JOURNAL OF BACTERIOLOGY, Aug. 1979, p. 544-551
0021-9193/79/08-0544/08$02.00/0

Vol. 139, No. 2

Cardiolipin Accumulation in the Inner and Outer Membranes of *Escherichia coli* Mutants Defective in Phosphatidylserine Synthetase

CHRISTIAN R. H. RAETZ,* GINA D. KANTOR, MASAHIRO NISHIJIMA, AND KARL F. NEWMAN

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received for publication 17 May 1979

Mutants of *Escherichia coli* defective in phosphatidylserine synthetase (*pss*) make less phosphatidylethanolamine than normal cells, and they are temperature sensitive for growth. We have isolated a new mutant, designated RA2021, which is better than previously available strains in that the residual phosphatidylethanolamine level approaches 25% after 4 h at 42°C. The total amount of phospholipid normalized to the density of the culture is about the same in RA2021 (*pss-21*) as in the isogenic wild-type RA2000 (*pss*$^+$). Consequently, there is a net accumulation of polyglycerophosphatides in the mutant, particularly of cardiolipin. The addition of 10 to 20 mM $MgCl_2$ to a culture of RA2021 prolongs growth under nonpermissive conditions and prevents loss of cell viability, but it does not eliminate the temperature-sensitive phenotype. Divalent cations, like Mg^{2+}, do not correct the phospholipid composition of the mutant, but may act indirectly by balancing the negative charges of phosphatidylglycerol and cardiolipin. To determine the effects of the *pss* mutation on membrane composition, we have examined the subcellular distribution of the polyglycerophosphatides that accumulate in these strains. All of the excess anionic lipids of RA2021 are associated with the envelope fraction and are distributed equally between the inner and outer membranes. The protein compositions of the isolated membranes do not differ significantly in the mutant and wild type. The fatty acid composition of RA2021 is almost the same as wild type at 30°C, but there is more palmitic and cyclopropane fatty acid at 42°C. These results demonstrate that the modification of the polar lipid composition observed in *pss* mutants affects both membranes and that cardiolipin, which is not ordinarily present in large quantities, can accumulate in the outer membrane when it is overproduced by the cell. The altered polar headgroup composition of the outer membrane in *pss* mutants may account, in part, for their hypersensitivity to the aminoglycoside antibiotics.

In the past few years, two general approaches have been developed for modifying the composition of polar phospholipid headgroups present in bacterial membranes (3, 22, 27). One strategy involves the fusion of exogenous lipid vesicles with deep rough mutants of *Salmonella typhimurium* (7, 8) and *Escherichia coli* (13), whereas the other utilizes mutants defective in specific enzymes of phospholipid biosynthesis (3, 22). As yet, the effects of such modifications on membrane structure and function have not been studied extensively because the membrane composition of the available strains has not been characterized in sufficient detail.

We previously reported that mutants defective in phosphatidylserine synthetase (*pss*) are temperature sensitive for growth (17, 21). At elevated temperatures these strains make much less phosphatidylethanolamine than normal cells (17, 21), and they accumulate polyglycerophosphatides, particularly cardiolipin (17, 21). This is compatible with the accepted pathways for phospholipid biosynthesis, in which CDP-diglyceride is a common precursor (3, 22). The exact ratio of phosphatidylethanolamine to the polyglycerophosphatides varies with temperature in the *pss* mutants, being somewhat closer to normal at 30°C (17, 21).

Mutants in phosphatidylserine synthetase are hypersensitive to hydrophilic antibiotics, especially to the aminoglycosides (23). The outer membrane is thought to provide a passive barrier to these substances (10, 15). The antibiotic hypersensitivity of the *pss* mutants, which is striking at all temperatures but especially so under nonpermissive conditions, suggests the

possibility that polar phospholipid headgroups play an important role in maintaining the barrier function of the outer gram-negative membrane (23). Such a hypothesis makes examination of outer membrane composition of interest. We now report that cardiolipin accumulates in both the inner and outer membranes of the *pss* mutants. In contrast to the polar headgroups, the membrane protein compositions of the purified envelope fractions do not differ significantly in the mutant and the wild type. The fatty acid composition of the membrane phospholipids has also been examined. It is very similar in the mutant and the wild type at 30°C. However, at 42°C there is a marked accumulation of cyclopropane fatty acids in the mutant, and the relative amount of palmitic acid is also increased. For the present study, we have employed strain RA2021 (*pss-21*), a newly isolated mutant that contains less residual phosphatidylethanolamine than mutants reported previously (17, 21).

MATERIALS AND METHODS

Materials. [^{32}P]phosphate and Triton X-100 were purchased from New England Nuclear Corp., Boston, Mass. Phosphatidylethanolamine labeled with ^{14}C in the fatty acid chains was prepared from cells that had been grown in the presence of [1-^{14}C]acetate, as reported previously (26). NADH was obtained from Sigma Chemical Co., St. Louis, Mo.

