JOURNAL OF BACTERIOLOGY, May 1982, p. 944–955
0021-9193/82/050944-12$02.00/0

Vol. 150, No. 2

Alcohol-Resistant Sporulation Mutants of *Bacillus subtilis*

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Received 15 June 1981/Accepted 30 November 1981

About 80% of *Bacillus subtilis* cells form spores when grown in nutrient broth. In medium containing various short-chain aliphatic alcohols, the frequency of sporulation was reduced to 0.5%. Mutants sporulated in the presence of alcohols at a frequency of 30 to 40%. Sporulation in the wild-type cells was sensitive to alcohol at the beginning of sporulation (stage zero). Sensitivity to alcohol in the mutants was also at stage zero, even though the sensitivity was considerably reduced. This sensitivity of sporulation to alcohol is the phenotypic expression of a genetic locus designated *ssa*. Mutations at this locus lead to a decreased sensitivity of sporulation to alcohol without modifying the sensitivity of growth. Genetic analysis by transduction with bacteriophage PBS1 revealed that *ssa* mutations are near the previously described *spo0A* locus. *ssa* mutants also differ from wild-type cells in the composition of membrane phospholipids. The relative amount of phosphatidylglycerol increased, whereas the relative amount of phosphatidylethanolamine and lysylphosphatidylglycerol decreased relative to the proportions in the wild type. The distribution of fatty acids in membrane lipids is the same as in the wild type. No differential sensitivity of phospholipid metabolism to alcohol could be detected in the mutant. This work therefore reveals that the extensive, pleiotropic changes in the membranes of *ssa* mutants are the phenotypic reflection of alterations at a specific gene locus.

Several observations suggest that the bacterial membrane is involved in the initiation of *Bacillus subtilis* sporulation. (i) Studies of *spo0* mutants have shown that the membrane-bound nitrate reductase (3) and the phospholipid metabolism in the membranes (35) of these mutants are altered; such membrane alterations could lead to the well-known pleiotropic phenotype of the *spo0* strains (29). (ii) Short-chain aliphatic alcohols, ethanol among them, preferentially inhibit sporulation at concentrations that lower the growth rate to 50% (4). Since short-chain aliphatic alcohols also inhibit various events associated with sporulation, they induce the appearance in normally sporogenous wild-type strains of phenocopies of *spo0* mutants (4).

The inhibition of sporulation by these alcohols can be correlated with their effect on the structural state of lipids in the biological membrane (7, 30). Ethanol induces significant perturbations in the lipid composition of animal cells (8, 27) as well as bacterial membranes (1, 6, 9, 21, 25, 45). In *B. subtilis* (34), this alcohol preferentially inhibits the synthesis of phosphatidylglycerol and causes a decrease in the relative amount of branched fatty acids.

One can thus hypothesize that these alcohols would then produce a general modification of membrane properties and consequently a change in activity of some membrane-bound enzymes. However, the direct inhibition of membrane-bound or cytoplasmic enzymes by alcohols cannot be ruled out. In addition, it is impossible to dissociate in the wild type the inhibition of growth from the inhibition of sporulation and, therefore, to rule out the possibility that the latter is the result of the former. Isolation and study of ethanol-resistant sporulation mutants may clarify the effects of alcohols on sporulation.

In mutants with alterations in the locus governing alcohol sensitivity of sporulation (*ssa* mutants), sporulation is resistant to short-chain aliphatic alcohol. The growth of *ssa* mutants, like that of the wild-type strain, remains sensitive to alcohols.

By their properties, *ssa* mutants can be defined as conditional mutants at stage zero of the sporulation process. They are physiologically similar and genetically linked to pleiotropic asporogenous mutants *spo0A* (29). *spo0A* bacteria are further characterized by a modification of their phospholipid composition (35). During exponential growth in defined conditions, they show a phosphatidylethanolamine deficiency. *ssa* mutants, which resist the action of drugs that disturb the structure of biological membranes,

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likewise are similar to *spo0A* mutants in the alteration of their membrane lipid composition, even when alcohol is absent.

There also exists the possibility that *ssa* resistance might result from a modified response of phospholipid metabolism to ethanol.

MATERIALS AND METHODS

Bacterial strains and cultures. Genotypes and origins of strains of *B. subtilis* employed in this investigation are listed in Table 1. *ssa* mutants were isolated from the wild-type strain 168. Growth and sporulation were routinely obtained in nutrient broth (38). Alcohol sensitivity of growth was tested in the medium of Sterlini and Mandelstam (43) modified as follows: Difco (Difco Laboratories) Casamino acids (vitamin free), 8.6 g; glutamic acid, 3 g; L-alanine, 1 g; D-L-aspartic acid, 2 g; KH_2PO_4, 0.5 g; $FeSO_4 \cdot 6H_20$, 0.6 mg; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_20$, 0.1 g; $Ca(NO_3)_2$, 20 mg; $MnCl_2$, 20 mg; and H_2O, 1 liter. The pH was adjusted to 7.1 with NaOH.

Cultures were incubated at 37°C under strong aeration, and growth was followed spectrophotometrically at 570 nm. The end of exponential growth was taken as time zero (T_0) of sporulation; T_1, T_{20}, etc., indicate the number of hours of subsequent incubation. Sporulation frequency was determined as previously described (4).

For the study of phospholipids, some precautions were taken to obtain reproducible results through the cell cycle. Glucose (2 g/liter) was therefore added to nutrient broth (then called NBG) for experiments with early exponential cultures. For experiments with postexponential bacteria, the following procedure was used. At $T_{0.5}$, a culture in regular nutrient broth was diluted sixfold in the same medium at 37°C (and containing ethanol if necessary); the growth then resumed for at least two generations before sporulation was committed.

Mutagenesis. Mutagenesis was performed by UV irradiation of spores to a survival of 0.1% or by ethylmethane sulfonate treatment of vegetative cells to a survival of 0.5% by the method of Guespin-Michel (16).

