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Isolation and Characterization of a Phosphatidylethanolamine-Deficient Mutant of *Bacillus subtilis*

JAMES L. BEEBE[1]

Department of Bacteriology, Rutgers—The State University, New Brunswick, New Jersey 08903

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A mutant of *Bacillus subtilis* ATCC 6051 deficient in phosphatidylethanolamine, an important membrane lipid, was isolated by a combination of nitrosoguanidine mutagenesis and penicillin concentration of auxotrophs employing phosphatidylethanolamine as a supplement. The mutant was compared to the parent strain with regard to lipid composition, growth, osmotic fragility, and staining character and differed substantially in each category. In addition to scant amounts of phosphatidylethanolamine, the mutant contained phosphatidylglycerol, cardiolipin, lysyl phosphatidylglycerol, and diglucosyldiglyceride, though in amounts differing from those found in the parent strain. The mutant was unable to grow appreciably on synthetic media, had enhanced osmotic fragility of protoplasts, and resisted decolorization in staining.

The diversity of lipid classes found in microorganisms (15) strongly suggests, at least to us, a concurrent diversity of function. However, the role of specific lipids in prokaryotic membrane function, especially transport, has been relegated largely to conjecture, primarily for the lack of appropriate mutants. The difficulties of supplementation as noted by Cronan et al. (9) appears to be the chief obstacle in this regard.

Advances have been made in attempts to define the role of lipids in general, mainly phosphoglycerides. Cronan et al. (9) reported the isolation and properties of a temperature-dependent fatty acyl transferase mutant of *Escherichia coli*. A mutant of *E. coli* which is unable to synthesize phosphatidic acid due to an alteration of fatty acyl transferase activity was reported by Kito, Lubin, and Pizer (18). Both mutants suffer a decline of various synthetic activities (ribonucleic acid, deoxyribonucleic acid, protein, phospholipid) when a lethal temperature is introduced as in the former or supplement is removed as in the latter.

Mindich (22, 23) has described similar results with deprivation of a glycerol-requiring mutant of *Bacillus subtilis*. These effects attest to the importance of lipids in normal cellular (membrane) function but contribute little to the knowledge of the role of individual lipid classes.

Vagelos and his group have also isolated unsaturated fatty acid auxotrophs of *E. coli* (31). Supplementation of these auxotrophs with various unsaturated fatty acids has been documented (14, 29, 30). Unsaturated fatty acid auxotrophs of *Saccharomyces cerevisiae* have also been isolated (17, 27, 38). To our knowledge none of these mutants have been characterized for transport alterations.

Evidence exists that implicates phosphatidylglycerol in sugar transport in *E. coli* (21) and in *S. cerevisiae* (11). These are the only instances in which a specific lipid has been shown to participate in membrane transport in microorganisms. Little is known regarding the role of such lipids as phosphatidylethanolamine (PE), cardiolipin, phosphatidylcholine, glycolipids, or amino acid-containing lipids.

In this paper we document the isolation and some pertinent properties of a mutant of *B. subtilis* ATCC 6051 which is incapable of optimal synthesis of PE. The effects of this mutation were profound and varied.

MATERIALS AND METHODS

Growth conditions. *B. subtilis* ATCC 6051 was grown at 37 C in media described by Gary and Bard (13). The concentration of glucose in the synthetic (S) medium was 0.25% (w/v). Cells were grown in 10-liter

[1] Present address: Department of Microbiology, Cornell University Medical College, New York, N.Y. 10021.

volumes in a New Brunswick model FS314 fermentor with sterile air delivered at a rate of 5 liters per min and an impeller speed of 200 rev/min, or in 1-liter flasks (250 ml of medium) in a New Brunswick Scientific controlled-environment incubator shaker at 250 rev/min. Cells were harvested from large volumes of medium by centrifugation in a supercentrifuge (Sharples Corp., Philadelphia, Pa.) and from smaller volumes in a Sorvall RC-2B superspeed centrifuge.

Mutant isolation. Late-log-phase cells of strain ATCC 6051 grown in Tryptic Soy Broth (TSB, Difco) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to the optimum conditions for nitrosoguanidine mutagenesis reported by Adelberg et al. (1). This was followed by penicillin concentration of mutants by a modification of the method of Davis et al. (10).