Conditions of cell growth and labeling. Bacteria were usually grown on LB broth (14) containing 10 g of Tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 10 g of NaCl per liter. For the purpose of preparing isolated inner and outer membranes, the organisms were grown on PPBE broth (18) consisting of 10 g of proteose peptone no. 3 (Difco), 1 g of beef extract (Difco), and 10 g of NaCl per liter. The phenotype and lipid composition of the *pss* mutant were similar on both media. Uniform labeling of the phospholipids was achieved by allowing the bacteria to grow for about eight generations in the presence of $^{32}P_i$ (1 to 2 mCi/ml). The inorganic phosphate concentration of LB broth is about 2.5 mM, whereas that of PPBE is 1.2 mM. In those experiments involving a shift of the culture from 30°C to 42°C, the cells were first uniformly labeled at 30°C and then shifted with continuous labeling to the nonpermissive temperature when the optical density of the culture at 550 nm had reached 0.1.

To test the effects of divalent cations on the temperature-sensitive phenotype, the cells were grown either on LB broth or on a minimal medium containing 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, pH 7.5), 0.3 mM potassium phosphate, 0.5 to 20 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 0.2 M NaCl, and 0.2% glucose. Thiamine and required amino acids were added at levels recommended by Miller (14).

Separation of inner and outer membranes. To separate inner and outer membranes, the method of Osborn and Munson was employed (18) as described previously for mutants defective in diglyceride kinase (26). Cells of strains RA2021 and RA2000 were rather fragile during spheroplast formation and tended to lyse spontaneously before the sonication step. However, this premature lysis did not interfere with subsequent membrane separation by sucrose gradient centrifugation. The yields of membrane phospholipid at each step of the separation procedure were comparable to those reported for other *E. coli* strains (18, 26).

Enzyme assays. NADH oxidase was determined spectrophotometrically (18), and phospholipase A was measured radiochemically by the method of Nishijima et al. (16). Phosphatidylserine synthetase was determined under the conditions described by Raetz and Kennedy (24).

Lipid extraction. Phospholipids were always extracted by the method of Ames (1), except that a phosphate-buffered saline solution was employed for the aqueous phase (5). The lower $CHCl_3$ layer was washed once with a pre-equilibrated upper (methanol-water) phase, and insoluble debris was removed from the $CHCl_3$ phase by passage of the extract through a Pasteur pipette stuffed with glass wool. In small-scale radiochemical extractions, 0.5 mg of *E. coli* phospholipid was included as carrier per ml of chloroform (22). Separation of the individual phospholipid species present in the $CHCl_3$ phase was accomplished by thin-layer chromatography, as described previously (21).

Bacterial strains. The experiments reported here were carried out with the temperature-sensitive strain RA2021 (HfrH *thi pss-21*). Both RA2021 and RA2000 (HfrH *thi pss*$^+$) were constructed by P1*vir* transduction of AT2471 (HfrH *thi tyrA pss*$^+$) (Table 1) to *tryA*$^+$, with *pss* as the unselected gene. The P1*vir* lysate used for this purpose was prepared by standard methods (14) on strain RA210 (*pss-21*), which is described more fully below and in Table 1.

TABLE 1. *Strains of Escherichia coli K-12*

Strain	Relevant characteristics	Source/reference
RA200	F$^-$ *pss-20 argH1 thi-1 gal-6 lacY1 mtl-2 xyl-7 malA1 ara-13 str-9 tonA2* λ^r; mutation *pss-20* is phenotypically silent	21
RA210	F$^-$ *pss-21 argH1*, etc.; otherwise same as RA200; temperature-sensitive derivative of RA200, which has a second mutation in the *pss* gene, designated *pss-21*	This work
AT2471	HfrH *tyrA thi-1 pss*$^+$	CGSC[a]
RA2000	HfrH *thi-1 pss*$^+$; constructed by transduction of AT2471 to *tyrA*$^+$ with a P1*vir* lysate prepared on RA210	This work
RA2021	HfrH *thi pss-21* (temperature-sensitive for growth); constructed by transduction of AT2471 with a P1*vir* lysate of RA210; isogenic with RA2000	This work
RA80	F$^-$ *nalA pss-8*	21

[a] *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

The *pss-21* mutation has not been reported previously and is probably in the structural gene for the phosphatidylserine synthetase. It was found among 200 temperature-sensitive colonies derived from strain RA200 (Table 1) by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The parent (RA200, F^- *pss-20*) already carries a partially defective synthetase (*pss-20*) (21). However, RA200 is not altered in lipid composition and is not temperature sensitive for growth (21), since the phosphatidylserine synthetase of *E. coli* appears to be present in excess (21, 25). The specific activity of the synthetase in extracts of RA200 is about 20% of wild type when assayed at 25°C (21).

RA210 was identified among the 200 temperature-sensitive derivatives of RA200 by means of a specific, rapid mapping technique. For this purpose, we used a donor organism carrying a hybrid ColE1 plasmid bearing the *pss* gene (and a few adjacent genes), which can transfer its hybrid plasmid to an F^- *pss* mutant and correct the temperature sensitivity of such an organism (25). The methodology for plasmid transfer, described previously (25), allowed us to eliminate 199 of the 200 possible mutant candidates.

