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Altered Phospholipid Composition in Mutants of *Escherichia coli* Sensitive or Resistant to Organic Solvents

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Mutants of *Escherichia coli* with altered resistance to low molecular weight organic solvents were isolated. Solvent-resistant mutants showed a decrease in the ratio of phosphatidylethanolamine to the anionic phospholipids (phosphatidylglycerol and cardiolipin) relative to the wild-type, whereas solvent-sensitive strains showed an increase. Reversion studies on representative mutants demonstrated that the phenotypic response to solvents and the changes in phospholipid composition were genetically associated. The fatty acid and lipopolysaccharide compositions of the various mutants showed no significant differences from the parental strain. The lesions in two of the solvent-sensitive mutants (DC7 and DC9) and one of the resistant mutants (DC11) were mapped by cotransduction with phage P1 and shown to lie very close to the *pss* locus at 56 min on the *Escherichia coli* map.

INTRODUCTION

Alcohols and other low molecular weight solvents have long been known to inhibit the growth of bacteria (Dagley *et al.*, 1950). Low concentrations of solvents are reversible in action whereas higher concentrations are lethal (Harold, 1970). The target for solvents appears to be the lipid bilayer of the cell membranes (Hugo, 1967). Low concentrations of solvents dissolve into lipid bilayers, increasing the bilayer fluidity (Hill, 1974), whereas higher concentrations cause irreversible disruption and, eventually, lysis (Harold, 1970; Hugo, 1967). These observations suggested that mutants with an altered sensitivity to organic solvents might have an altered lipid composition which would change their membrane fluidity. We therefore isolated mutants of *Escherichia coli* K12 with altered sensitivity to ethanol and examined their lipid compositions.

METHODS

Bacterial strains and media. Bacterial strains are listed in Table 1. Strain UB1005 is a spontaneous nalidixic acid-resistant derivative of W1655F$^-$ (Bachmann, 1972) and is the parent of mutants DC7 to DC11. Strain KY2670 and its phenylethyl alcohol-resistant/azide-sensitive derivative KY2671 were received from Dr T. Yura (Kyoto University, Japan).

The nutrient broth has been described previously (Beard & Conolly, 1975). The minimal salts medium contained (g l^{-1}): K_2HPO_4, 7; KH_2PO_4, 3; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·25; sodium citrate, 0·5; glucose, 4. It was supplemented with necessary L-amino acids at 20 μg ml^{-1}. Solid media contained 1·65% (w/v) agar.

Mutagenesis and isolation of mutants. Mutagenesis was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 100 μg ml^{-1}) or ethyl methanesulphonate (EMS; 2·5%, w/v) under standard conditions (Adelberg *et al.*, 1956). Solvent-sensitive strains were isolated by replica-plating of mutagenized cells on to minimal agar containing 4% (v/v) ethanol. Solvent-resistant mutants were selected on minimal agar containing 5% (v/v) ethanol after enrichment of mutagenized cells in liquid medium containing 5% (v/v) dimethyl sulphoxide (DMSO).

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Table 1. *Strains of Escherichia coli*

Strain	Relevant characteristics	Reference or source*
UB1005	F⁻ *nalA37 metB1*	*nalA* of W1655F⁻
DC11	Solvent-resistant mutant of UB1005	This paper
DC7, DC9	Solvent-sensitive mutants of UB1005	This paper
DC76	Lac⁻ derivative of DC7	This paper
OS2101	F⁻ *strA thi-1 pyrD34 thyA33 pss-1*	CGSC5759
H680	F⁻ *strA thi-1 tyrA2 his-68 trp-45 purB51*	CGSC5038
JC1552	F⁻ *strA argG6 metB1 his-1 leu-6 trp-31 mtl-2 xyl-7 malA1 gal-6 lacY1*	CGSC4273
KA197	HfrKL16 *thi-1 pheA97*	CGSC5243
PA3306	F⁻ *strA thi-1 purI66 nadB4 argH1*	CGSC4537
KY2670	F⁻ *strA his met thi*	Yura & Wada (1968)
KY2671	F⁻ *strA thi-1 his pea/azi*	Yura & Wada (1968)
NH4104	F42*lac*⁺/*thr-1 leu-6 thi-1 his-4 proA2 lacY1 ara-14 uvrA6*	CGSC4349

* CGSC, Coli Genetic Stock Center, Yale University, U.S.A.

Sensitivity to organic solvents. Minimal inhibitory concentrations (m.i.c.) were measured by plating bacteria as single colony-forming units on minimal agar containing various concentrations of the appropriate solvent. The less volatile solvents gave more reproducible results.

Antibiotic sensitivity. This was measured using antibiotic discs. For tests with nitrofurans, nutrient agar was used; for sulphonamides, minimal agar with a reduced (0·25 mM) concentration of Mg^{2+} was used. Revertants of strain DC11 were selected on nutrient agar containing 0·1 μg furazolidone ml^{-1}.

Genetic mapping. Initial mapping was done using F′-mediated mobilization of the chromosome as described by Low (1973). Use of F42*lac*⁺ and F15*thy*⁺ was found sufficient to cover the whole chromosome. Counter-selection against the donors was by means of streptomycin (200 μg ml^{-1}). Cotransduction experiments were performed using phage P1vir, as described by Cronan *et al.* (1972).

