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Isolation and Characterization of Fusidic Acid-Resistant, Sporulation-Defective Mutants of *Bacillus subtilis*

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Fusidic acid-resistant, sporulation-defective mutants were isolated from *Bacillus subtilis* 168 *thy trp*. About two-thirds of the fusidic acid-resistant (*fus*^{r}) mutants were defective in sporulation ability and fell into three classes with respect to sporulation character. The representative mutants FUS426 and FUS429 were characterized in detail. FUS426 [*fus*^{r} *spo*(Ts)], a temperature-sensitive sporulation mutant, grew well at 30 and 42°C but did not sporulate at 42°C. FUS429 [*fus*^{r} *spo*(Con)], a conditional sporulation mutant, grew and sporulated normally in the absence of fusidic acid, but its sporulation and growth rates decreased in the presence of fusidic acid, depending on the concentration of the drug. Although electron microscopic observation showed that both mutants were blocked at stage I of sporulation, the physiological analyses indicate that these mutants belong to the SpoOB class. Both mutants formed a thickened cell wall as compared with that of the parental strain. Genetic and in vitro protein synthesis analyses led to the conclusion that the sporulation-defective character of mutants FUS426 and FUS429 resulted from an alteration in elongation factor G caused by a single lesion in the *fus* locus. The possible role of elongation factor G in sporulation is discussed.

The sporulation process of *Bacillus subtilis* depends on sequential gene expression. Many investigators have been trying to clarify the sporulation mechanism at the transcriptional and translational levels. Transcriptional control must play an important role in the process (22) and has been studied extensively. Gene expression at the translational level has been invoked to explain diverse genetic phenomena. Translational control of sporulation has been suggested by studies on the relationship between ribosomes and sporulation (6, 7, 10, 17, 18, 21). Kobayashi (17) previously found defective ribosomal subunits in *Bacillus cereus* dormant spores and suggested that ribosomal alteration was somehow related to bacterial sporulation (17, 18). Leighton (21) also suggested that the 30S ribosomal subunit specifies a sporulation-specific message. Furthermore, Domoto et al. (6) recently reported that the 50S ribosomal subunit plays a regulatory role in sporulation.

However, there is little evidence on the relationship between sporulation and factors participating in protein synthesis, except for studies on initiation factors (5). Elongation factor G (EF-G) plays a very important role in protein synthesis (25). In addition, Atherly (1), Rabbani and Srinivasan (27) (*Escherichia coli*), and Kimura et al. (16) (*B. subtilis*) reported recently that it functions in the regulation of stable ribonucleic acid (RNA) synthesis. Furthermore, Fortnagel and Bergmann (7) found that sporulating cells become resistant to fusidic acid, an inhibitor of EF-G. These facts prompted us to investigate the role of EF-G in sporulation.

In the present paper we report the isolation and characterization of fusidic acid-resistant, sporulation-defective mutants of *B. subtilis*. In addition, the possible role of EF-G in *B. subtilis* sporulation is discussed.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* 168 *thy trp* was used as a parental strain. *B. subtilis* Marburg CSL (*cysA strA leu*) was used as a recipient in the transformation test.

Isolation of mutants. Fusidic acid-resistant mutants were isolated spontaneously from *B. subtilis* 168. Cells grown for 6 to 7 h at 36°C in 10 ml of modified nutrient broth (8 g of Difco nutrient broth, 5 g of tryptose, and 5 g of NaCl per liter) were centrifuged and then suspended in 0.3 ml of 0.85% NaCl, and 0.1 ml of cell suspension was plated on Schaeffer sporulation medium (29) containing 10 μg of fusidic acid per ml. Incubation was continued for 4 to 5 days at 45°C. The translucent, asporogenous colonies appearing on the plate were picked and

used for further investigation. Each mutant was replicated on Schaeffer sporulation plates with or without fusidic acid and incubated at 30 and 45°C, respectively. The sporulation character was judged by the color of the colony on the plate.

Sporulation ability. Cells were incubated, with shaking, in Schaeffer sporulation medium containing thymine (10 μg/ml) or containing thymine and fusidic acid (at the appropriate concentration) at 30 or 42°C, respectively. Samples (0.1 ml each) were harvested at T_{24}. Heat-resistant colony-forming units were counted on nutrient broth plates after the cell suspension was heated for 10 min at 80°C.