TABLE 1. Bacterial strains

Strain	Genotype	Origin
168	*trpC2*	P. Schaeffer (4)
ETH7	*trpC2 ssa-7*	This work
ETH15	*trpC2 ssa-15*	This work
MO213	*spo0A47 acf-1*[a]	P. Schaeffer (22)
QB395	*trpC1 aroD120 lys-1*	R. A. Dedonder (10)
S23	*strC23*[b]	J. A. Hoch (42)
MO210	*strC23 aroD120*	Product of the transduction of QB395 by an S23 lysate.
11ONA	*spo0A trpC2*	P. Schaeffer (22)
6Z	*spo0B34 trpC2*[c]	P. Schaeffer (22)
9V	*spo0C49 trpC2*	P. Schaeffer (22)
3U	*spo0D41 trpC2*[c]	P. Schaeffer (22)

[a] The previous designation of this strain was *spo0A5NA acf*.

[b] The streptomycin-resistant strains *strC* are also unable to use glycerol as a carbon source.

[c] 6Z and 3U loci were previously designated as $spo0B_1$ and $spo0B_2$, respectively (28).

Electron microscope examination. Bacteria were fixed, embedded in araldite, and stained as described by Ryter (36).

Screening for the Ssa phenotype. It would be helpful to be able to distinguish *ssa* mutants from wild-type colonies on plates. Ethanol was not suitable for such use on plates, since its concentration significantly decreased during the formation of colonies. The less volatile 1-propanol (0.5 M) was added to nutrient agar, but on these plates the size of colonies was very heterogenous. Sporulated colonies normally show a typical brown color (38), but of the brown-colored colonies on such plates, few showed the Ssa phenotype when tested in liquid medium. These experimental conditions were not useful in genetic experiments, since they did not allow a good enumeration of *ssa* clones. However, 4 clones that sporulated in nutrient broth with added ethanol (0.7 M) were isolated from among 20 to 30 brown colonies (8,400 total colonies) obtained on nutrient agar plus 1-propanol after ethylmethane sulfonate mutagenesis. This method has made the discovery of new mutants easier.

Genetic crosses. The transduction experiments with PBS1 bacteriophage were performed by the method of Jamet and Anagnostopoulos (23). These crosses were made by transducing an ssa^+ spo^+ auxotrophic recipient by phage lysate from an *ssa* spo^+ or ssa^+ *spo0A* prototrophic donor. Prototrophic transductants were isolated on minimal agar plates (41), reisolated on the same medium, and then transferred to nutrient agar or suspended in 1 ml of nutrient broth plus ethanol (0.7 M). After a 24-h incubation at 37°C, the spo^+ and *spo0A* colonies can be easily recognized on nutrient agar plates (38). At the same time, it was possible to determine by phase-contrast microscopy the number of *ssa* mutant clones among the cultures containing ethanol.

Acriflavine-resistant clones (Table 1) were tested as described (22). The *strC* phenotype was determined through the inability of *strC* bacteria to use glycerol as the sole source of carbon (42).

Extraction and identification of phospholipids. Before the bacteria were extracted they were grown for at least three to four generations in medium (1.6 mM phosphate) to which 50 to 100 μCi of $^{32}PO_4^{2-}$ per ml (from Centre d'Etudes Atomiques, Gif-sur-Yvette, France) had been added. Cells were quickly diluted in an equal volume of perchloric acid (0.5 M, containing 50 mM NaH_2PO_4), and the pellet was then treated for 25 min at 4°C with a methanol-chloroform-water mixture as described by Bligh and Dyer (2). The final chloroform solution was used for the estimation of phospholipid content and for the fatty acid analysis as previously described (34). The lipid components had been identified by Rigomier and Lubochinsky (35). In these growth conditions, the cardiolipin content was negligible.

RESULTS

Isolation of *ssa* mutants. Mutagen-treated bacteria (see Materials and Methods) were used to start a series of parallel cultures in nutrient broth

at 37°C. In mid-log phase, ethanol was added (final concentration 0.7 M), and incubation was resumed. Overnight cultures were diluted five times in the same medium and heated for 10 min at 80°C. Ethanol was then added, and the cultures were reincubated at 37°C. This dilution-heating-reincubation cycle was repeated three times. After these four cycles, the cultures were plated on nutrient agar to obtain isolated colonies. Each colony was tested in nutrient broth plus ethanol (0.7 M), and its capacity to form spores in this medium was observed by phase-contrast microscopy. The same result was obtained with two different types of mutagenesis (see Materials and Methods). Mutant bacteria were isolated from 3 of 10 cultures. One clone from each culture, ETH7 (UV) and ETH15 (ethylene sulfonate) in particular, was kept for further study.

Growth of *ssa* mutants. Each strain of the 17 tested grew normally in nutrient broth. The growth of each was slowed, as was growth for the wild-type strain, when ethanol (0.7 M) was added. The growth rates of the wild type and the ETH7 and ETH15 mutant strains were compared in Casamino acid medium to which various concentrations of ethanol were added. The growth of the mutants was as sensitive as that of the wild-type strain (Fig. 1). Moreover, the concentrations of different short-chain aliphatic alcohols (methanol, 1 M; ethanol, 0.7 M; 1-propanol, 0.3 M; and 2-propanol, 0.4 M) that lowered the growth rate of the wild type to about 50% in nutrient broth produced the same effect on mutants (data not shown).

Sporulation of *ssa* mutants. Every mutant strain sporulated normally in the absence of alcohol. However, the sporulation frequency of *ssa* mutants (>90%) showed a tendency to be higher than that of the wild type (<80%, Table 2). When ethanol (0.7 M) was added during exponential growth, the sporulation of the wild-type strain was lowered to about 0.5%, whereas the sporulation of mutant strains was much higher (10 to 60%). Variations of these frequencies were observed from day to day, but the ratio of mutant sporulation in ethanol to the wild-type sporulation in the same experiment was relatively constant. Strains ETH7 and ETH15 sporulated at an average frequency of 30 and 40%, respectively, when methanol, 1-propanol, 2-propanol, or ethanol was added (Table 2). Spores were formed in the cultures of *ssa* strains in a normal period of 8 h after T_0, whether they were grown with or without alcohol. Fig. 2 shows the effect of various concentrations of ethanol, which was present during exponential growth, upon sporulation of the wild-type strain and the ETH7 *ssa* mutant, measured at T_{20}. In both cases, similar curves were observed which presented a break at 0.4 M ethanol for the wild type, but at only 0.8 M for mutant ETH7. Moreover, the sporulation frequency of the mutant was always higher than that of the wild type at any concentration.