Usually, 4×10^8 cells were incubated statically at room temperature with NTG at a concentration of 100 μg/ml in 0.1 M phosphate buffer (*p*H 6.0) for 15 min, washed, and placed in 15 ml of S medium containing 25 μg of PE (Nutritional Biochemicals Corp., Cleveland, Ohio) per ml but no glucose. The cells were incubated with shaking for 1 hr, and then the cells were harvested, washed, and placed in fresh S medium (15 ml) which contained 0.25% glucose and penicillin G (300 units/ml). Further incubation resulted in a 95% reduction in optical density within 3 hr. The cells were harvested and resuspended in fresh S medium, and samples were spread plated to master Tryptic Soy Agar (TSA) plates (TSA:TSB plus 1.75% agar). In some trials, the penicillin treatment was omitted and the NTG-treated cells were washed and plated as described. The master plates (some of which were also supplemented with PE) were incubated for 12 hr at 37 C and then replicated with sterile velveteen (19) to S agar plates and S agar plates supplemented with PE (25 μg/ml). Replicas were incubated for 12 to 24 hr and compared. Possible mutants were tested by streaking on supplemented and unsupplemented S agar plates.

Lipid extraction and purification. Cells were extracted exhaustively with chloroform-methanol (2:1, v/v). The extract was purified by a combination of a modified Folch wash (12) and passage through Sephadex G25 by the procedure of Wells and Dittmer (36). Extraction and purification were performed under an atmosphere of nitrogen gas.

Column and paper chromatography. Quantities of individual lipids sufficient for characterization were obtained by a combination of silicic acid and diethylaminoethyl cellulose column chromatography. Purified lipid (approximately 12 mg of lipid phosphorus) dissolved in chloroform was loaded on a 1.3 by 15 cm silicic acid column (Mallinckrodt, 100 mesh). The column was discontinuously eluted with chloroform to elute neutral lipid, acetone to elute glycolipid (Y1), chloroform-methanol (94:6, v/v) to elute cardiolipin, and chloroform-methanol (1:1, v/v) to elute the remaining polar lipids. The last fraction was reduced to dryness, dissolved in chloroform-methanol (9:1, v/v), and loaded on a 1.3 by 15 cm diethylaminoethyl cellulose (Schleicher & Schuell Co., Keene, N.H.) column and eluted with chloroform-methanol (9:1, v/v) followed by chloroform-methanol (7:3, v/v) to remove amino phosphatides (PE, Y2) and chloroform-methanol (4:1, v/v, plus 20 ml of concentrated ammonium hydroxide per liter and made 0.5 M with respect to ammonium acetate) to elute phosphatidylglycerol. All fractions were rechromatographed on silicic acid-impregnated paper (Whatman SG81) on solvent systems described by Marinetti (20). Additionally, total separation of all the lipid components of a whole extract was performed by two-dimensional paper (SG81) chromatography, using chloroform-methanol-water (65:25:4, v/v/v) in the first direction followed by diisobutyl ketone-glacial acetic acid-water (40:25:5, v/v/v) in the second direction.