Growth rate. Growth rate was estimated by determining the reciprocal of the doubling time of exponentially growing cells: μ (per hour) = 1/T, where μ is the growth rate and T is the doubling time (hours). Growth was monitored at 660 nm with a Klett-Summerson photometric colorimeter.

Electron microscopy. Electron microscopy was carried out by the method of Domoto et al. (6).

Transformation and mapping. Strain CSL (*cysA strA leu*) was used as a recipient. Transforming deoxyribonucleic acid was prepared by phenol extraction according to the method of Saito and Miura (28). Transformation was carried out at 36°C by the method of Bott and Wilson (4) with a slight modification. Competent cultures were grown in a minimal medium supplemented with the mixture of nine amino acids described by Wilson and Bott (32) and 0.05% yeast extracts. Two to three hours after the logarithmic growth phase, cells were incubated with deoxyribonucleic acid (1 μg/ml) for 30 min with rapid shaking. Transformants carrying *cysA*$^+$ were selected on minimal plates supplemented with leucine; those carrying *fus*^{r} were selected on Schaeffer sporulation plates containing fusidic acid (10 μg/ml).

Mapping was carried out by three-factor transformation crosses (12) involving *cysA*, *strA*, and *fus* markers. Each transformant selected was replicated on the appropriate plates to test the unselected markers of the transformant. The sporulation ability of transformants carrying the *fus*^{r} marker were tested on Schaeffer sporulation plates with or without fusidic acid.

Preparation of washed ribosomes and supernatant (S-150). Cells grown in Schaeffer sporulation medium at 36°C until the logarithmic growth phase were harvested, poured over ice, and collected by centrifugation at 10,000 rpm. Harvested cells were washed twice with buffer I (20) [10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5; 10 mM $MgCl_2$; 1 M KCl; 5 mM Mg-Titriplex; 10 mM 2-mercaptoethanol; and 10% glycerol] and once with buffer II (buffer I with the KCl concentration reduced to 50 mM). The following procedures were carried out at 4°C. Cell pellets were ground with quartz sand and extracted with extraction buffer containing 5 μg of deoxyribonuclease per ml, 10 mM Tris-hydrochloride buffer (pH 7.8), 10 mM magnesium acetate, 60 mM NH_4Cl, 1 mM Mg-Titriplex, 7 mM 2-mercaptoethanol, and 3.5 mM phenylmethylsulfonyl fluoride. The extract was clarified by centrifugation at 10,000 rpm for 10 min. The supernatant fraction was centrifuged again at 22,000 rpm (30,000 × *g*) for 30 min. The ribosomes were sedimented from the supernatant by centrifugation at 150,000 × *g* for 3 h and then washed twice with 1 M NH_4Cl by the method of Guha and Szulmajster (11). The upper one-third of the 150,000 × *g* supernatant liquid was dialyzed overnight at 4°C against standard buffer (10 mM Tris-hydrochloride buffer, pH 7.8; 10 mM magnesium acetate; 60 mM NH_4Cl; 6 mM 2-mercaptoethanol) and used as the supernatant fraction (S-150). The S-150 and ribosomes were frozen quickly and stored, until use, at −20°C. The protein concentration of the S-150 was determined by the Lowry et al. method (23). Albumin was used as the standard.

Assay of in vitro protein synthesis. Assay of polyuridylate-dependent polyphenylalanine synthesis was carried out in a 125-μl reaction mixture containing: Tris-hydrochloride buffer (pH 7.8), 60 mM; magnesium acetate, 8 mM; NH_4Cl, 80 mM; 2-mercaptoethanol, 6 mM; spermidine, 0.8 mM; adenosine 5′-triphosphate, 1 mM; guanosine 5′-triphosphate, 0.2 mM; phosphoenolpyruvate, 4 mM; pyruvate kinase, 2 μg; [^{14}C]phenylalanine, 0.025 μCi (100 μCi/μmol); 19 amino acids except phenylalanine, 0.04 mM each; *B. subtilis* transfer RNA, 25 μg; polyuridylate, 25 μg; washed ribosomes, 50 μg; S-150, 60 μg; and fusidic acid, 3 μg. S-150 was added just before incubation. The reaction mixture was incubated at 30 or 42°C for 40 min. After the addition of 10% cold trichloroacetic acid, the reaction mixture was heated at 90°C for 15 min. The precipitates were collected on glass-fiber disks and washed with 5% trichloroacetic acid. Radioactivity was counted in toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)benzene with a liquid scintillation spectrometer.