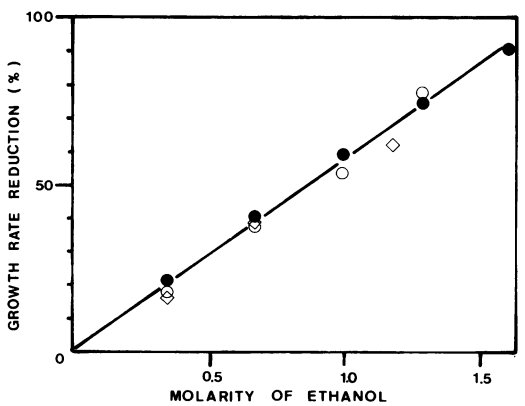

FIG. 1. Ethanol sensitivity of *ssa* mutant growth. Bacteria were grown in Casamino acid medium at 37°C. Symbols: ●, wild-type strain 168; ○, ETH7; and ◇, ETH15.

TABLE 2. Effects of various alcohols on the sporulation of *ssa* and wild-type bacteria

Alcohol added	Concn (M)	Sporulation frequency (%)[a]		
		168	ETH7	ETH15
None		77	95	92
Methanol	1.0	0.5	39	40
Ethanol	0.7	0.4	28	37
1-Propanol	0.3	ND[b]	25	49
2-Propanol	0.4	0.8	34	ND

[a] Sporulation frequency was measured in nutrient broth at 37°C as described in the text.
[b] ND, Not determined.

Phenethyl alcohol (PEA) inhibits sporulation in *B. megaterium* (40) and *B. cereus* (32) at a concentration which only slightly affects their growth. Since PEA seemed to act mainly on the membrane of these bacteria (39), its effects upon *ssa* mutants were examined. The growth rate, the yield, and the sporulation of strain 168 of *B. subtilis* were lowered to about 50% when PEA (0.25%) was added to cultures in nutrient broth (data not shown). In these conditions *ssa* mutants were similar to the wild type. Aliphatic and aromatic alcohols must therefore act in different ways.

Sporulation sensitivity period persists in *ssa* mutants. To select and characterize *ssa* mutants, alcohol was added at the time of inoculation of the cultures. Indeed, when ethanol (0.7 M) was added at T_0 only, the sporulation of *ssa* strains was no longer resistant to alcohol (Fig. 3). Thus,

the resistance of sporulation appeared only if the *ssa* bacteria had previously grown in the presence of alcohol. Moreover when added after T_2, ethanol no longer inhibited the formation of mature spores by *ssa* or wild-type cells (Fig. 3). In wild-type cultures, when exposure to alcohol was restricted to 45-min periods (4), sporulation was inhibited only when such treatments were made between T_0 and T_2 (Fig. 4). Therefore the period of sensitivity of sporulation to alcohols that persists in *ssa* mutants is the same as in the wild type.

Stage at which sporulation was partly blocked in *ssa* alcohol-treated cultures. Some six cytologically defined stages have been recognized during spore formation (36). When wild-type cells are exposed to ethanol (0.7 M) from the beginning of their growth, 75% of the cells are in stage zero of sporulation; at T_7, the 25% remaining cells show abnormal septation (4). Electron microscopic examination of mutant strains (thin section and negative contrast) revealed that the cells which did not sporulate were blocked at stage zero (see Fig. 10). Moreover, 10% of the cells in strain ETH7 showed abnormal septation similar to those observed with the wild type (see Fig. 10). These abnormalities were not seen with mutant ETH15.

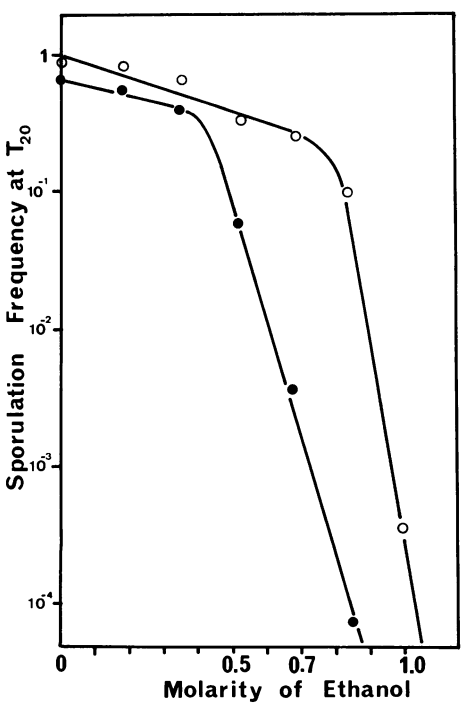

FIG. 2. Resistance of sporulation in strain ETH7. Bacteria were grown and sporulated in nutrient broth at 37°C. Ethanol was added at the beginning of growth. Symbols: ●, wild-type strain 168; and ○, ETH7.

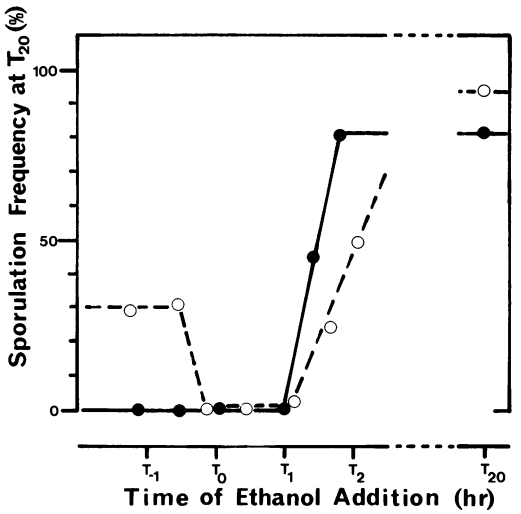

FIG. 3. Time course of ethanol (0.7 M) resistance of strain ETH7 compared with that of the wild type. Bacteria were grown and sporulated in nutrient broth at 37°C. Symbols: ●, wild-type strain 168; and ○, ETH7.