RA210 was then confirmed as a second-step mutant in the *pss* gene by direct assay of cell-free extracts (24) at 25°C. This revealed no detectable activity (data not shown), in contrast to the parent RA200 which contained 20% of the *pss*$^+$ level (21).

Several lines of evidence strongly support the view that RA210 (*pss-21*) is temperature sensitive for growth because of a second lesion in the *pss* gene. First, the temperature sensitivity of *pss-21* cannot be separated from the synthetase defect (i.e., its complete absence in vitro) by transduction. Second, upon spontaneous reversion of RA2021 to the temperature-resistant state (approximate frequency, 10^{-9}), most of the colonies regain the synthetase activity present in the parent RA200 (*pss-20*). This level of enzyme is sufficient to permit growth at 42°C and normal lipid synthesis (21). Third, the altered lipid composition (see below) of strains carrying the *pss-21* lesion (i.e., RA210 and RA2021) is consistent with a major defect in the synthetase (Fig. 1) and closely resembles the single step *pss* mutation, *pss-8*, reported previously (21). However, RA2021 (*pss-21*) should be more useful for studies of membrane physiology than RA80 (*pss-8*), since RA2021 contains less residual enzymatic activity in vitro, and its phospholipid content is altered more dramatically (Fig. 1). Furthermore, RA2021 does not revert to a temperature-resistant state as frequently as RA80.

Miscellaneous methods. Protein concentration was measured by the procedure of Lowry et al. (11). Methods for liquid scintillation counting and gel electrophoresis of membrane proteins have been reported elsewhere (9, 19). Fatty acid compositions were determined by gas chromatography after conversion to methyl esters (28). Methyl ester standards were purchased from Sigma, and cyclopropane fatty acids were generously provided by John Law, University of Chicago, Chicago, Ill.

RESULTS

Effect of temperature on the membrane lipid composition and growth of RA2000 (*pss*$^+$) and RA2021 (*pss-21*).

As with two of the previously reported mutants defective in phosphatidylserine synthetase, RA2021 is temperature sensitive for growth on a variety of media, including LB broth (14), PPBE broth (18), minimal A salts (14) supplemented with 0.2% glucose, and the minimal HEPES medium described above. Temperature sensitivity can be demonstrated for cells growing on agar plates as well as for shaking cultures. The residual level of phosphatidylethanolamine at 42°C in RA2021 is lower than in other mutants of this type (17, 21). The headgroup composition of *pss*$^+$ cells does not change dramatically after a shift to 42°C (Fig. 1A), whereas the phosphatidylethanolamine level of RA2021 falls to about 25% after 4 h (Fig. 1B). This is accompanied by a gradual cessation of growth and filamentation under some conditions (21). The accumulation of cardiolipin in RA2021 under nonpermissive conditions is especially striking (Fig. 1B). At 30°C, the phosphatidylethanolamine content of RA2021 is also reduced significantly (Fig. 1), ranging between 45 and 55%, depending on the medium. However, the cells are able to tolerate this perturbation of the membrane composition.

Table 2 shows the total lipid content of

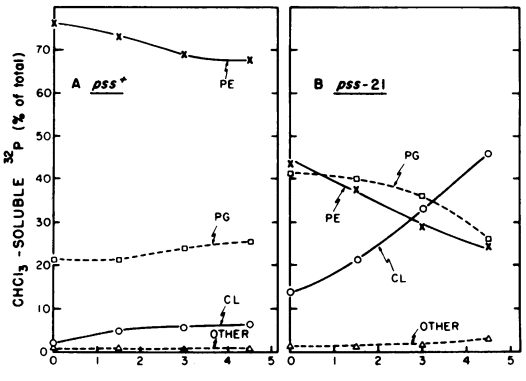

FIG. 1. *Modification of the phospholipid composition of RA2000 and RA2021 (pss-21) after a shift from 30 to 42°C. Cells growing in shaking culture at 30°C were shifted to 42°C when the absorbance at 550 nm ≅ 0.1, designated as time zero. Cells were labeled continuously with $^{32}P_i$ (1 to 2 μCi/ml) before and after the shift to nonpermissive conditions. The concentration of inorganic phosphate is 2.5 mM in this medium. At the times indicated, a 5- to 10-ml portion of the culture was withdrawn, and the cells were rapidly harvested by a 5-min centrifugation at 8,000 × g. After suspension in 0.8 ml of saline (5), the phospholipids were extracted from the cells by the method of Ames (1). However, 0.5 mg of crude E. coli phospholipid was included as carrier per ml of chloroform. Phospholipids were then separated by thin-layer chromatography and quantitated radiochemically, as described previously (21).*

TABLE 2. *Phospholipid content of pss⁺ and pss-21*

Mutant	Acyl ester content (nmol/μg of protein)[a]	Phospholipid content (% dry weight)[b]
Expt 1: 30°C		
pss⁺ (RA2000)	0.46	8.0
pss-21 (RA2021)	0.38	6.7
Expt 2: 42°C[c]		
pss⁺ (RA2000)	0.38	6.7
pss-21 (RA2021)	0.36	6.3

[a] Each value represents the average of two determinations (29).