RESULTS

Isolation and classification of fusidic acid-resistant, sporulation-defective mutants. It is well known that some mutants resistant to ribosome-directed antibiotics are simultaneously defective in sporulation (3). Fusidic acid inhibits EF-G, one of the factors participating in protein synthesis, in *E. coli* (19, 31) and *B. subtilis* (9). If *B. subtilis* sporulation is regulated at the translational level, it is possible that some fusidic acid-resistant mutants carry a sporulation-defective character simultaneously. In fact, Fortnagel and Bergmann (7) isolated several fusidic acid-resistant mutants that are defective in sporulation. However, no detailed genetic and biochemical characterization has been carried out.

We isolated spontaneously about 200 fusidic acid-resistant mutants of *B. subtilis* 168. The frequency of these mutants (1.5×10^{-7}) was higher than that of other antibiotic-resistant mutants (spiramycin, streptomycin, erythromycin, and siomycin [10^{-8}; data not shown]). Furthermore, the ratio of sporulation-defective

mutants was high (68%); the remainder sporulated normally.

Fusidic acid-resistant, sporulation-defective mutants fell into three classes with respect to their sporulation phenotypes. Group I includes temperature-sensitive sporulation mutants in which sporulation is independent of the presence of fusidic acid. Sporulation is inhibited at 42°C in this group. Group II includes conditional sporulation mutants in which sporulation is reduced proportionately to an increase of fusidic acid concentration. In addition to these two groups, asporogenous mutants were obtained (group III). In the following experiments, two typical mutants, FUS426 (group I) and FUS429 (group II), were used.

Effect of fusidic acid on sporulation and growth rate of strains FUS426 and FUS429. Table 1 summarizes the sporulation character of strains FUS426 and FUS429. Strain FUS426 is a temperature-sensitive sporulation mutant, whereas sporulation of strain FUS429 is suppressed in the presence of fusidic acid. Figure 1 shows the relationship between sporulation and growth rate of FUS426 in relation to fusidic acid concentration. FUS426, independent of fusidic acid concentration, sporulated at 30°C but not at 42°C. Its growth rate was constant. FUS429 sporulated normally in the absence of fusidic acid, but, in the presence of increasing amounts of the drug, the sporulation efficiency gradually decreased. Its growth rate declined as the drug concentration increased (Fig. 2). There was good parallelism between the sporulation and growth rate of FUS429. On the contrary, sporulation of the parental strain was not affected when the growth rate was gradually decreased by the addition of increasing concentrations of fusidic acid, so as to cause a comparable change in growth rate as observed in mutant FUS429 (Fig. 3). This result suggests strongly that the conditional sporulation character of FUS429 is not due to mere sensitivity to fusidic acid, but to a unique character caused by the mutation.

TABLE 1. *Sporulation character of strains FUS426 and FUS429*[a]

Strain	Spores/ml			
	30°C		42°C	
	Without FA	With FA[b]	Without FA	With FA
FUS426	1.6×10^8	1.2×10^8	1.5×10^4	6.0×10^3
FUS429	1.2×10^8	1.4×10^6	1.5×10^8	6.6×10^4
B. subtilis 168	4.9×10^8		1.1×10^8	

[a] FA, Fusidic acid. Maximal number of viable cells was as follows: *B subtilis* 168, 5.1×10^8/ml; FUS426, 5.3×10^8/ml; FUS429, 3.7×10^8/ml.

[b] Schaeffer sporulation medium containing 25 μg of fusidic acid per ml.