Genetic mapping of *ssa* mutations. To complement this physiological study, an attempt was made to localize two *ssa* mutations on the genetic map of *B. subtilis* by PBS1-mediated transduction (23). Such localization is not easy since the *ssa* phenotype cannot be recognized on plates (see Materials and Methods). It seemed likely however that *ssa* mutations might be located between the markers *phe* and *lys*, like many *spo* mutations and some *spo0* loci especially (18, 22, 31). As shown on Table 3, *ssa-7*

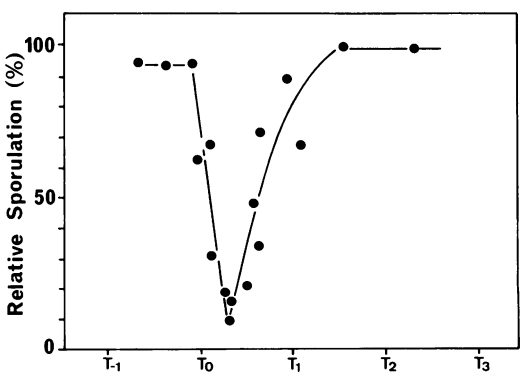

FIG. 4. Time course of the wild-type sporulation sensitivity to ethanol when exposure was limited to 45-min periods. Ethanol exposures were in nutrient broth culture, ending with filtration and resuspension in the filtrate of an ethanol-free parallel culture. Sporulation measured at T_{20} is expressed relative to that in a control culture.

TABLE 3. Two-factor transduction crosses involving *ssa* markers

Recipient genotype[a]	Donor genotype[a]	Recombinant phenotypes		No.	Recombination (%)
		Selection	Ssa		
lys-1	*ssa-7*	Lys^+	Ssa^+	61	65
		(94)	Ssa^-	33	
lys-1	*ssa-15*	Lys^+	Ssa^+	62	65
		(96)	Ssa^-	34	
ssa-15	*acf-1*	Acf^r	Ssa^+	28	81
		(150)	Ssa^-	122	
aroD120	*ssa-15*	Aro^+	Ssa^+	54	68
		(80)	Ssa^-	26	

[a] The relevant markers only are shown.

(ETH7) and *ssa-15* (ETH15) were in fact both located at 65% recombination from the *lys-1* marker and were close to each other. The *ssa* mutations were also linked to *acf-1* (81% recombination) and to *aroD120* (68% recombination). These results suggested that *ssa* mutations are near the *spo0A* locus (22, Fig. 5). Crosses between *ssa* and *spoOA* mutations were not done, because these two types affected in different ways the sporulation ability of the bacteria that carried them. The *strC* marker, located next to *spo0A* and between *aroD* and *spo0A*, was used as the only one which was not involved in sporulation (19).

Recombination between markers was studied by transferring individual colonies onto selective medium as required to score for the *ssa* phenotype (see Materials and Methods). It can be seen in Table 4 and Fig. 5 that the linkage observed between *aroD120* and *strC23*, between *spo0A47* and *lys-1*, and between *lys-1* and *spo0A47* were in good agreement with published results (22, 42).

The results of two marker crosses led to two possible orders for the *ssa* mutations: *aroD-ssa-strC* or *aroD-strC-ssa*. However, three-factor transduction crosses did not permit ordering these loci. As shown in Table 5, minority recombinants were actually not observed in one class, but in two quite important classes (about 10% each). Such results could reflect some interaction between the studied markers, the nature of which remains unknown.

Phospholipid composition of various strains of *B. subtilis*. During exponential growth the relative amount of each phospholipid may vary considerably, especially if growth is biphasic as may be the case in nutrient broth (35). To compare various strains, it is thus necessary to employ strictly controlled conditions of growth. A conditioned nutrient broth has been used in this way (35). In the present work we resorted to different methods: addition of glucose (medium NBG) or dilution of culture at $T_{0.5}$ (see Materials and Methods).

Growth of *ssa* and ssa^+ strains in NBG medium was identical (data not shown). For both types, ethanol reduced the growth rate in the same proportions as in nutrient broth (4). Nevertheless inhibition of growth was immediate in NBG, whereas it was delayed in nutrient broth (4, 34). During early exponential growth in NBG, the wild-type membrane contained three main phospholipid components (Table 6): phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were in equal proportion, each 40% of the total phospholipids, and lysylphosphatidylglycerol (LPG) reached 20%. This high amount of LPG might result from acidification of the medium produced by glucose consumption (20);

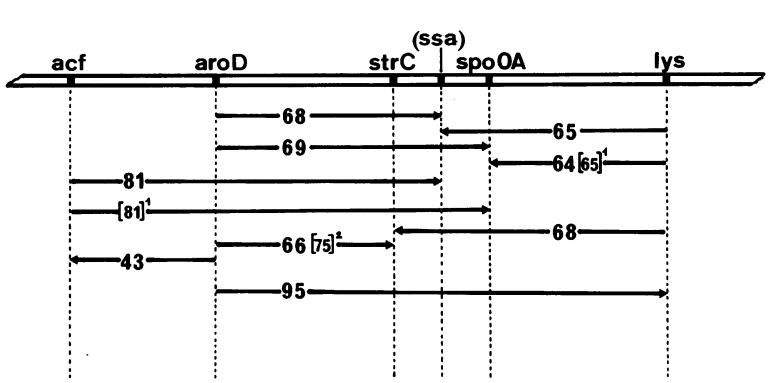

FIG. 5. Map of the *acf-lys* chromosomal segment. Distances are expressed as the percentage of recombination in PBS1 transduction. The heads of the arrows point to the unselected markers. Values are taken from Tables 3 to 5, except for the values between brackets, which are from Ionesco et al. ([1]; [22]) and Staal and Hoch ([2]; [42]).

TABLE 4. Two-factor transduction crosses involving no *ssa* markers

Recipient genotype[a]	Donor genotype[a]	Recombinant phenotypes[b]		No.	Recombination (%)
		Selection	Classes		
aroD120	*acf-1*	Aro$^+$ (260)	Acf^s	110	43
			Acf^r	150	
aroD120	*spo0A47*	Aro$^+$ (260)	Spo$^+$	179	69
			Spo$^-$	81	
aroD120 strC23	Wild type	Aro$^+$ (104)	StrC$^+$	35	66
			StrC$^-$	69	
aroD120 lys-1	Wild type	Aro$^+$ (260)	Lys$^+$	13	95
			Lys$^-$	247	
lys-1	*spo0A47*	Lys$^+$ (208)	Spo$^+$	134	64
			Spo$^-$	74	

[a] The relevant markers only are shown.
[b] Recombinant phenotypes were determined by picking the colonies in the particular way required for Ssa recognition.

yet it remained steady in the growth period during which phospholipids were extracted (optical density, 0.10 to 0.35).

