrophoretic mobility. On the other hand, Halling et al. (11) found that the alteration of the largest polypeptide, which they called β, was responsible for rifampin resistance. We analyzed the mutant RNA polymerase by electrophoresis, but could not detect any differences between the RNA polymerase of FUS426 and that of RIF122FUS426 (data not shown).

RNA synthesis rate and sporulation. The growth rates of FUS426 and RIF122FUS426 at 42°C were very similar (Fig. 3). However, the apparent RNA synthesis rate ([^{3}H]uracil incorporation into cold trichloroacetic acid-insoluble fraction) of both strains was remarkably different. After $t_{0.5}$, the RNA synthesis rate of RIF122FUS426 increased and reached a maximum at about $t_{1.5}$, whereas that of FUS426 continued to decrease. The RNA synthesis rates of the wild-type, RIF122, and FUS426 strains (at the permissive temperature) also increased after $t_{0.5}$ (Fig. 3b; 17). Similar results were obtained in experiments using [^{3}H]uridine. These results suggest that the higher rate of RNA synthesis of RIF122FUS426 strain observed after $t_{0.5}$ seems to be related to sporulation.

[^{3}H]UTP incorporation into RNA. There are several factors that influence the efficiency of [^{3}H]uridine incorporation into RNA, such as the difference in the precursor ([^{3}H]uridine) uptake into cells, intracellular precusor synthesis, UTP and CTP pool sizes, and RNA polymerase activity. Therefore, to determine the actual rate of RNA synthesis, it is necessary to measure the specific activity of [^{3}H]UTP and [^{3}H]CTP in the cells and to calculate the rate of the incorporation of UMP into RNA (36) (Fig. 4). Contrary to our expectation, the actual RNA synthesis rate of RIF122FUS426 at 42°C was lower than that of FUS426 at $t_{1.5}$ (Fig. 4d). The actual RNA synthesis rate of FUS426 at 30°C was almost the same level as that of RIF122FUS426 at 42°C (data not shown). The high specific activity of [^{3}H]UTP in RIF122FUS426 cells (Fig. 4a) is not due to a small intracellular UTP pool size, because the UTP pool size in RIF122FUS426 cells is nearly the same as that in FUS426 (data not shown).

To verify the above results, RNA polymerases were partially purified from FUS426 and RIF122FUS426, and the activity was compared.

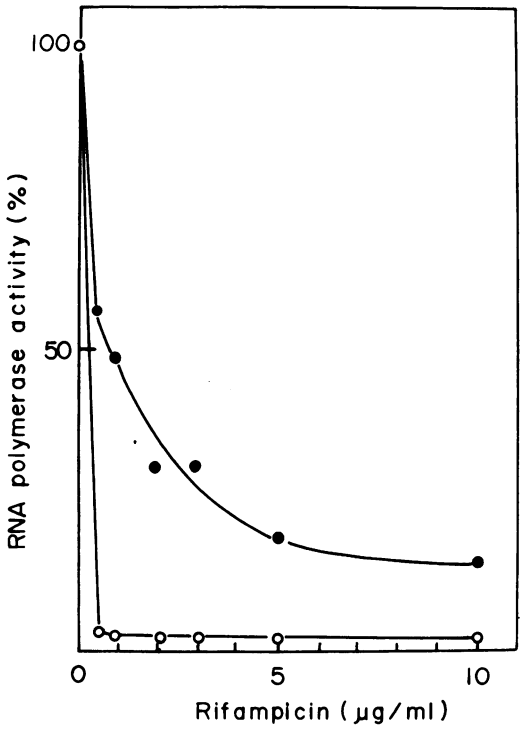

FIG. 2. *Effect of rifampin on RNA synthesis in vitro. Preparation of extracts and assay of RNA polymerase were as described in the text. RNA polymerase activities are shown as a percentage of the control without drug for each enzyme preparation employed. Symbols:* (○) *FUS426;* (●) *RIF122FUS426.*

The activity of RNA polymerase from FUS426 was about twofold higher than that from RIF122FUS426 (Fig. 5a). Similar results were obtained for the RNA polymerases of the logarithmic-growth-phase cells (data not shown) and by the experiments using the permeabilized cells (Fig. 5b). These results indicate clearly that the RNA synthesizing activity of RIF122FUS426 was reduced by *rfm* mutation, and the apparent increase in RNA synthesis observed at $t_{1.5}$ (Fig. 3) was not due to an actual increase in the RNA synthesis.