Analysis of lipids. Bacteria were grown in nutrient broth to mid-exponential phase and washed by centrifugation. For measurement of the phospholipid content, a known weight of washed, lyophilized cells (100 to 200 mg) was resuspended in 5 ml deionized water and the lipids were extracted quantitatively as described by Radin (1972). A sample of the lipid extract was then assayed for phosphorus by the molybdate method (Chen *et al.*, 1956).

For other analyses, lipids were extracted by the procedure of Bligh & Dyer (1959). The proportions of the three phospholipid classes were examined by labelling cells with [2-^{3}H]glycerol (5 μCi ml^{-1}) and separating the extracted lipids on silica gel thin-layer plates using chloroform/methanol/water (65:25:4, by vol.) as solvent. The components were visualized with iodine vapour and the incorporation of ^{3}H was measured by liquid scintillation counting of the appropriate areas of the silica gel.

Fatty acid methyl esters were prepared from phospholipids by transmethylation with sodium methoxide in methanol (Polacco & Cronan, 1977). The esters were analysed by isothermal gas–liquid chromatography (g.l.c.) on a diethylene glycol succinate column (1·8 m × 4 mm) in a Varian 2100 Aerograph with dual-flame ionization detectors.

Analysis of lipopolysaccharides (LPS). The fatty acids of lipid A were obtained by acid hydrolysis of whole cells from which phospholipids and free fatty acids had previously been extracted (Spencer *et al.*, 1977). These fatty acids were analysed by g.l.c. after methylation using diazomethane (Schlenk & Gellerman, 1960).

Complete lipopolysaccharide was extracted using the method of Galanos *et al.* (1969) and its component sugars were separated by g.l.c. of the trimethylsilyl derivatives (Bhatti *et al.*, 1970). The g.l.c. of sugars was kindly performed by Dr J. Clamp and staff of the Clinical Research Laboratory of Bristol University.

The quantity of LPS was estimated by measuring the amount of ketodeoxyoctonate per unit protein in purified envelope fractions prepared as described previously (Beard & Conolly, 1975). Ketodeoxyoctonate was measured by the thiobarbituric acid reaction (Aminoff, 1961) using LPS from *E. coli* 026:B6 (Difco) as a standard. Protein was measured by Lowry's method and total carbohydrate by the phenol/sulphuric acid method of Norris & Ribbons (1971).

Gel electrophoresis of envelope proteins. Cell envelopes were prepared and their constituent proteins were separated by polyacrylamide gel analysis as described previously (Beard & Conolly, 1975).

Fluorescence measurements. Purified envelope material was dispersed at approximately 1 mg envelope protein ml^{-1} in fluorescence buffer (45 mM-NaCl, 30 mM-sodium acetate, 1 mM-NaN_3, 7·5 mM-sodium phosphate, pH 7·0). Then 1·0 mM-1-anilino-8-naphthalene sulphonate (ANS), 1·0 ml of envelope suspension

and an appropriate amount of ethanol were mixed thoroughly and made up to 5·0 ml with fluorescence buffer. After equilibrating for 10 min at room temperature, the sample was transferred to the cuvette of a fixed filter fluorimeter constructed by Dr R. W. G. Berkeley of this department. The excitation wavelength was 390 nm and the detection wavelength was 480 nm. Control samples (envelope material omitted) were also prepared and the fluorescence readings observed (always very low) were subtracted from those of the corresponding experimental samples. The fluorescence of samples of envelope material plus ANS, but without ethanol, was assigned an arbitrary value of 100. At the concentrations used, the fluorescence was proportional to the amount of envelope protein in the sample. Furthermore, equal amounts of membrane material from the various strains examined gave equal fluorescence readings when no ethanol was present.

RESULTS

Isolation and characterization of mutants

The wild-type strain UB1005 formed single colonies on minimal agar containing 4% ethanol (but not 5% ethanol) after 48 h incubation at 37 °C. Solvent-sensitive mutants were found by replica-plating cells mutagenized with NTG on to minimal agar containing 4% ethanol. Resistant mutants were selected by an enrichment step in liquid minimal medium containing 5% DMSO followed by plating on minimal agar containing 5% ethanol.

The frequency of ethanol-resistant mutants was approximately 1 in 10^4 following the mutagenesis and enrichment steps. We failed to select alcohol-resistant mutants spontaneously since resistance to organic solvents is extremely dependent on the inoculum and successful selections required relatively small inocula. Three classes of mutants were obtained. (1) Mutants showing an altered phospholipid composition and mapping very close to *pss*, the gene for phosphatidylserine synthetase (Ohta & Shibuya, 1977); these were either solvent-sensitive (e.g. DC7) or -resistant (e.g. DC11). (2) Mutants showing an altered phospholipid composition and also alterations in envelope protein composition; these were all solvent-resistant. (3) One solvent-resistant strain which contained nearly twice as much total phospholipid as the wild-type. The remainder of this paper is concerned with mutants of class 1.