The phospholipids of four *spo0* strains, isogenic with wild-type strain 168 and bearing mutations in four distinct loci, were measured in these new conditions. Our results (Table 6) are in agreement with those of Rigomier and Lubochinsky (35). *spo0A* and *spo0B* mutants contained slightly more phospholipid than the wild type did and differed from it by a relative deficiency of PE and LPG, whereas *spo0C* and *spo0D* were little modified. Both *ssa* strains revealed a similar and even more pronounced modification of phospholipid composition than did the *spo0A* and *spo0B* mutants. The phospholipid imbalance, observed in NBG medium (Table 7), consisted of an increase in PG (+90%) and a decrease in LPG (−50%), whereas the amount of PE remained unchanged. However in other physiological conditions, *ssa* strains contained a lower amount of PE (for instance, −25% in nutrient broth; Table 8).

The development of the phospholipid composition through subsequent generations and sporulation was the same in *ssa* and *ssa*$^+$ strains. It essentially consisted of stopping PE accumulation in the membrane soon after T_0, in an enrichment in PG until about T_2 to T_3, and in a

TABLE 5. Three-factor transduction crosses for the mapping of *ssa* marker

Recipient genotype[a]	Donor genotype[a]	Recombinant phenotypes		No.	Implied order
		Selection	Classes		
aroD120 strC23	*ssa-7*	Aro$^+$ (104)	Ssa$^+$ StrC$^-$	59	*aroD-ssa-strC* or *aroD-strC-ssa*
			Ssa$^+$ StrC$^+$	11	
			Ssa$^-$ StrC$^-$	10	
			Ssa$^-$ StrC$^+$	24	

[a] The relevant markers only are shown.

TABLE 6. Phospholipid composition of various *B. subtilis* strains

Phenotype	Strain	Percentage of each phospholipid[a]			PG/PE	Phospholipid concn (mutant/wild-type)
		PE	PG	LPG		
Wild type	168	38	42	20	1.1	—
Spo0A	110NA	34	51	15	1.5	1.3
Spo0B	6Z	29	58	13	2.0	1.3
Spo0C	9V	36	45	19	1.3	1.0
Spo0D	3U	36	47	17	1.3	0.9
Ssa	ETH7	28	63	9	2.2	1.2
	ETH15	31	57	13	1.9	1.2

[a] Extractions were performed from early exponential cultures in NBG medium (see text). PE, Phosphatidylethanolamine; PG, phosphatidylglycerol, and LPG, lysylphosphatidylglycerol.

TABLE 7. Phospholipid composition of the wild-type strain and an *ssa* mutant grown with or without ethanol

Strain	Culture without alcohol		Culture with alcohol		Phospholipid concn with/without alcohol
	nmol[a]	%[b]	nmol	%	
168					
PL[c]	2.39		1.82		0.76
PE	0.95	40	0.88	48	0.93
PG	0.95	40	0.69	38	0.73
LPG	0.49	20	0.25	14	0.51
PG/PE	1.0		0.8		
ETH7					
PL	2.95		2.32		0.79
PE	0.91	31	0.96	41	1.05
PG	1.81	61	1.21	52	0.67
LPG	0.23	8	0.15	7	0.65
PG/PE	2.0		1.3		

[a] Results are expressed as nanomoles of phospholipid per ml of culture per optical density unit (at 570 nm).
[b] Percentage for each phospholipid (PE, PG, and LPG) in relation to the total (PL).
[c] Extractions were performed from early exponential cultures in NBG medium at least 1 h after 0.7 M ethanol was added (after the case).

TABLE 8. Phospholipid composition of the wild-type strain and an *ssa* mutant in mid-log culture in nutrient broth

Strain	Phospholipid composition				
	PL	PE	PG	LPG	PG/PE
168					
nmol[a]	6.12	1.13	4.24	0.75	3.7
%[b]		18	69	12	
ETH7					
nmol	6.29	0.86	4.91	0.52	5.7
%		14	78	8	

[a] and [b], See footnotes to Table 7.

drastic decrease in LPG (Fig. 6). Thus, the differences observed between both types during exponential growth persisted during sporulation.

Whereas *ssa* and *ssa*$^+$ bacteria differed in phospholipid composition, such was not the case for their fatty acid substituents. For instance at the end of exponential growth ($T_{-0.5}$), branched fatty acids 12-methylhexadecanoic (a-C_{15}, 45% of the total fatty acids) and 15-methylhexadecanoic (i-C_{17}, 26%) were the major constituents in *ssa* mutants as in the wild type (data not shown). There were also no differences in fatty acids between both types of bacteria observed at T_3 during sporulation (results not shown).

Effect of ethanol on phospholipid composition of bacteria. When grown in NBG with 0.7 M ethanol, the bacteria contained 20% less phospholipids (Table 7). The decrease of phospholipids resulted from a differential effect of ethanol on each species. A reduction of PG (−30%) and LPG (−50%) was observed, but there was no effect on PE. Ethanol had an identical effect quantitatively upon *ssa* or *ssa*$^+$ strains (Table 7). It was the same for the kinetic aspect of this inhibition; in fact, when ethanol was added, the growth of both bacterial types was immediately slowed down, and phospholipid accumulation stopped. This lasted about 20 min, then, after a short 10-min period of increase in synthesis necessary to reach a new balance, incorporation resumed at a rate commensurate with the new growth rate (Fig. 7). This phenomenon essentially concerned PG (Fig. 7) and LPG (results not shown); PE was very slightly affected. From this point of view, the mutant and the wild type also had identical behavior (Fig. 7).

Development of the amount of each major phospholipid during growth and sporulation (if any) in nutrient broth with 0.7 M ethanol added is shown in Fig. 8. With these conditions, the sporulation frequency of *ssa* strain was 30%, that of the wild type was 0.5%. As in cultures without alcohol, LPG drastically decreased after T_0; PE, which was stable when alcohol was absent, also tended to decrease then. The behavior of *ssa* and *ssa*$^+$ types for these two phospholipids was identical, whereas important differences existed in PG. In the wild type, which did not sporulate, the amount of PG was significantly reduced after T_1, whereas it slightly increased in the mutant, which partially sporulated. It appears that a rise in PG after T_0 is connected with the progress of sporulation, at least under the given conditions.