Analysis of RNA synthesized by the mutants. It is of special interest to know what kind of RNA is synthesized at $t_{1.5}$. To determine the proportion of rRNA in the total RNA synthesized during sporulation, the cells were pulse-labeled for 2 min with [^{3}H]uridine at $t_{1.5}$ or $t_{2.0}$. The labeled RNA was then extracted and hybridized with denatured *B. subtilis* DNA in the presence or absence of excess amounts (75 μg per assay) of unlabeled rRNA, and the proportion of rRNA in the total RNA was determined by the method of Kimura (14). The relative amounts of rRNA synthesized in the wild-type, RIF122, FUS426, and RIF122FUS426 strains were almost the same (Table 4), indicating that the proportion of rRNA and mRNA was not altered significantly by the *rfm* mutation.

There is a possibility that FUS426 has a defect in making a certain sporulation-specific mRNA and that this defect is suppressed by the *rfm* mutation. To test this possibility, DNA-RNA hybridization-competition experiments were carried out. Qualitative differences were not detected (Fig. 6). Although we cannot rule out the possible existence of a very small qualitative difference between FUS426 and RIF122FUS426 messages, these results indicate that there is almost no significant difference in the properties of RNA synthesized in FUS426 and RIF122FUS426 at $t_{1.5}$.

DISCUSSION

In the present paper, we have shown that the temperature-sensitive sporulation of the fusidic acid-resistant mutant of *B. subtilis* is suppressed by a second mutation, *rfm*. It is evident from the following facts that the *rfm* mutation is caused by a single mutation in RNA polymerase: (i) by the genetic analysis, these mutations map in the *rfm* locus, which codes for the β-subunit of RNA polymerase (11, 12, 20); (ii) the RNA polymerase of the mutant (RIF122FUS426) is resistant to rifampin; (iii) the rifampin-resistant mutants are isolated spontaneously, and the rifampin resistance is not separated from the suppressive character by transformation.

The *rfm122* mutation suppresses the temperature-sensitive sporulation of FUS426 and conditional sporulation of FUS429, but does not suppress the asporogeny caused by erythromycin, glucose, or the asporogenous mutation (*spo0*) blocked at an early stage of sporulation. Since the mutation to fusidic acid resistance causes an alteration of EF-G (16), these results indicate a close functional relationship between EF-G and RNA polymerase during sporulation (for more detailed discussion, see ref. 17).

Although we cannot detect any significant difference in the RNA synthesized in FUS426 and RIF122FUS426, we cannot rule out the possibility that the sporulation suppression is a secondary consequence of the *rfm* mutation that primarily affects the transcription of the genes essential for normal sporulation, which are defectively transcribed at nonpermissive temperature in FUS426. Several investigators have reported that EF-G plays an important role in RNA synthesis (15, 34) and that the β-subunit of RNA polymerase is involved in promoter recognition (32). Therefore, as we have already