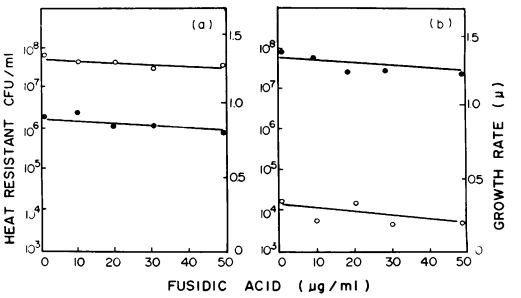


FIG. 1. *Effect of temperature and fusidic acid on sporulation and growth rate of FUS426. Cells were grown, with shaking, in Schaeffer sporulation medium with or without fusidic acid and harvested at* T_{24}. (○) *Spores (heat-resistant colony-forming units [CFU] per milliliter) and* (●) *growth rate* (μ) *were estimated as described in the text. (a) 30°C; (b) 42°C.*

Blocked stage of sporulation and thickened cell wall of strains FUS426 and FUS429. To ascertain which sporulation stage was blocked, electron microscopic observations were carried out. Sporulation of FUS426 at the nonpermissive temperature was blocked at stage I (Fig. 4a), as characterized by an axial filament deoxyribonucleic acid, and didn't proceed further. FUS429 harvested at T_6 was also blocked at stage I in the presence of fusidic acid (Fig. 4b).

Physiological analyses also indicated that both mutants are blocked at an early process of sporulation, since they produced extracellular protease but didn't produce antibiotics. Therefore, these mutants seem to belong to the SpoOB class.

Electron microscopic observation also showed that the cell wall of both mutants harvested at T_6 is thicker than that of the parental strain. FUS429 formed a winding cell with a thickened wall even during logarithmic growth in the presence of fusidic acid (Fig. 4c). Overproduction or disorganization of the cell wall in the logarithmic growth phase may inhibit normal sporulation. In the absence of the drug, there was no morphological difference between FUS429 and the parental strain.

Genetic analysis. It is of interest to know whether fusidic acid resistance and the sporulation-defective character are derived from a single mutation and whether the genetic locus causing fusidic acid resistance coincides with the *fus* locus reported previously (9). To clarify these points, the chromosomal location of the *fus*^{r} marker was determined by three-factor transformation crosses. The mutants and the CSL strain were used as the donor and recipient, respectively. All transformants having the

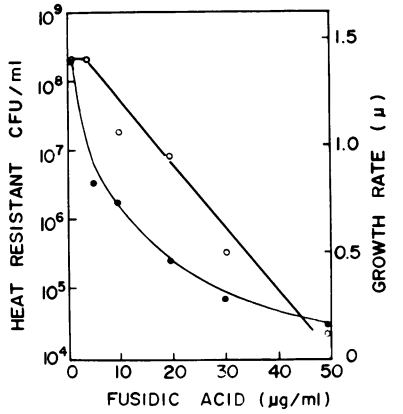


FIG. 2. *Effect of fusidic acid on sporulation and growth rate of FUS429 at 36°C. The methods and symbols are as for Fig. 1.*

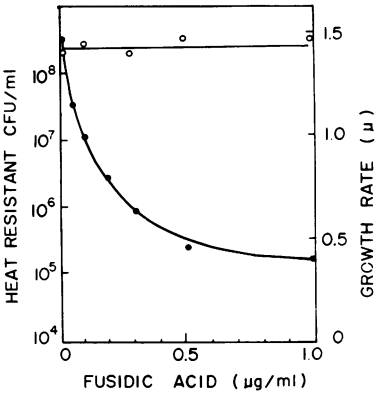


FIG. 3. *Effect of low fusidic acid concentrations on sporulation and growth rate of B. subtilis 168 thy trp at 36°C. The methods and symbols are as for Fig. 1.*

fus^r marker (indicated as 1* in Table 2) are sporulation defective: 258/258 for FUS426 and 61/61 for FUS429. This fact suggests that fusidic acid resistance and the sporulation-defective character are tightly linked. The possibility of double mutation is small, since the mutants arose spontaneously. Therefore, it is strongly suggested that both characters are caused by a single mutation.

Table 2 also shows that the *fus*^{r} character of both mutants lies in the same region of the *B. subtilis* chromosome as that of the *fus* locus reported previously (9). It is well known that the *fus* locus is the structural gene of EF-G in *E. coli* (19, 31) and in *B. subtilis* (2, 16). Therefore, we conclude that the sporulation-defective character is derived from an EF-G mutation.