Ethanol had the same effect on the fatty acid composition of *ssa* or *ssa*$^+$ bacteria. It was mainly a decrease in the relative amount of 12-methylhexadecanoic acid (a-C_{15}) to the benefit of 15-methylhexadecanoic (i-C_{17}) and hexadecanoic (n-C_{16}) acids (results not shown). It proved the same for both strains at T_3 (results not shown).

Phospholipid turnover with or without alcohol. The differences in phospholipid composition observed between *ssa* and *ssa*$^+$ strains and for a given strain, depending on the presence of alcohol, could be the result of modifications of either phospholipid synthesis or degradation. Phospholipid turnover of both bacterial types did not differ very much (Fig. 9A); PE was always stable, whereas the renewal rate of PG was particularly fast, since 50% of the $^{32}PO_4^{2-}$ previously incorporated in this lipid was lost within 11

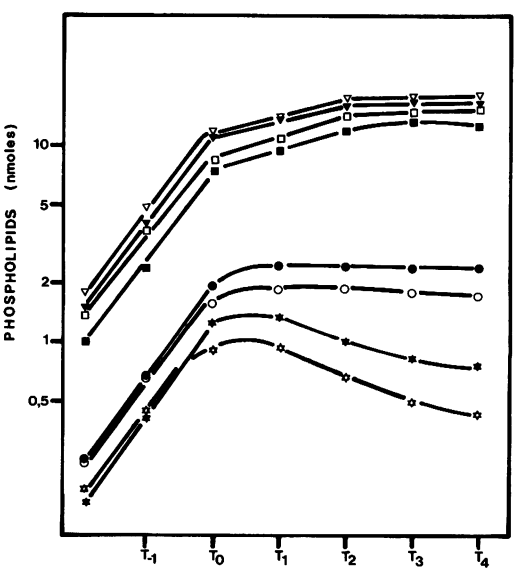

FIG. 6. Phospholipid composition of bacteria at the end of exponential growth and, later, in nutrient broth without alcohol. Filled symbols, wild type; empty symbols, ETH7. Symbols: (▼, ▽) PL, or total phospholipid, (■, □) PG, (●, ○) PE, and (★ ☆) LPG. Phospholipid concentrations were measured in 1 ml of culture (see text).

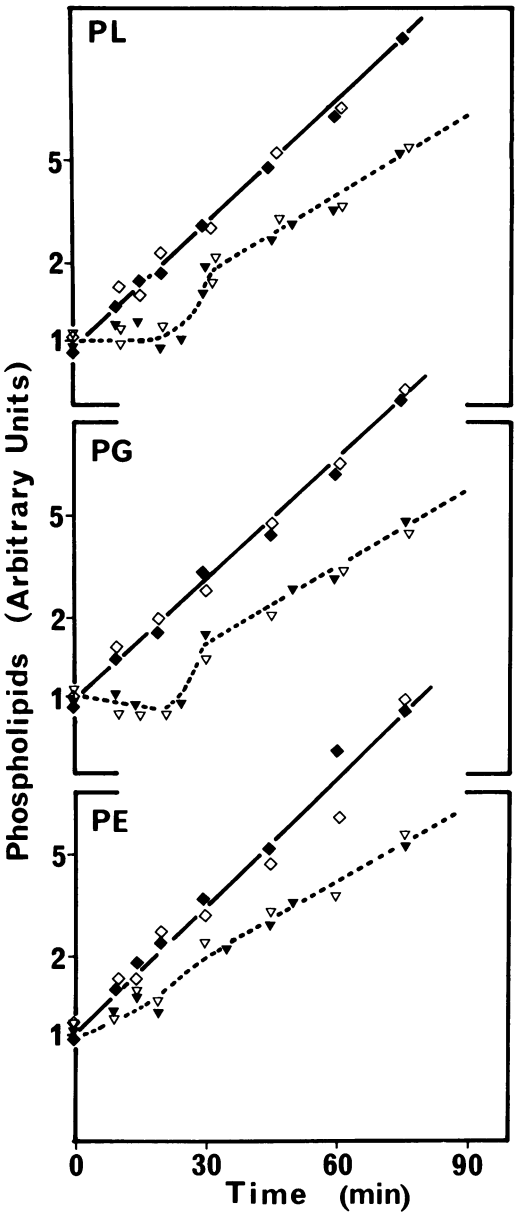

FIG. 7. Kinetics of phospholipid accumulation in bacteria. Ethanol (0.7 M) was added to early exponential cultures in NBG (optical density at 570 nm ≈0.1) at time zero of the experiment. Filled symbols, wild type; empty symbols, ETH7. —, Cultures without alcohol; ---, cultures with alcohol. Extractions were performed from 1 ml of culture (see text). To simplify the comparison between the two strains and the compilation of four independent experiments, the amounts of PL (total phospholipid), PG, and PE were arbitrarily chosen as unitary at time zero.

min of chase. As for LPG, it appeared less stable in *ssa*$^+$ than in *ssa* bacteria. The addition of ethanol at the beginning of chase did not modify the final apparent rate of turnover of PG and PE, but stimulated that of LPG (Fig. 9B). This was the same for both strains.

DISCUSSION

In nutrient broth containing 0.7 M ethanol, *B. subtilis* wild-type strain 168 grows at a doubling rate of 0.9 per h and sporulates at a frequency of only 0.5%. *ssa* mutants have been isolated from this wild-type strain. The growth of the mutants is equally sensitive to ethanol, but they sporulate at 30 to 40% under these conditions. The sporulation of *ssa* mutants is resistant not only to ethanol but also to other short-chain aliphatic alcohols. Thus, in *ssa* strains, the sporulation sensitivity to alcohols is clearly dissociated by mutation from the sensitivity of growth. At least one necessary step in sporulation is abolished by adding alcohol to the wild type, but not to the mutant strains. Moreover, the fact that the resistance induced by *ssa* mutations is not only directed to the ethanol used in their selection but also to other alkanols suggests that they act upon sporulation by perturbing the bilayer structure rather than by interacting with a particular protein.