[b] Calculated from the ester content, assuming an average phospholipid molecular weight of 700. Protein represents 50% of the dry weight of *E. coli* (30).

[c] Cells were shifted from 30 to 42°C at an absorbance at 550 nm of 0.1. RA2000 was harvested when the absorbance at 550 nm was 0.7. RA2021 was incubated at 42°C for 3 h, and the absorbance at 550 nm at harvesting was 0.49.

RA2000 and RA2021 by chemical ester determination (29) and normalized to the protein content of the culture. The slight differences observed between the mutant and the wild type are barely significant. Taken together with Fig. 1, these results prove that there is a net accumulation of polyglycerophosphatides in RA2021, particularly of cardiolipin.

Unlike previously reported mutations in the *pss* gene (17, 21), the phenotype of the *pss-21* lesion cannot be suppressed completely by the addition of salts or divalent cations to the growth medium. However, 20 mM $MgCl_2$ added to the standard LB broth does prolong the growth and survival of RA2021 at 42°C (Fig. 2B). This level of Mg^{2+} has no effect on the growth of the wild type (Fig. 2A). Higher or lower levels of Mg^{2+} are not as effective as 20 mM Mg^{2+} in sparing the growth of the mutant, and a similar Mg^{2+} effect can be demonstrated with cells of RA2021 grown on the minimal HEPES medium described in Materials and Methods. The prolongation of growth at 42°C observed with Mg^{2+} (Fig. 2B) cannot be attributed to changes in the osmolarity or ionic strength of the medium and must represent a more specific effect, possibly a neutralization of the excess negative charges in the mutant membranes. Other multivalent cations, such as Ca^{2+} and spermidine, closely resemble Mg^{2+} in their effect on RA2021. However, neither Mg^{2+} nor the other divalent cations correct the phospholipid headgroup composition of RA2021 back to that of RA2000.

Isolation of inner and outer membranes. Membrane fragments were prepared by mild sonic disruption of spheroplasts and were separated by isopycnic sucrose gradient centrifugation as described in Fig. 3. Experiments were carried out with cells of RA2000 and RA2021 that either were growing at 30°C or had been shifted to 42°C for several hours. The results shown in Fig. 3 pertain only to cells that had been shifted to 42°C, but very similar results were also obtained with cells grown at 30°C.

As demonstrated in Fig. 3A and 3C, outer membrane fragments were detected by assay of phospholipase (16), whereas inner membranes were located by means of a spectrophotometric assay for NADH oxidase (18). Satisfactory resolution of these enzymes was obtained both with the mutant and with the wild type, although the proportion of intermediate, unseparated membrane material was somewhat greater in the mutant (Fig. 3C). As shown in the lower panels (Fig. 3B and 3D), the membranes of RA2000 and RA2021 were resolved into two major peaks of phospholipid-containing material, corresponding to the peaks of the marker enzymes (cf. Fig. 3A with 3B, and 3C with 3D). The phospholipid material was detected radiochemically, since the cells had been grown for many generations in the presence of $^{32}P_i$ before the membrane isolation. Recoveries of lipid and marker enzymes from the gradients were about the same for RA2000 and RA2021, varying between 50 and 70% relative to the cell-free extracts. The specific activities of NADH oxidase and phospholipase A were comparable in both strains. The inner and the outer membranes of RA2000 and RA2021 floated at about the same respective densities (cf. Fig. 3B and Fig. 3D) and had

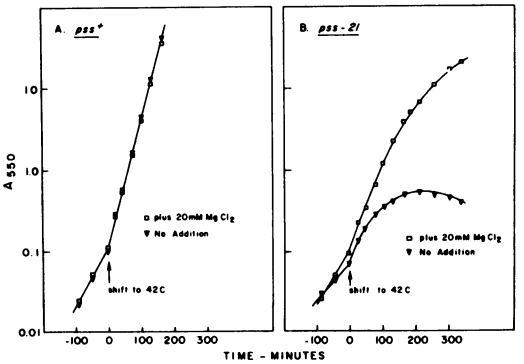

FIG. 2. *Effect of $MgCl_2$ on the growth of RA2000 (pss⁺) and RA2021 (pss-21) at 30 and 42°C. Cells growing on LB broth at 30°C were shifted to 42°C at time zero. The optical density at the time of the shift was 0.08 to 0.1. To observe continued exponential growth in the case of the wild type (and RA2021 plus $MgCl_2$), we intermittently diluted the cultures to maintain an absorbance at 550 nm between 0.05 and 0.8. Thus, above a density of 0.8, the absorbance at 550 nm shown in figure represents the total growth yield and not the actual density of the culture.*