In vitro resistance to fusidic acid. To test whether the EF-G's of both mutants were resistant to fusidic acid, a cell-free, protein-synthesizing system was prepared, and fusidic acid resistance was examined. S-150 from the parental strain was sensitive to fusidic acid, whereas S-150's from the mutants were resistant (Table 3). Resistance of strain FUS429 to fusidic acid was slightly weaker than that of FUS426. This in vitro result suggests that FUS426 and FUS429 have altered EF-G's. Similar results were obtained at 30°C.

Thus, it is concluded that the sporulation-defective character of FUS426 and FUS429 is derived from an EF-G mutation, suggesting that EF-G plays a role in sporulation.

DISCUSSION

Fortnagel and Bergmann (7) reported that sporulating cells of *B. subtilis* become resistant to fusidic acid by the alteration of ribosomes. However, Guha and Szulmajster (11), showing that S-150 fractions and ribosomes of sporulating cells were sensitive to fusidic acid, disagreed. At present, Fortnagel and his co-workers (8) think that ribosomes of sporulating cells contain a protein factor that is synthesized de novo in sporulating cells and that such a ribosomal alteration may result in fusidic acid resistance. So far, a direct relationship between EF-G function and sporulation has not been proven. The present paper showed that a mutation affecting EF-G altered the sporulation ability of *B. subtilis*.

Sporulation can be considered asymmetric cell division caused by cellular adaptation to environmental changes such as nutrient limitation (14). Pitel and Gilvarg (26) observed that there is an unbalanced synthesis between cell wall and cell membrane during the initial stage of sporulation. These facts suggest that cell wall and/or cell membrane metabolism plays an important role in sporulation.

As shown in Fig. 4, mutants FUS426 and FUS429 are blocked at an early process of sporulation (stage I), and the cell walls of both mutants are thicker than that of the parental strain. The synthesis of cell wall and cell membrane components, i.e., peptidoglycan (15) and phospholipid (24, 30) as well as ribosomal RNA, is regulated by stringent control. Recently, it was reported that EF-G participates in the regulation of ribosomal RNA synthesis in *B. subtilis* (16) and *E. coli* (1, 27). Our preliminary experiment showed that regulation of FUS426 RNA synthesis at the nonpermissive temperature is disordered during an early stage of sporulation (13). Therefore, it is quite likely that the mutation affecting EF-G results in a dis-