Characterization of lipids. The lipids were identified by the use of several criteria. The chromatographic mobility of whole and deacylated lipids was determined. The following solvent systems were used for diacyl lipids: (i) diisobutyl ketone-glacial acetic acid-water, (40:20:3, v/v/v), (ii) diisobutyl ketone-glacial acetic acid-water (40:25:5, v/v/v), and (iii) chloroform-methanol-water (65:25:4, v/v/v) (20). Deacylated forms were chromatographed on Whatman no. 1 paper by using (i) 75% phenol and (ii) *n*-butanol-propionic acid-water (142:71:100, v/v/v) (6). Diacyl lipids were revealed by reaction with rhodamine 6G (20), ninhydrin (20), ammonium molybdate for phosphorus (3), and periodate-Schiff (34) and periodate-benzidine (7) for vicinal hydroxyls and aniline-acid-phthalate (4). Ninhydrin and ammonium molybdate (6) were used to detect water-soluble phosphate esters. Acid hydrolysis of the glycolipid and the amino acyl phosphatidylglycerol were performed by the procedures described by White et al. (37). Dinitrophenyl derivatives of the amino acid moiety of the amino acyl phosphatidylglycerol and authentic dinitrophenyl amino acid standards were made by the method of Sanger (28). The acid hydrolysis products of the glycolipid and authentic carbohydrate standards were chromatographed on Whatman no. 1 paper on (i) isopropanol-glacial acetic acid-water (3:1:,v/v/v, ascending) and (ii) isopropanol-water (4:1, v/v, ascending). Amino acids and the acid hydrolysis products of the amino acyl phosphatidylglycerol were chromatographed on Whatman no. 1 paper on (i) *n*-butanol-glacial acetic acid-water (4:1:5, v/v/v, upper phase, ascending) and (ii) phenol-water, (160:40, w/v, ascending). Dinitrophenyl derivatives were chromatographed in water-saturated *n*-butanol in the descending mode. Deacylation was carried out by the procedure of White et al. (37).

Analytical procedures. Molar ratios for the various constituent moieties of each isolated lipid were determined. Values were obtained for phosphorus (2), glycerol (26), fatty acid ester (32), and carbohydrate by anthrone (25).

Quantitation of lipids. The relative amount of each lipid was measured as percentage of ^{14}C radioactivity incorporated. D-Glucose-*UL*-^{14}C (specific activity, 180 μCi/μmole) was added to cultures at a rate of 0.1 μCi/ml. Lipid extracts were obtained from cells grown on these labeled media and chromatographed in the two-dimensional system described previously. Lipids were detected and eluted with 95% methanol, and the activity of each was determined by liquid scintillation

spectrometry (Nuclear-Chicago Unilux II Liquid Scintillation Spectrometer). The amount of each lipid (including neutral lipid fraction) was recorded as percentage of ^{14}C activity of the sample chromatographed.

Osmotic fragility. Protoplasts of both strains were obtained by the lysozyme treatment of Weibull (35). The stable protoplasts were subjected to an osmotic fragility test modeled on that used by Kappy and Metzenberg (16). Samples (0.1 ml) of protoplast suspensions were placed in 1.0-ml volumes of mannitol solutions of varying molarity (0.0 to 0.06 M). After 0.5 hr of incubation at 37 C, the number of intact protoplasts in samples of each solution was enumerated in a Petroff-Hausser counting chamber, and the percentage of lysis was determined.

Growth measurement. Both strains were grown to stationary phase in TSB, harvested, and washed with S medium. Test medium (100 ml in 250-ml culture flasks) was inoculated with 0.1 ml of washed cell suspensions (optical density = 10). The flasks were incubated at 37 C with shaking (200 rev/min) in a New Brunswick Scientific gyratory water-bath shaker. The optical density of the growing cultures was measured at intervals with a Bausch & Lomb Spectronic 20 spectrophotometer at 540 nm. Dry weight determinations were conducted on 18-hr-old cultures.

Chemicals. The lipid standards used were cardiolipin, PE, and Tween 80, products of Nutritional Biochemicals Corp., Cleveland, Ohio. Phosphatidylserine was a product of Pierce Chemical Co., Rockford, Ill. Chloroform, methanol, acetone, and phenol were distilled before use in chromatography or extraction. The D-glucose-^{14}C used in lipid quantitation experiments was a product of New England Nuclear Corp., Boston, Mass.

RESULTS

Characterization of the lipids. Purified lipid extracts of *B. subtilis* ATCC 6051 were found to contain four phospholipids and one glycolipid. The phospholipids were designated PE, phosphatidylglycerol, cardiolipin, and Y1. The glycolipid was designated Y2. Table 1 summarizes the data obtained in characterization of these lipids. Table 2 shows the chromatographic mobility of the deacylated water-soluble products of these lipids and authentic standards in two solvent systems.

On the basis of the data presented in these tables, the lipids were identified as PE, phosphatidylglycerol, and cardiolipin.