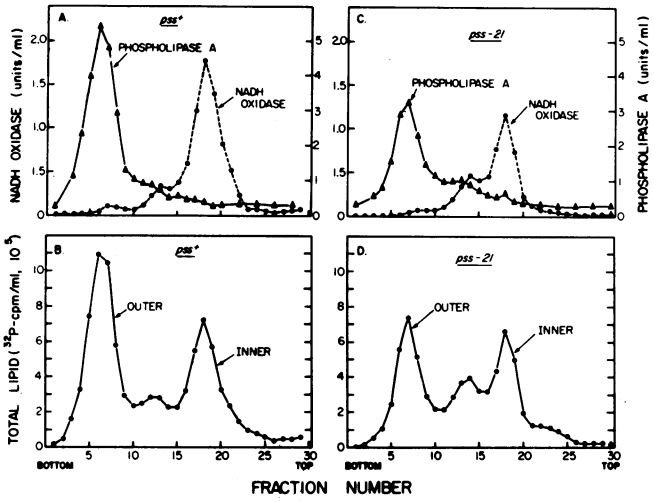

FIG. 3. *Separation of inner and outer membrane from RA2000 (pss$^+$) and RA2021 (pss-21). A culture of 200 ml of cells, growing on PPBE, was continuously labeled with $^{32}P_i$ (2 to 4 μCi/ml). At an absorbance at 550 nm ≅ 0.15, the cells were shifted to 42°C. The wild-type cells were harvested by centrifugation (10 min, 5,000 × g) when the absorbance at 550 had reached 0.8. The mutant was allowed to grow for 3 h, and the final absorbance at 550 was about 0.6. The cells were then treated with lysozyme and EDTA and disrupted by mild sonic oscillation, as described elsewhere (18, 26). The membranes were washed and separated by isopycnic sucrose gradient sedimentation, using the method of Osborn and Munson (18, 26). Briefly, a sample of membranes (0.8 ml) was layered on top of a six-step sucrose gradient, the bottom of which contained 55% sucrose (wt/wt) and the top of which was 30% sucrose (wt/wt). The gradients were centrifuged for 14 to 16 h at 36,500 rpm, using a Beckman SW41 Ti rotor at 2°C. Fractions of about 0.4 ml each were collected. A and B show the distribution of marker enzymes and lipids, respectively, for a gradient separation of membranes obtained from RA2000, whereas C and D show similar results for the membranes of RA2021. The number of fractions collected was not sufficient to resolve the inner membrane into the L_1 and L_2 subfractions (18).*

similar lipid-to-protein ratios. This is consistent with the results in Table 2, which demonstrates that there is little difference in the lipid-to-protein ratios of whole cells of mutant and wild type.

Phospholipid composition of isolated inner and outer membranes. Table 3 shows the polar headgroup compositions of the phospholipids extracted from the peak fractions of the isolated inner and outer membranes (Fig. 3). In experiment 1 (Table 3), the cells were grown to late-log phase at 30°C, whereas in experiment 2 the cells were incubated for 3 h at 42°C, as in Fig. 3. In all instances, the phospholipid composition of the isolated membranes (Table 3) represents an average of the four peak fractions (see Fig. 3). Accumulation of cardiolipin is observed both in the inner and in the outer membranes (Table 3). The relative buildup of cardiolipin is most pronounced in the outer membrane, representing about a 10-fold excess over the wild type. After 3 h at 42°C in PPBE broth, cardiolipin constitutes about 30% of the outer-membrane phosphoglyceride in RA2021. The amount of phosphatidylglycerol does not increase as dramatically. In general, a slightly higher percentage of phosphatidylethanolamine is always present in the outer membrane, (Table 3, experiments 1 and 2). This is in agreement with other studies of the lipid composition of wild-type *E. coli* K-12 (12, 26).

Other properties of isolated membranes. The peaks from the gradients of Fig. 3 were analyzed for their protein composition by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (12). No striking differences in the major proteins of the isolated membranes could be observed by visual comparison of stained polyacrylamide gels (data not shown). Furthermore, sensitive two-dimensional thin-layer chromatography (M. Nishijima and C. R. H. Raetz, J. Biol. Chem., manuscript in preparation) of the phospholipids of RA2021 did not reveal any appreciable differences in the amount of minor lipid intermediates.

Table 4 shows the fatty acid compositions of the unfractionated phospholipids of RA2000 and RA2021 grown at various temperatures. At 30°C, there is little difference in the fatty acids, despite the twofold increase in the polyglycerophosphatide content of RA2021. However, at 42°C, the mutant contains much more of cyclopropane fatty acid than wild type, and the amount of palmitic acid is also consistently higher.

TABLE 3. *Phospholipid composition of outer and inner membranes isolated from RA2000 (pss⁺) and RA2021 (pss-21)*

Strain and membrane	% of total ^{32}P[a]			
	PE	PG	CL	Other
Expt 1: 30°C[b]				
RA2000, outer	82.5	11.8	0.6	5.1
RA2000, inner	67.3	28.3	2.7	1.7
RA2021, outer	69.1	21.6	6.7	2.6
RA2021, inner	52.7	28.6	16.9	1.8
Expt 1: 42°C[b]				
RA2000, outer	78.8	16.2	2.8	2.2
RA2000, inner	59.4	33.4	5.8	1.4
RA2021, outer	49.8	18.9	29.6	1.7
RA2021, inner	39.3	33.3	26.4	1.0

[a] Each determination represents the average of the four peak fractions (Fig. 3). Other lipids include lysophosphatidylethanolamine and several minor, unidentified species. The standard deviation on each value is approximately ±2%. PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

[b] In this experiment, cells were grown on PPBE broth (18) instead of LB broth (14). RA2000 and RA2021 were harvested after incubation at 42°C as described in Table 2.