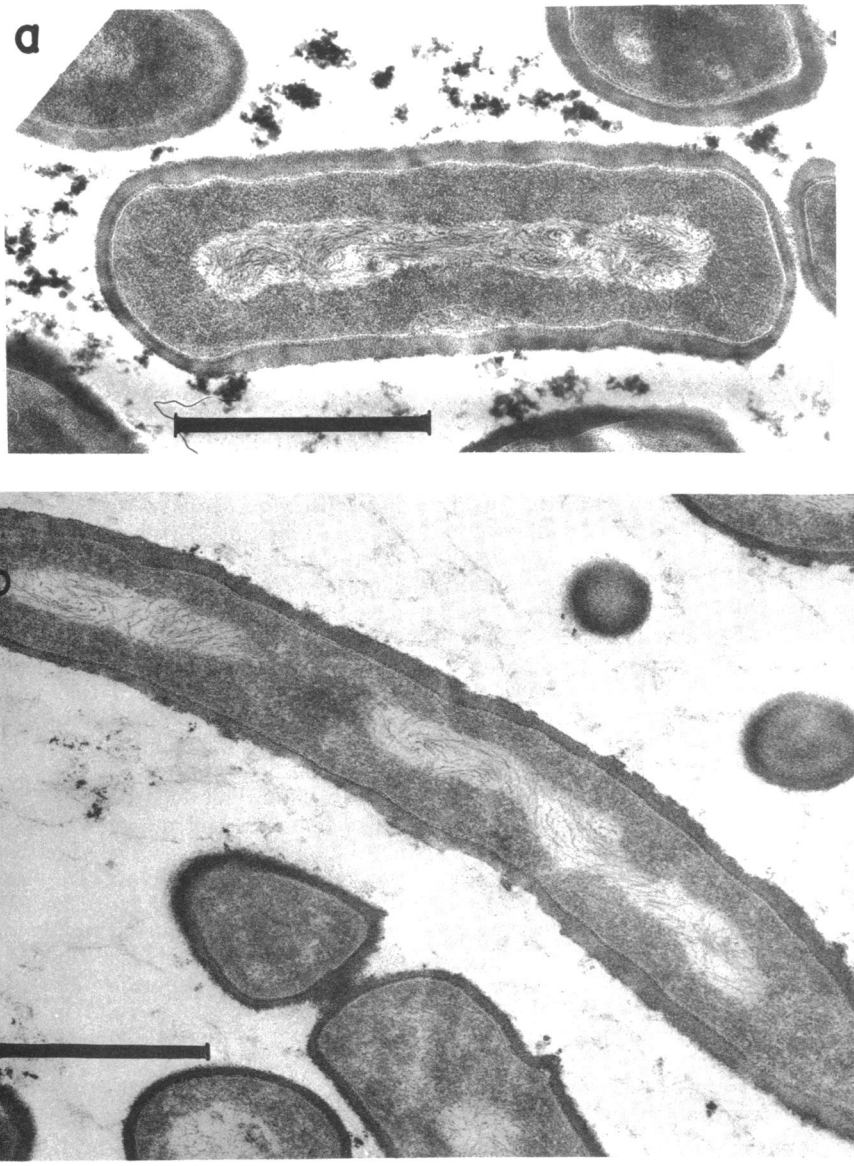


FIG. 4. *Electron micrographs of strains FUS426 and FUS429. Thin-sectioned samples were prepared from a culture growing in Schaeffer sporulation medium. (a) FUS426 cells harvested at T_6 (42°C); (b) FUS429 cells cultured in the presence of 20 μg of fusidic acid per ml at 36°C and harvested at T_6; (c) FUS429 cells cultured in the presence of 25 μg of fusidic acid per ml at 36°C and harvested in the logarithmic growth phase; (d) B. subtilis 168 thy trp cells harvested at T_6 (42°C). Bars indicate 0.5 μm.*

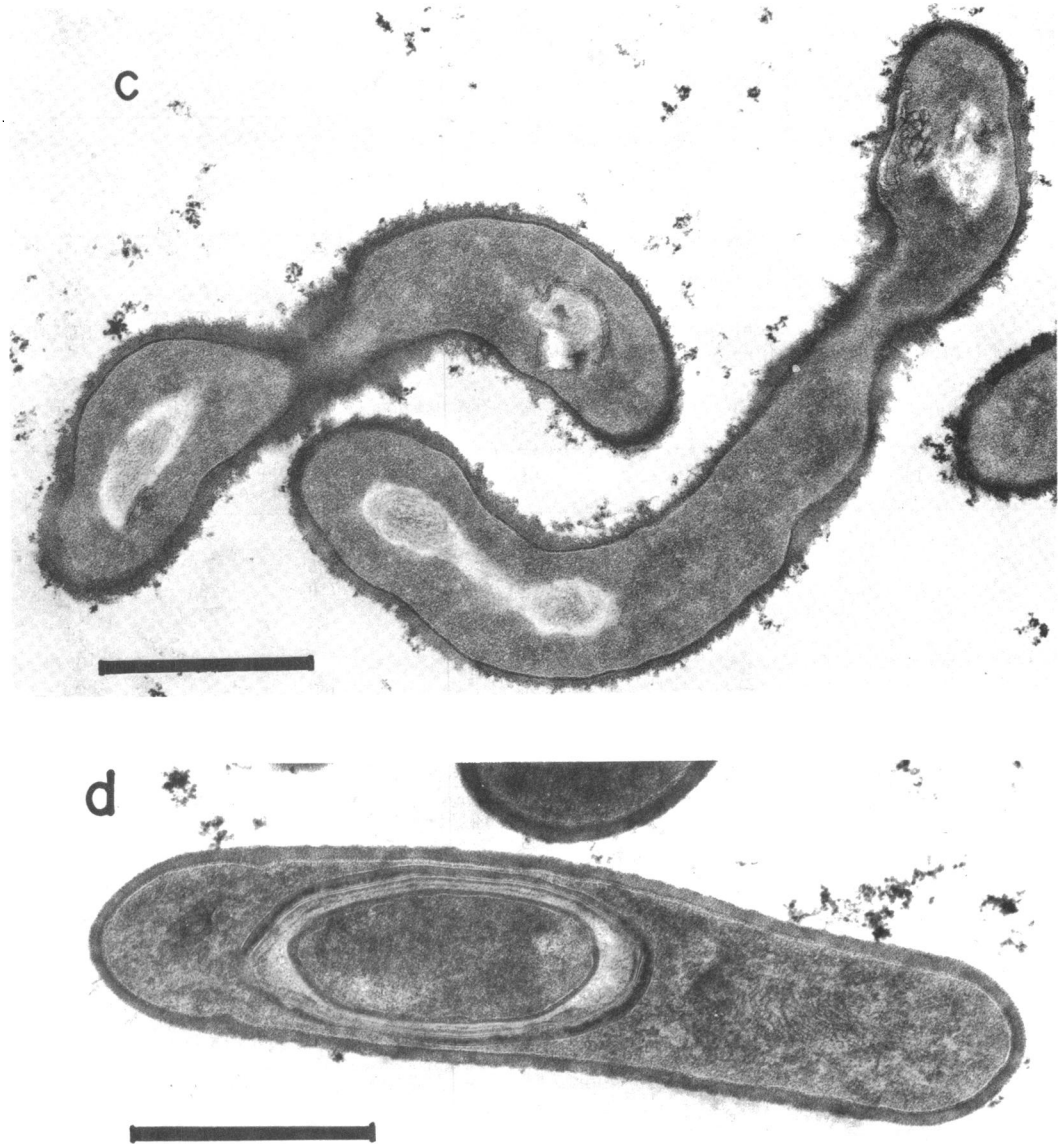
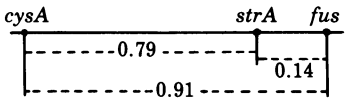
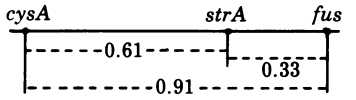
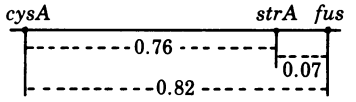