Chromatography of the acid hydrolysis products of Y2 produced a ninhydrin-positive spot with a chromatographic mobility identical to L-lysine. Reaction of the acid hydrolysis products of Y2 with 1-fluoro-2,4-dinitrobenzene produced a colored derivative with a chromatographic mobility identical to dinitrophenyl-lysine. Deacylated Y2 produces two components, one with the mobility of L-lysine and a second ninhydrin-negative spot with an R_F similar to glyceryl-phosphorylglycerol. On the basis of the preceding data, Y2 is identified as lysyl phosphatidylglycerol. Chromatography of the acid hydrolysis products of Y1 yielded two periodate-sensitive spots. R_F values of 0.39 and 0.63 were obtained for isopropanol-glacial-acetic acid-water (3:1:1, v/v/v, ascending). Glycerol and glucose had R_F values of 0.63 and 0.39 on the same system. Detection with aniline acid phthalate (4) indicates that the slower-moving component was hexose. Accordingly, Y1 is identified as diglucosyldiglyceride. The identity of these lipids agrees with the reports of Bishop et al. (5) and Op Dan Kamp et al. (24).

Isolation of mutant. Among the possible mutants tested, a single strain exhibited negligible growth on S agar but reasonable growth on S agar supplemented with PE. This strain, designated 6051-4, was capable of limited growth in liquid S medium. A preliminary extract indicated a deficiency of PE in this strain. *B. subtilis*

TABLE 1. *Properties of the lipids of Bacillus subtilis ATCC 6051*

Lipid[a]	Molar ratios[b]				R_F values[c]			Reactions[d]			
	P	G	FA	CHO	1	2	3	RG6	nin	P	IO_4
Y2	1.00	1.88	2.03		0.07	.16	.10	yel	+	+	−
Y1	0.00	0.89	2.10	2.00	0.23	.39	.90	yel	−	−	+
PG	1.00	1.68	1.95		0.30	.36	.67	bl	−	+	+
PE	1.00	1.03	2.31		0.33 (.33)	.44 (.45)	.62 (.62)	pk	+	+	−
CL	1.00	1.61	2.47		0.60 (.60)	.63 (.63)	.95 (.95)	bl	−	+	−

[a] PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin.

[b] P, phosphorus; G, glycerol; FA, fatty acid ester; CHO, carbohydrate by anthrone.

[c] R_F values: 1, diisobutyl ketone-glacial acetic acid-water (40:20:3, v/v/v); 2, diisobutyl ketone-glacial acetic acid-water (40:25:5, v/v/v); 3, chloroform-methanol-water (65:25:4, v/v/v). R_F values for phospholipid standards in parentheses.

[d] Reactions: R6G, rhodamine 6G; nin, ninhydrin; P, phosphate; IO_4, periodate benzidine for vicinal hydroxyls; yel, yellow; bl, blue; pk, pink.

ATCC 6051 contains two colonial variants, a smooth form and a rough form. The rough colonial variant was used in these investigations. This character remains unchanged in the mutant.

The use of PE as a supplement in liquid or solid media, although far from satisfactory, proved to be the only system amenable in the isolation of a PE-deficient mutant. Phosphatidylserine, L-serine, and monoethanolamine proved fruitless as supplements in numerous trials.

TABLE 2. *Chromatography of water-soluble products of deacylation of lipids of Bacillus subtilis*

Lipid[a]	R_F[b]	
	75% Phenol	BPW
Y2[c]	0.39, 0.60 n	0.14, 0.56 n
PG[c]	0.37	0.17
PE[c]	0.60 n	0.18 n
CL[c]	0.17	0.10
GPGPG	0.16	0.09
GPE	0.65 n	0.23 n
GPG	0.40	0.17

[a] PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; GPGPG, 1,3 diglycerylphosphorylglycerol; GPE, glycerylphosphorylethanolamine; GPG, glycerylphosphorylglycerol.

[b] R_F values for GPGPG, GPE, and GPG are those quoted by Card et al. (6). BPW, *n*-butanol-propionic acid-water (142:71:100, v/v/v); n, ninhydrin-positive.

[c] Water-soluble products of deacylation of these lipids.