Since PEA equally inhibits growth and sporulation of the wild type and *ssa* mutants, this alcohol must act upon the bacterial membrane (40) in a different way from that of the short-chain aliphatic alcohols studied here. This sug-

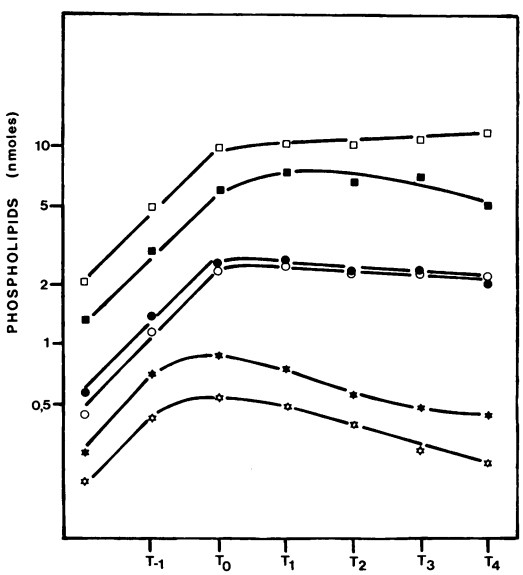

FIG. 8. Phospholipid composition of bacteria at the end of exponential growth and, after, in nutrient broth with 0.7 M ethanol added. For details see the legend to Fig. 2.

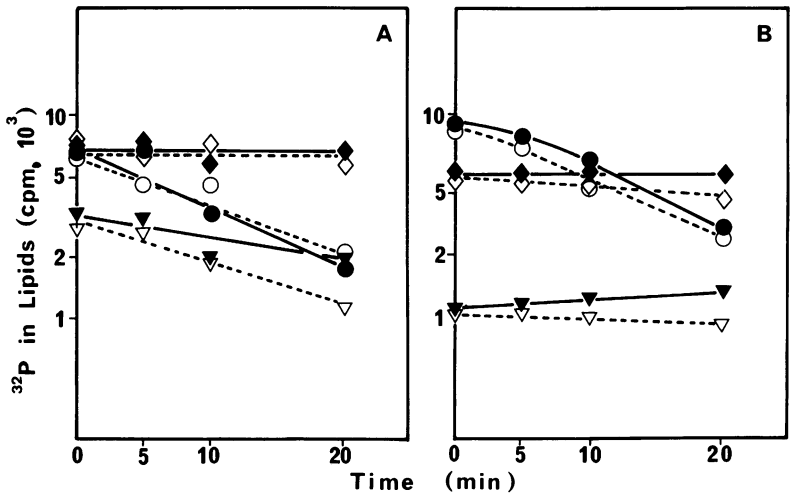

FIG. 9. Phospholipid turnover in the wild-type strain (A) and in mutant ETH7 (B) with (empty symbols) or without (filled symbols) 0.7 M ethanol. The bacteria were grown for three or four generations in NBG (without alcohol) containing $^{32}PO_4^{2-}$, and then they were quickly filtered, washed, and suspended in the filtrate of a nonradioactive culture in NBG medium (optical density at 570 nm $\simeq$0.2). Phospholipid concentrations were measured in 1 ml of culture.

gests the necessity of a more systematic study on the effects of alcohols by their hydrophobicity upon the growth and sporulation of *B. subtilis*.

When various concentrations of alcohol were added to the growth medium, sporulation was always higher in an *ssa* mutant than in the wild type; the sporulation frequencies of both types of bacteria diminished in the same way when the alcohol concentration increased. Therefore, *ssa* mutations induce a shift of the bacterial response towards the high concentrations of alcohol, although the mechanism(s) of inhibition probably remains the same.

Like the wild-type strain, *ssa* mutants remain sensitive to the addition of alcohol during the period T_0 to T_2. This further indicates that sensitivity may be of the same kind in the *ssa* and *ssa*$^+$ strains. This result suggests a structural state of the membrane at the end of exponential growth, which makes it more sensitive to alcohol than during the growth before T_0. This particular state would not be realized if alcohol was present in the medium before T_0.

Like the wild-type cells, all of the sensitive mutant cells appear to be blocked by alcohol in stage zero of sporulation. This suggests that only a single step is sensitive to alcohol. If many steps were sensitive, one would expect to find cells blocked at various stages.

As previously reported (9), it is difficult to make a genetic analysis of mutations in which the phenotype is not easily recognizable; therefore, our experiments remain incomplete. However, if the results presented here do not enable us to answer the question of identity between *ssa* and *spo0A* genes, at least they show that these mutations are closely located on the chromosome.

ssa mutants also differ from the wild-type strain from which they were derived in the phospholipid composition of their membranes during exponential growth without alcohol and during sporulation. In *ssa* strains, only the balance of phospholipid polar head groups is modified, whereas the fatty acid composition remains unchanged. Thus, in nutrient broth with added glucose, *ssa* bacteria contain twice as much PG as do wild-type bacteria and half as much LPG. Nevertheless this difference seems to be relative, since under various growth conditions, phospholipid composition significantly changes and since the imbalance of head groups may then result from a deficit of PE. In fact we always observed an enrichment of the *ssa* membrane in negatively charged phospholipids (i.e., PG at neutral pH) in comparison with neutral or positively charged ones.

A similar phenomenon had been observed in asporogenous mutants (35) especially in *spo0A* strains in which no alteration of any phospholipid biosynthetic enzyme could be detected (D. Rigomier, Thèse de Doctorat d'Etat, Université de Poitiers, France, 1977). Thus we can say that the physiological similarity and genetic linkage we have shown between *ssa* and *spo0A* mutants