FIG. 4 c and d

TABLE 2. *Analysis of three-factor transformation crosses involving cysA, strA, and fus markers and genetic map of each marker in strains FUS426 and FUS429*[a]

Donor deoxyribonucleic acid	Selected marker	Recombinant class			No. of recombinants	Gene order and distance
		cysA	*strA*	*fus*		
FUS426	*cysA*$^+$	1	1	1*	32	
		1	1	0	38	
		1	0	1*	11	
		1	0	0	252	
FUS426	*fus*^{r}	1	1	1*	19	
		0	1	1*	125	
		1	0	1*	6	
		0	0	1*	65	
FUS429	*cysA*$^+$	1	1	1*	59	
		1	1	0	21	
		1	0	1*	2	
		1	0	0	244	

[a] 1, Donor marker; 0, recipient marker. Donor, FUS426 or FUS429 (*cysA*$^+$ *str*^{s} *fus*^{r}); recipient, CSL (*cysA str*^{r} *fus*^{s}). Map distance is defined as 1 − frequency of cotransfer of the markers.

TABLE 3. *Localization of fusidic acid resistance in cell-free extracts derived from strains FUS426 and FUS429*[a]

B. subtilis 168		FUS426		FUS429		[^{14}C]phenylalanine incorporated (cpm)		% of control
R	S	R	S	R	S	−FA	+FA	
+	+					1,925	950	49
		+	+			719	783	108
				+	+	795	725	91
+			+			783	837	110
+					+	926	874	86
	+	+				1,861	1,073	57
	+			+		2,138	824	47

[a] R, Washed ribosomes; S, S-150; −FA, without fusidic acid; +FA, with 25 μg of fusidic acid per ml. The reaction mixture (total volume, 125 μl) contained 50 μg of washed ribosomes and 60 μg of S-150. The reaction was carried out at 42°C for 40 min.

turbance of RNA, phospholipid, and peptidoglycan synthesis, causing the overproduction of cellular envelope and the arrest of sporulation.

At present, we can't explain the EF-G function during sporulation, but further analyses of EF-G mutants should give more information on the relationship between EF-G function and RNA or cell envelope metabolism during sporulation. Experiments along this line are now in progress.

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