Attempts to calculate percentage of reversion of the mutant were not made because the mutant was able to grow, however poorly, on unsupplemented S agar medium, prohibiting accurate identification of revertants.

Quantitation of individual lipids. Total lipid of ATCC 6051 accounted for 4.03% of the dry weight of the cells on the basis of gravimetric determinations. Colorimetric phosphorus determination showed the presence of 57.2 ± 8.2 μmoles of lipid phosphorus per g of cells (dry weight) in the same strain. When the individual lipids of both strains were quantitated on the basis of radioactivity of incorporated ^{14}C label, the results shown in Table 3 were obtained. Both strains were assayed at 6 hr of growth on TSB and on S medium and at 12 and 18 hr on TSB alone.

The most striking feature of the data is the difference in the amounts of PE in the parent strain (7 to 8% ^{14}C activity) and the mutant strain (0.2 to 0.9%). This great difference is not altered significantly by the choice of growth medium or the age of the culture.

Table 3 also shows a number of differences in the relative amounts of the various lipid classes when either strain is cultured on TSB compared to that grown on S medium. The parent has less neutral lipid and only half the cardiolipin when grown on the synthetic medium. The mutant has more neutral lipid and less phosphatidylglycerol under similar conditions. Both strains have more glycolipid and nearly double the level of lysyl

TABLE 3. *Changes in the lipid composition of Bacillus subtilis ATCC 6051 and 6051-4 with time*

Lipid	Per cent ^{14}C radioactivity						Medium[a]
	6051			6051-4			
	6 hr	12 hr	18 hr	6 hr	12 hr	18 hr	
Neutral lipid	33.4	40.0	40.8	28.4	35.8	25.0	TSB
Diglucosyldiglyceride	9.8	1.9	3.4	12.6	8.4	19.7	
Cardiolipin	17.0	14.0	15.3	12.6	19.6	17.7	
Phosphatidylglycerol	30.1	34.7	32.3	41.8	29.6	35.2	
Phosphatidylethanolamine	7.1	5.2	5.6	0.5	0.9	0.8	
Lysyl phosphatidylglycerol	2.2	4.2	1.9	3.9	5.6	1.4	
Neutral lipid	27.5	ND[b]	ND	35.1	ND	ND	S
Diglucosyldiglyceride	11.9			13.8			
Cardiolipin	8.5			19.1			
Phosphatidylglycerol	37.6			28.6			
Phosphatidylethanolamine	7.9			0.2			
Lysyl phosphatidylglycerol	7.1			6.2			

[a] TSB, Tryptic Soy Broth; S, synthetic.

[b] ND, Not determined.

phosphatidylglycerol when grown on the synthetic medium.

Figure 1 depicts graphically the changes that occur in the lipid composition of the two strains with time (6-, 12-, and 18-hr-old cells on TSB).

The data obtained for *B. subtilis* ATCC 6051 show little change in the amounts of phosphatidylglycerol and PE, a small decrease in cardiolipin, and an increase in neutral lipid with time. The amounts of the individual lipids are similar to those of *B. natto*, a closely related species (33).

In contrast to the parent, the mutant is subject to marked fluctuation in lipid composition with time. Neutral lipid increases to maximum at 12 hr, and then sharply falls to a minimum at 18 hr. Phosphatidylglycerol and diglucosyldiglyceride fall and rise reciprocally to the neutral lipid changes. Cardiolipin increases slightly in total content, whereas lysyl phosphatidylglycerol decreases after an initial increase. PE is present in such small quantities in the mutant that changes are impossible to evaluate.

Growth. A comparison was made of the ability of the two strains to grow on a variety of media. Table 4 shows the data obtained on the basis of dry weight, protein, and optical density on 18-hr-old cells grown on a complex medium (TSB) and four types of minimal, S medium. The results indicate that the mutant is metabolically handicapped. On the basis of dry weight, the mutant averages ca. 1.0% of parental levels on S media but attains 80% of the level of the parent on TSB.