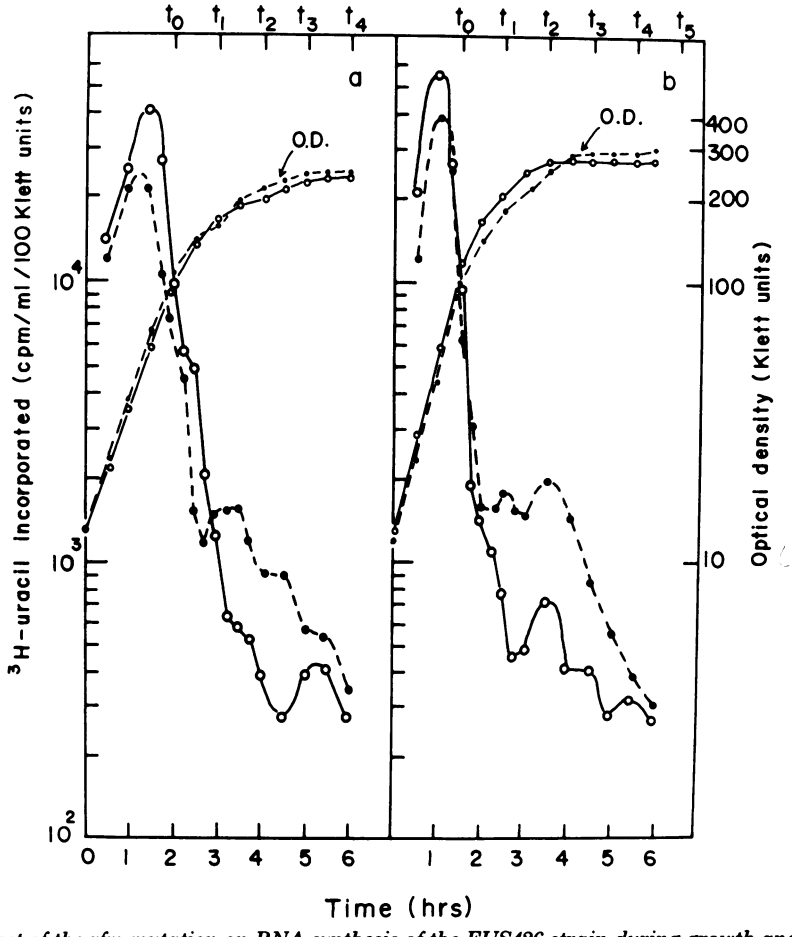

FIG. 3. *Effect of the rfm mutation on RNA synthesis of the FUS426 strain during growth and sporulation. Cells were grown in the sporulation medium at 42°C and labeled for 2 min with [^{3}H]uracil at intervals. Symbols: (a): ○, FUS426; ●, RIF122FUS426. (b): ○, 168; ●, RIF122.*

discussed in detail elsewhere (17), the direct or indirect interaction of RNA polymerase and EF-G at the transcriptional level would be possible during sporulation. This interaction may determine the specificity of the promoter recognition by RNA polymerase, causing the suppression of the temperature-sensitive sporulation.

At the nonpermissive temperature, FUS426 cannot proceed to stage II of sporulation (16). This means that asymmetric septation is inhibited in this mutant. The cell envelope of FUS426 is thicker than that of the wild-type strain at the nonpermissive temperature (16), and RIF122FUS426 has a normal envelope (unpublished data). Furthermore, antibiotic resistance is enhanced in FUS426 in vivo, whereas in vitro protein-synthesizing activity of FUS426 is as sensitive as the parental strain. When membrane proteins are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, several membrane proteins are altered at the nonpermissive temperature in FUS426, and the defects are recovered by *rfm* mutation (manuscript in preparation). These results suggest that the mutation is somehow related to the membrane alteration.

There are several reports indicating that membrane alterations may play an important role during early sporulation stages. For example, Bohin et al. (1) found that the sporulating cells of *B. subtilis* are more sensitive to ethanol than the vegetative cells and that sporulation is blocked at stage 0-I in the presence of ethanol. Therefore, it is possible that sporulation-specific membrane changes are somehow disordered in FUS426 and that this defect is suppressed by *rfm* mutation. The presence of β and β' subunits of RNA polymerase in the membrane fraction (23; unpublished data) would support this possibility. Furthermore, in a study of flagella for-