TABLE 4. *Fatty acid composition of the total cellular phospholipid fraction as a function of growth temperature*[a]

Fatty acid[b]	% of total[c]					
	RA2000 (*pss⁺*)			RA2021 (*pss-21*)		
	30°C	37°C	42°C	30°C	37°C	42°C
14:0	3	2	2	2	2	3
16:0	27	29	30	30	34	37
16:1	35	32	27	30	28	20
17:Δ	1	2	7	2	8	17
18:0	1	1	1	1	1	—
18:1	32	33	32	34	28	20
Other	1	1	1	1	—	3

[a] At 30 and 37°C, cells were grown into late-log phase on LB broth (absorbance at 550 nm = 0.6 to 0.8). The values given for 42°C were obtained by shifting the cells to this temperature for 3 h, as in Fig. 1.

[b] Acids are designated as follows: 14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 17:Δ, *cis*-9,10-methylenehexadecanoic acid; 18:0, stearic acid; 18:1, *cis*-vaccenic acid. Other species include lauric acid and lactobacillic acids.

[c] Values indicate the average of two determinations. Precision is approximately ±1%. —, None detected.

Table 5 presents the fatty acid composition of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, isolated from cultures of RA2000 and RA2021, which were shifted to 42°C for 3 h (Fig. 1). The fatty acid composition of phosphatidylethanolamine differs from that of phosphatidylglycerol and cardiolipin in that it contains less *cis*-vaccenate. All of the separated lipids from the mutant are characterized by a striking accumulation of the cyclopropane derivatives of palmitoleate and *cis*-vaccenate, and there is a higher percentage of palmitate relative to that found in wild type.

TABLE 5. *Fatty acid composition of total and separated phospholipids at 42°C*[a]

Fatty acid[b]	% of total[c]							
	RA2000 (*pss⁺*)				RA2021 (*pss-21*)			
	Total	PE	PG	CL	Total	PE	PG	CL
12:0	0.2	0.1	—	—	0.4	0.1	—	—
14:0	1.6	1.8	1.0	—	2.7	2.5	1.7	1.8
16:0	29.6	30.9	28.1	28.3	36.5	35.8	38.3	37.5
16:1	27.5	27.7	25.4	22.1	19.7	15.7	19.6	20.8
17:Δ	6.7	8.0	3.0	2.8	16.7	23.9	16.1	14.1
18:0	0.8	0.6	0.9	1.7	—	—	—	—
18:1	32.2	29.8	40.9	45.1	19.7	17.3	20.0	21.5
19:Δ	0.5	0.5	0.6	—	3.0	3.0	3.4	3.2

[a] Cells were grown for 3 h at 42°C as described in Fig. 1 and Table 4.

[b] Designations and abbreviations as in Tables 3 and 4, except that 12:0 designates lauric acid and 19:Δ designates lactobacillic acid. In each column an additional 1 to 2% was unidentified.

[c] —, None detected.

DISCUSSION

The phospholipids of all biological membranes are a heterogeneous class of molecules, differing not only with regard to their polar headgroups but also with respect to their fatty acid substituents (3, 4, 22, 27). The functional significance of this structural diversity, particularly the role of the polar headgroups, remains largely unexplained (3, 22, 27).

Modification of the membrane lipid composition by genetic methods affords a powerful and relatively unexploited approach to this problem (3, 22). At the very least, those structural features which are essential or nonessential for cell growth may be identified. For instance, in *E. coli* some saturated and some unsaturated (i.e., fluid) hydrocarbon moieties are required at all temperatures, and the ratio of zwitterionic to anionic polar headgroups cannot be varied by more than a factor of two in either direction without disturbing cell growth (22). On the other hand, cardiolipin appears to be nonessential under ordinary conditions (20). These generalizations are based on recent genetic studies (3, 22) and will be refined as additional methods for the perturbation of the membrane lipid composition become available.

The effects of compositional modifications on

specific membrane functions, such as active transport, ATP generation, or O_2 consumption, have not been investigated systematically in mutants with altered polar headgroup synthesis. As a first step in this direction, it is necessary to determine the composition of the inner and outer membranes isolated from these mutants, since each membrane carries out a unique set of functions.

In the present work we have demonstrated that the reduction of the phosphatidylethanolamine level in RA2021 (*pss-21*) is accompanied by a net increase in the polyglycerophosphatide content, especially in cardiolipin. The absolute amount of phospholipid normalized to the total cellular protein concentration remains about the same (Table 2), and the buoyant density of the isolated inner and outer membranes does not differ greatly in the mutant and the wild type (Fig. 3). All of the excess polyglycerophosphatide of RA2021 appears to be associated with the membrane fraction and is not sequestered elsewhere within the cell.