The mutant shows a gradation of growth response to a variety of supplements on S medium (Table 5). There are substantial increases in growth when glyceryl phosphorylethanolamine and PE are used as supplements. There is nearly a fourfold increase in growth when the deacylated form of PE is used; somewhat less growth is observed at the maximal stimulating concentration of PE. The small increases observed when neutral lipid of *B. subtilis* ATCC 6051 (shown by chromatography to contain diglycerides and free fatty acids), phosphatidylserine, and Tween 80 are employed as supplements were not significantly different from control values.

Since the mutant is not truly supplemented by growth on TSB, i.e., provided with PE or a precursor which is incorporated into cell membrane,

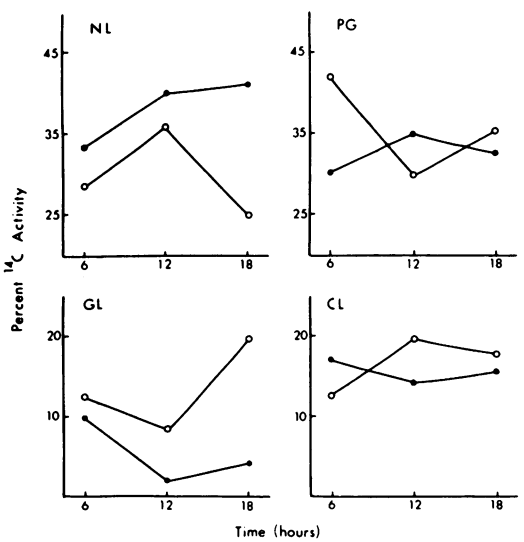

FIG. 1. *Lipid composition changes with time (6-, 12, 18-hr-old cells) of Bacillus subtilis ATCC 6051 (●) and 6051-4 (○) with regard to neutral lipid (NL), phosphatidylglycerol (PG), diglucosyldiglyceride (GL), and cardiolipin (CL).*

TABLE 4. *Growth of parent and mutant strains on various media*

Medium[a]	Dry wt (μg/ml)		Protein (μg/ml)		Optical density[b] (540 nm)	
	6051	6051-4	6051	6051-4	6051	6051-4
TSB	1,250.0	1,009.0	640.0	520.0	3.33	2.72
S	587.0	4.5	420.0	3.6	1.57	0.058
SE	577.0	8.3	360.0	5.2	1.83	0.066
SPE	565.0	11.9	380.0	6.6	1.71	0.077
SS	542.0	11.7	390.0	6.4	1.70	0.071

[a] TSB, Tryptic Soy Broth (Difco); S, synthetic medium of Gary and Bard (13), SE, SPE, and SS: synthetic medium supplemented by monoethanolamine, phosphatidylethanolamine, and L-serine, respectively (all at 15 μg/ml).

[b] Measured with a Bausch & Lomb Spectronic 20 spectrophotometer.

TABLE 5. *Supplementation of Bacillus subtilis 6051-4*

Supplement	Maximal growth[a]	Concn of supplement (μg/ml)
None	4.5	
Phosphatidylethanolamine	14.1	35[c]
Phosphatidylserine	5.4	100
Glyceryl phosphorylethanolamine	18.7	100
Neutral lipid[b]	5.8	100
Tween 80	6.3	100

[a] Micrograms of cells (dry weight) per milliliter.

[b] Isolated from *B. subtilis* ATCC 6051.

[c] Inhibitory at higher concentrations.

the nature of this ability to grow on TSB is curious. We speculate that TSB provides a rich source of a battery of nutrients which the mutant cannot synthesize or retain at optimal levels in a synthetic medium. This was borne out by nutritional studies on the mutant which revealed no single entity in TSB that could be classified as the one growth stimulant. Only by inclusion of all major categories of compounds from TSB could maximal growth of the mutant be obtained.

Figure 2 shows the growth curves of the two strains on S medium. The parent grows logarithmically for nearly 3 hr to a maximum optical density of ca. 1.8. The mutant grows logarithmically for a little more than 90 min, reaching a maximum optical density of 0.23. After a short stationary phase, there is a substantial decrease in optical density which may be indicative of autolysis.