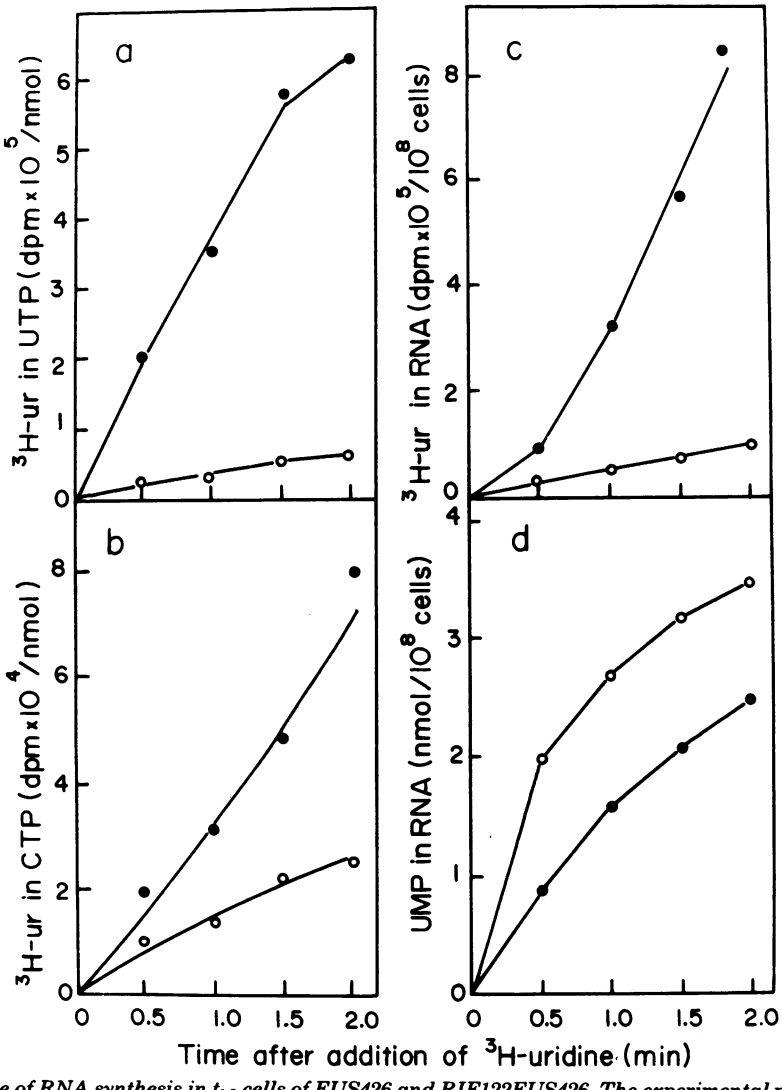

FIG. 4. *Rate of RNA synthesis in $t_{1.5}$ cells of FUS426 and RIF122FUS426. The experimental procedures are described in the text. (a) Specific activity of UTP. (b) Specific activity of CTP. (c) Incorporation of [^{3}H]uridine into RNA. (d) Incorporation of UMP into RNA calculated according to Segall et al. (28). Symbols:* (○) *FUS426;* (●) *RIF122FUS426.*