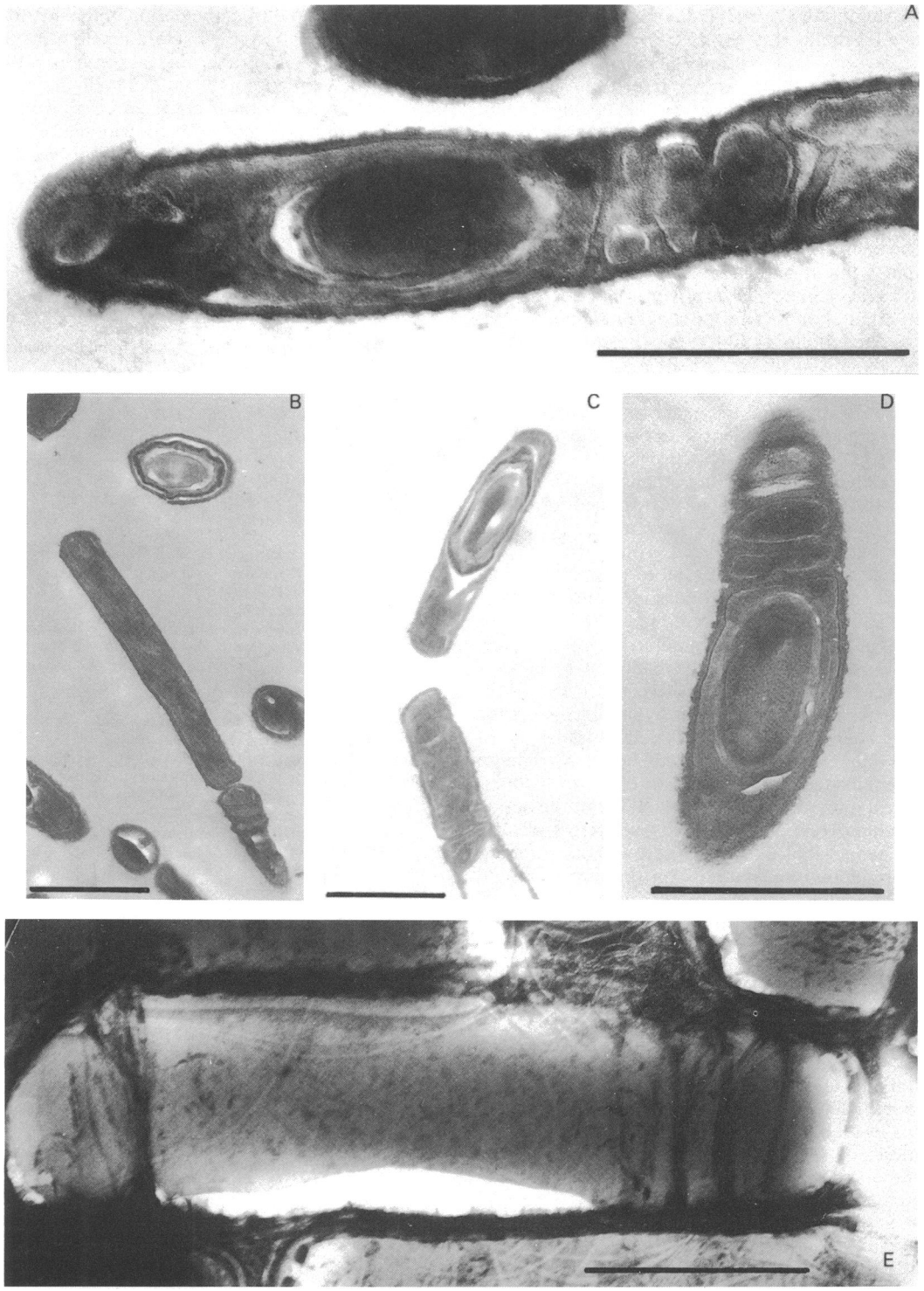

FIG. 10. ETH7 cells were incubated in nutrient broth, to which ethanol was added four generations before cessation of exponential growth (T_0). (A) to (D), Thin sections of cells harvested at T_7; (E), cells examined at T_7 (negative contrast). Bar, 1 μm.

are completed by a biochemical analogy at the membrane level.

The fact that mutants in which sporulation resists ethanol are impaired in the balance of their various phospholipids is in good agreement with the hypothesis that the primary effect of this short-chain aliphatic alcohol is at the phospholipid polar head group level (34). However, it is striking that such *ssa* mutants react to addition of alcohol in the same way as do ssa^+ strains. The bacteria of both types reduce their phospholipid content and modulate the amount of PG and LPG. Since PG turnover is not accelerated by ethanol and since the alcohol cannot solubilize phospholipids (34), it may be concluded that in *ssa* and ssa^+ bacteria the synthesis of PG itself is inhibited by alcohol. With LPG, its faster turnover and the decrease of its precursor (PG) could be sufficient to explain the inhibition of its accumulation. Kinetic study showed that immediately after adding ethanol to the growth medium, the synthesis of PG stopped for nearly one generation and then resumed, creating a new balance of polar head groups in the membrane. This process was more pronounced and faster in NBG than in conditioned nutrient broth (34). Such a difference could be explained by a smaller amount of phospholipid in cells grown in NBG medium, where the bacterial membrane thus would be more sensitive to alcohol addition. As previously shown, ethanol acted at the fatty acid level also (34). The membranes of *ssa* and ssa^+ bacteria tended to be more rigid to compensate for the fluidizing effect of alcohol. (For a more detailed discussion about the action of ethanol on *B. subtilis* lipids, see reference 34.)

The fact that, normally (i.e., without alcohol), sporogenous *ssa* mutants on the one hand and asporogenous *spo0A* mutants on the other hand show a common character makes the interpretation and understanding of the mode of action of the altered genes more difficult. When ethanol was present, *ssa* bacteria, which were able to make spores, showed a phospholipid composition more closely related to that of the wild-type bacteria without alcohol. This composition is unlikely to be the origin of the ability to make spores. The observed phospholipid composition in the mutants might be the reflection of alterations of regulation mechanisms of the membrane and thus of the physicochemical state of the bacterial membrane.

In the wild type, various reorganizations of membrane proteins occur between T_0 and T_2 (12, 15); they are concomitant with changes in activity of several membrane-bound enzymes (13, 14, 24, 26, 33). Moreover, modifications in the composition or activity of enzymes located in the membrane were observed in stage-zero asporogenous mutants (3, 5, 11, 17, 37, 44). One can consider that during the period of the institution of differentiation, the bacterial membrane is the seat of a considerable rearrangement of its different constituents and thus of their interactions. The correct progress of the process would depend upon mechanisms for the modulation of those interactions, and these mechanisms would be disturbed by alcohols or by certain mutations.

ACKNOWLEDGMENTS

We are grateful to C. Anagnostopoulos and P. Schaeffer for their helpful discussion and criticisms and to E. P. Kennedy for critical reading of the manuscript. We thank A. Bohin and C. Besse for their excellent technical assistance.

This work was supported by a grant from the Centre National de la Recherche Scientifique (L. A. 290).

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