Osmotic fragility. Lysozyme treatment resulted in the formation of protoplasts from both strains which was complete within 90 min. The lysis of the protoplasts in the mannitol solutions of varying molarity is shown in Fig. 3. The results indicate the mutant is more osmotically fragile than the parent. A 50% lysis of parent protoplasts is incurred with 0.26 M mannitol, whereas the same result is obtained with mutant protoplasts by using a 0.37 M mannitol solution.

Microscopy. The ability to produce spores did not appear to be affected by the mutational event, at least from a qualitative viewpoint.

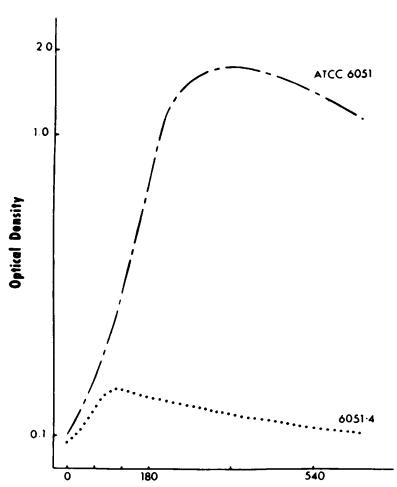

FIG. 2. *Growth curves of Bacillus subtilis ATCC 6051 (broken line) and 6051-4 (dotted line) on synthetic medium of Gary and Bard (13). Optical density 0.1 = 15.4 ± 2.1 μg of cells (dry weight) per ml.*

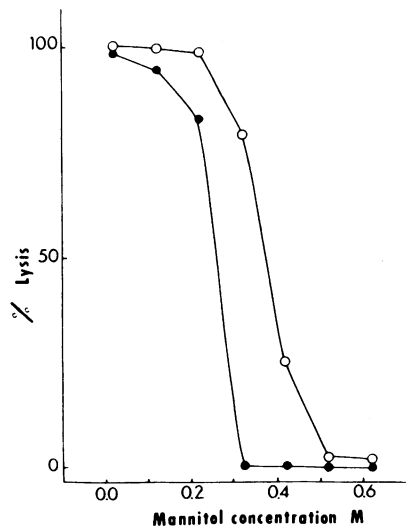

FIG. 3. *Osmotic fragility of protoplasts of Bacillus subtilis ATCC 6051 (●) and 6051-4 (○).*

However, irregularities in mutant cell size and shape were observed in stained smears of cells taken from S medium. The mutant decolorized less readily than the parent in the Gram staining procedure.

DISCUSSION

The inability to synthesize PE, or, more accurately, to produce optimal amounts of this phospholipid, has profound repercussions on many aspects of the physiology of this mutant of *B. subtilis*. The low levels of PE found may indicate a "leaky" mutant or a capacity of the mutant for synthesis of PE by a secondary and poorly efficient route. A precedent for the latter possibility exists in certain phosphatidylcholine mutants of *Neurospora crassa* (8).

The PE-deficient mutant has lost to a great measure the ability to grow on synthetic media. It can grow quite well on a complex medium but does not synthesize normal amounts of PE, i.e., is not supplemented by a component present in the complex medium. Nutritional studies attest to the absence of a single supplement in complex medium, providing impetus to the theory of a generalized membrane alteration with multiple effects. Among these effects may be the inability to take up or concentrate essential metabolites. The activities of membrane-bound enzymes may be altered by such a mutational event. In fact, the relative amounts and the pattern of change of the other lipids present in the mutant are substantially different from the parent strain. The deletion of a basic phosphatide from the mem-

brane may incur a charge imbalance within the membrane substructure, resulting in stress and increased protoplast fragility. In fact, the mutant has increased protoplast fragility. The lesion appears to have effects of such a magnitude that the cellular morphology and staining character of the mutant are altered as well.

Growth of the mutant is minimally but significantly increased when glyceryl phosphorylethanolamine or PE are used as supplements. Since other lipids have no such effect, it is probable that glyceryl phosphorylethanolamine and PE are incorporated into the membrane and relieve the effects of the lesion. It is of great interest to us to establish the nature of this phenomenon and to evaluate the activities of L-serine-cytidine monophosphate phosphatidyl transferase and phosphatidylserine decarboxylase in the mutant.

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