The substantial accumulation of cardiolipin in the outer as well as the inner membrane is compatible with the view that certain phospholipids can move freely between the two membranes, whereas proteins and lipopolysaccharide are restricted to one site or the other (7, 8, 22). However, the biosynthesis of phospholipids occurs on the inner membrane, presumably on its cytoplasmic surface (2, 31). The notion of unrestricted mobility of certain phospholipids between the two membranes was first advanced by Jones and Osborn (7, 8), who observed that phospholipids could be fused with the outer membrane of deep rough mutants of *S. typhimurium* and subsequently could be recovered in both membranes. However, fusion studies may not accurately reflect normal cellular events, since some lipids are taken up and assembled into nonphysiological membrane-like structures, which coil up within the interior of the cell (13).

The distribution of unusual lipids which build up in various mutants has not been studied in detail. Strains defective in phosphatidylserine decarboxylase contain large amounts of phosphatidylserine (6), which resembles cardiolipin in that it is recovered in both membrane fractions (E. Hawrot and E. P. Kennedy, personal communication). In contrast, the 1,2-diglyceride, which accumulates in mutants lacking diglyceride kinase, is predominantly associated with the inner membrane (26). Perhaps the mechanism(s) responsible for lipid translocation between the membranes does not recognize diglyceride molecules. In this regard it would be of interest to examine the distribution of phosphatidic acid, which accumulates in strains lacking CDP-diglyceride synthetase (B. R. Ganong et al., Fed. Proc. 38:471, 1979). An in vitro system capable of lipid translocation between the two membranes would also be very helpful. It should be noted that the conditions of membrane separation described by Osborn and co-workers appear to cause little (if any) rearrangement of membrane lipids and proteins (18, 26).

At 30°C, the amount of anionic phospholipid in RA2021 is about two times greater than in isogenic wild-type strains. However, the fatty acid composition (Table 4) and the membrane protein profile (not shown) do not differ appreciably. Furthermore, the chromatographic properties of the lipopolysaccharide of the *pss* mutants is not altered in cells grown at 30°C (23). Consequently, it is possible that the striking antibiotic hypersensitivity of mutants such as RA2021 (23), which is observed at 30°C as well as higher temperatures, is somehow related to the change in polar headgroup composition. The outer membrane of *E. coli* is a passive barrier to substances of intermediate molecular weight and thus confers considerable resistance to certain hydrophilic antibiotics, such as the aminoglycosides (10, 15). The observation (Table 3) that the cardiolipin content of the outer membrane is increased 10-fold relative to wild type at both 30°C and 42°C makes it necessary to consider the possibility that a proper balance of polar headgroups is essential for the maintenance of the outer-membrane permeability barrier.

Unlike the results obtained at 30°C, the fatty acid composition of RA2021 (*pss-21*) differs from that of RA2000 (*pss*$^+$) when the cells are shifted to 42°C for 3 h. This perturbation of the hydrocarbon moieties must be considered in any future studies of membrane function. It might be desirable to combine the *pss* mutation with certain lesions in fatty acid synthesis (3, 27), to prevent the changes in fatty acid distribution that seem to accompany the polar headgroup alterations under nonpermissive conditions. Although other explanations are possible, the effects observed in Table 5 suggest that the *pss* lesion causes an inhibition both of the elongation and of the unsaturation of the hydrocarbon chains, since the mutant contains more palmitate and less *cis*-vaccenate than the wild type. Furthermore, there is a striking accumulation of the cyclopropane derivatives of palmitoleate and *cis*-vaccenate in RA2021 at 42°C.

The results of Fig. 2 demonstrate that Mg^{2+} ions significantly stimulate the growth of RA2021, especially at 42°C. Since the $MgCl_2$ promotes the growth without correcting the phospholipid composition, it must act indirectly. This might occur through the binding of the divalent cations to the excess negative polar

headgroups that accumulate in RA2021. In any case, the Mg^{2+} stimulation shown in Fig. 2 cannot be attributed to effects on osmolarity or ionic strength. The finding that Mg^{2+} prevents loss of cell viability at 42°C while permitting a dramatic reduction in the phosphatidylethanolamine content should be useful for certain physiological studies of the role of polar phospholipid headgroups in membrane function. In this regard, it should be noted that the specific role of phosphatidylethanolamine in the membrane is unknown, and it is uncertain whether the lack of phosphatidylethanolamine or the excess of polyglycerophosphatides (or both) contribute to the temperature-sensitive phenotype of RA2021. The prolongation of growth at 42°C by Mg^{2+} indicates that the excess of polyglycerophosphatides may be an important factor.

ACKNOWLEDGMENTS

We thank Barbara Bachmann of the *E. coli* Genetic Stock Center, Yale University, for some of the strains shown in Table 1, and R. Knibbs for help with the gas chromatography.

This research was supported in part by grants AM-19551 and 1K04-AM-00584 from the National Institute of Arthritis, Metabolism and Digestive Diseases, by Hatch Project 2269 from the Cooperative State Research Service, and by a Harry and Evelyn Steenbock Career Advancement Award to C.R.H. Raetz.

LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833–843.
2. **Bell, R. M., R. D. Mavis, M. J. Osborn, and P. R. Vagelos.** 1971. Enzymes of phospholipid metabolism: localization in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*. Biochim. Biophys. Acta **249**:628–635.
3. **Cronan, J. E., Jr.** 1978. Molecular biology of bacterial membrane lipids. Annu. Rev. Biochem. **47**:163–189.
4. **Cronan, J. E., Jr., and E. P. Gelmann.** 1975. Physical properties of membrane lipids: biological relevance and regulation. Bacteriol. Rev. **39**:232–256.
5. **Dulbecco, R., and M. Vogt.** 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. **99**:167–182.
6. **Hawrot, E., and E. P. Kennedy.** 1975. Biogenesis of membrane lipids: mutants of *Escherichia coli* with temperature-sensitive phosphatidylserine decarboxylase. Proc. Natl. Acad. Sci. U.S.A. **72**:1112–1116.
7. **Jones, N. C., and M. J. Osborn.** 1977. Interaction of *Salmonella typhimurium* with phospholipid vesicles: incorporation of exogenous lipids into intact cells. J. Biol. Chem. **252**:7398–7404.
8. **Jones, N. C., and M. J. Osborn.** 1977. Translocation of phospholipids between the outer and inner membranes of *Salmonella typhimurium*. J. Biol. Chem. **252**:7405–7412.
9. **Laemmli, V. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
10. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–129.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Lugtenberg, E. J. J., and R. Peters.** 1976. Distribution of lipids in cytoplasmic and outer membranes of *Escherichia coli* K12. Biochim. Biophys. Acta **441**:38–47.
13. **McIntyre, T. M., and R. M. Bell.** 1978. *Escherichia coli* mutants defective in membrane phospholipid synthesis: binding and metabolism of 1-oleoylglycerol-3-phosphate by a *plsB* deep rough mutant. J. Bacteriol. **135**: 215–226.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Nakae, T., and H. Nikaido.** 1975. Outer membrane as a diffusion barrier in *Salmonella typhimurium*: penetration of oligo- and polysaccharides into isolated membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. **250**:7359–7365.
16. **Nishijima, M., S. Nakaike, Y. Tamori, and S. Nojima.** 1977. Detergent-resistant phospholipase A of *Escherichia coli* K-12: purification and properties. Eur. J. Biochem. **73**:115–124.
17. **Ohta, A., and I. Shibuya.** 1977. Membrane phospholipid synthesis and phenotypic correlation of an *Escherichia coli pss* mutant. J. Bacteriol. **132**:434–443.
18. **Osborn, M. J., and R. Munson.** 1974. Separation of inner (cytoplasmic) and outer membranes of gram-negative bacteria. Methods Enzymol. **31**:642–653.
19. **Patterson, M. S., and R. C. Green.** 1965. Measurement of low β-emitters in aqueous solution by liquid scintillation counting in emulsions. Anal. Chem. **37**:854–857.
20. **Pluschke, G., Y. Hirota, and P. Overath.** 1978. Function of phospholipids in *Escherichia coli*: characterization of a mutant deficient in cardiolipin synthesis. J. Biol. Chem. **253**:5048–5055.
21. **Raetz, C. R. H.** 1976. Phosphatidylserine synthetase mutants of *Escherichia coli*: genetic mapping and membrane phospholipid composition. J. Biol. Chem. **251**: 3242–3249.
22. **Raetz, C. R. H.** 1978. Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. Microbiol. Rev. **42**:614–659.
23. **Raetz, C. R. H., and J. Foulds.** 1977. Envelope composition and antibiotic hypersensitivity of *Escherichia coli* mutants defective in phosphatidylserine synthetase. J. Biol. Chem. **252**:5911–5915.
24. **Raetz, C. R. H., and E. P. Kennedy.** 1972. The association of phosphatidylserine synthetase with ribosomes in extracts of *Escherichia coli*. J. Biol. Chem. **247**: 2008–2014.
25. **Raetz, C. R. H., T. J. Larson, and W. Dowhan.** 1977. Gene cloning for the isolation of enzymes of membrane lipid synthesis: phosphatidylserine synthetase overproduction in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:1412–1416.
26. **Raetz, C. R. H., and K. F. Newman.** 1979. Diglyceride kinase mutants of *Escherichia coli*: inner membrane association of 1,2-diglyceride and its relation to synthesis of membrane-derived oligosaccharides. J. Bacteriol. **137**:860–868.
27. **Silbert, D. F.** 1975. Genetic modification of membrane lipid. Annu. Rev. Biochem. **44**:315–339.
28. **Silbert, D. F., F. Ruch, and P. R. Vagelos.** 1968. Fatty acid replacements in a fatty acid auxotroph of *Escherichia coli*. J. Bacteriol. **95**:1658–1665.
29. **Stein, I., and B. Shapiro.** 1953. A rapid and simple method for the determination of esterified fatty acids and for total fatty acids in blood. J. Clin. Pathol. (London) **6**:158–161.
30. **Watson, J. D.** 1970. Molecular biology of the gene, 2nd ed., p. 85. W. A. Benjamin, Inc., New York.
31. **White, D. A., F. A. Albright, W. J. Lennarz, and C. A. Schnaitman.** 1971. Distribution of phospholipid-synthesizing enzymes in the wall and membrane subfractions of the envelope of *Escherichia coli*. Biochim. Biophys. Acta **249**:636–642.