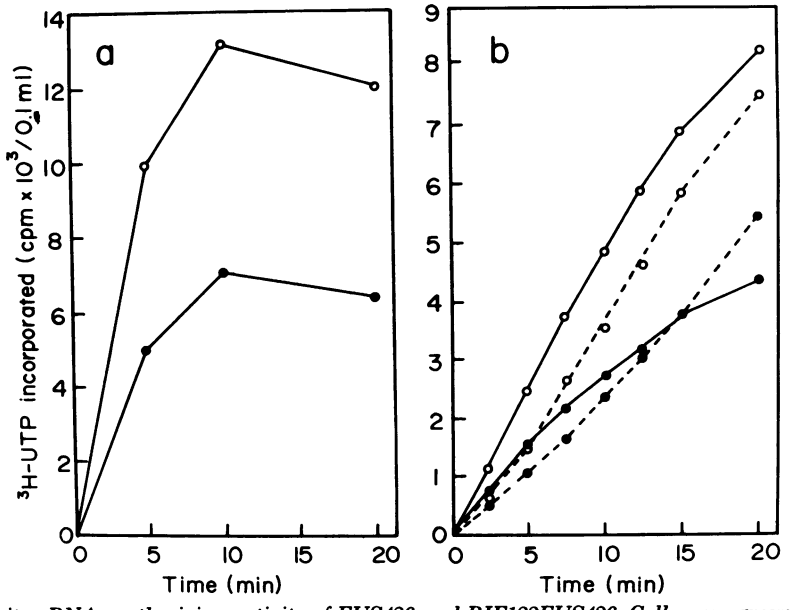

FIG. 5. *In vitro RNA-synthesizing activity of FUS426 and RIF122FUS426. Cells were grown at 42°C and harvested at $t_{1.5}$. Preparation of RNA polymerase, toluenization, and assay for RNA synthesis are described in the text. (a) RNA polymerase activity. (b) RNA synthesis in toluenized cells. Symbols: (○——○) FUS426 assayed at 42°C; (●——●) RIF122FUS426 assayed at 42°C; (○- - -○) FUS426 assayed at 32°C; (●- - -●) RIF122FUS426 assayed at 32°C.*

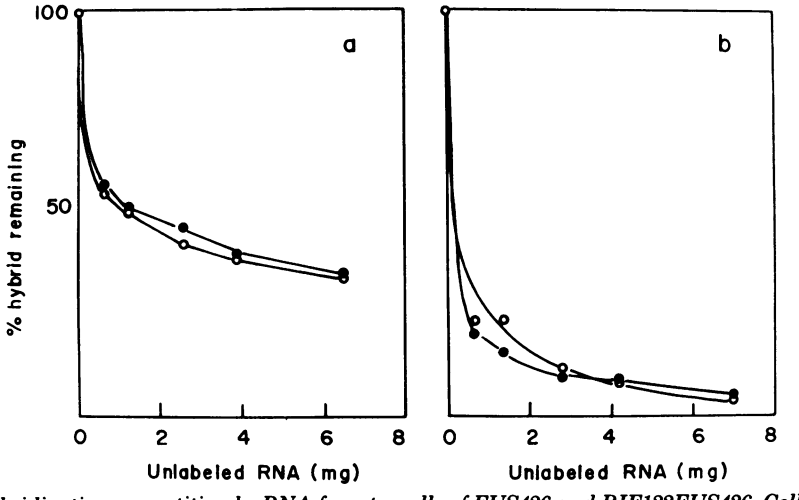

FIG. 6. *Hybridization competition by RNA from $t_{1.5}$ cells of FUS426 and RIF122FUS426. Cells were grown at 42°C in the sporulation medium. RNA was extracted, and the hybridization competition was performed as described in the text. (a) Competition by unlabeled RNA from $t_{1.5}$ cells of FUS426. (b) Competition by unlabeled RNA from $t_{1.5}$ cells of RIF122FUS426. Symbols: (○) [^{3}H]RNA of FUS426; (●) [^{3}H]RNA of RIF122FUS426. 100% values were 1,700 cpm for [^{3}H]RNA of FUS426 and 11,000 cpm for [^{3}H]RNA of RIF122FUS426.*

mation, Yamamori et al. (37) suggested the possibility that *rfm* mutations affect membrane architecture.

Whatever the mechanisms underlying the observations reported here, the present experiments clearly indicate the functional interaction between EF-G and RNA polymerase. Further analysis of the mutant, especially with respect to the coupling of RNA and protein syntheses and the membrane alterations, may provide a clue to the general mechanism that controls sporulation.

TABLE 4. *Relative amount of rRNA in RNA synthesized during sporulation*

Source of labeled RNA	Input (cpm/0.5 μg)	RNA hybridized (cpm)[a]		% rRNA
		Uncompeted	Competed by rRNA[b]	
168 ($t_{1.5}$)	3,823	1,455	1,008	31 ± 5
RIF122 ($t_{1.5}$)	18,696	5,983	3,944	34 ± 4
FUS426 ($t_{2.0}$)	5,127	1,758	1,210	31 ± 5
RIF122FUS426 ($t_{2.0}$)	3,311	1,184	794	33 ± 3

[a] Counts per minute (cpm), mean of three experiments.
[b] The amount of rRNA was 75 μg per assay.

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