Properties of class 1 *mutants*

Some properties of a representative solvent-sensitive strain (DC7) and a solvent-resistant strain (DC11) are given in Table 2. Sensitivity to organic solvents was assayed with a variety of alcohols, linear and branched (up to C_6), and with DMSO, acetone and dimethylformamide. In all cases, strain DC7 was more sensitive than UB1005 whereas DC11 was more resistant. In rich media, all strains were more solvent-resistant than in minimal media, but the relative differences between the mutants and the wild-type were retained. Neither of the mutants was cold-sensitive (at 30 °C) or temperature-sensitive (at 42 °C) nor were they altered in sensitivity to high concentrations of salt or sucrose. Unlike many envelope mutants, including the alcohol-resistant strains of Fried & Novick (1973), the mutants described here were of normal morphology when grown in nutrient broth at 37 °C.

Effect of ethanol on duration of the lag phase. Alcohol is known to increase the lag phase of growth when bacteria are inoculated into fresh media (Dagley *et al.*, 1950). We observed that solvent-resistant strains emerged from the lag phase more rapidly than did UB1005, even without ethanol; with ethanol, these differences were more pronounced. The duration of the observed lag depended both on the type of inoculum and on the alcohol concentration (data not shown). A converse effect was seen in solvent-sensitive mutants.

The enrichment procedure for solvent-resistant mutants depended to a considerable extent on the effect of solvents on the duration of the lag phase. In the enrichment medium (which contains 5% DMSO), the solvent-resistant mutants did not grow significantly faster than the wild-type (data not shown). However, the resistant mutants started to divide long before the parent emerged from the lag phase, and when the cultures were

Table 2. *Phospholipid composition and antibiotic sensitivity of wild-type and mutant strains of E. coli*

Strain	M.i.c. of *tert*-butanol (%, v/v)	Lipid phosphorus [μg (g dry wt)$^{-1}$]	Phospholipid composition * (%)			Sensitivity to†	
			PE	PG	CL	SMZ	FZ
UB1005	3	772	71·8	10·4	17·5	21·1	16·6
DC7	1·5	797	80·8	5·1	12·3	21·5	17·0
DC11	4	743	62·7	21·9	14·0	25·2	21·7

* Percentage incorporation of [^{3}H]glycerol into phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) for bacteria grown in nutrient broth.

† Sensitivity is expressed as the diameter (mm) of the inhibition zone around an antibiotic disc containing 200 μg sulphamethizole (SMZ) or furazolidone (FZ).

sampled at a bacterial density of 2×10^8 to 3×10^8 ml^{-1}, considerable enrichment was achieved.

Effect of phenylethyl alcohol (PEA). PEA inhibits phospholipid synthesis (Nunn *et al.*, 1977). The solvent-sensitive mutant DC7 was more sensitive to PEA than was UB1005, whereas the solvent-resistant mutant DC11 showed no increased resistance, Moreover, in liquid medium the growth rate of solvent-resistant strains was decreased less than that of UB1005 by 0·1% PEA, but all strains were severely and equally inhibited by 0·2% PEA. These results suggested that PEA might have two separate antibacterial actions. In support of this idea, we found that a PEA-resistant mutant, KY2671, isolated by Dr T.Yura (Yura & Wade, 1968) was as sensitive to aliphatic alcohols as its parent KY2670. The *pea/azi* mutation in KY2671 also resulted in increased sensitivity to azide, but UB1005, DC7 and DC11 showed identical responses to this inhibitor.

Sensitivity to antibiotics. Many mutants with altered cell envelopes show changes in sensitivity to various antibiotics (Raetz & Foulds, 1977; Sanderson *et al.*, 1974; Tamaki & Matsuhashi, 1973). Approximately 40 different antibiotics were tested against strain UB1005 and mutants DC7 and DC11. The only significant difference observed was that DC11 was more sensitive to nitrofurans (e.g. furazolidone) and sulphonamides (e.g.sulphamethizole), both very hydrophobic groups of antibiotics (Table 2).

Lipid composition. The lipid contents and phospholipid fatty acid compositions of the mutant strains were not markedly different from those of UB1005 (Table 2). The ratio of zwitterionic phospholipid species [phosphatidylethanolamine (PE)] to anionic species [phosphatidylglycerol (PG) and cardiolipin (CL)] correlated well with the response to organic solvents. The solvent-sensitive strain DC7 has significantly more PE than the wild-type, whereas the resistant mutant DC11 had considerably less. Since PG is converted into CL as cultures age (Cronan & Vagelos, 1972), the sum of these components is considered here.

To demonstrate further the relationship between phospholipid composition and solvent resistance, reversion studies were performed. Butanol-resistant revertants of DC7 were selected on minimal agar plates containing 2·5% (v/v) *tert*-butanol, after EMS treatment (without which no revertants could be isolated). Revertants of strain DC11 were selected for spontaneous resistance to furazolidone (0·1 μg ml^{-1} in nutrient agar) and then screened for solvent resistance. Four revertants of DC7 (85% PE) had an average PE content of 80·3% and four revertants of DC11 (75% PE) had an average PE content of 81·9% when grown in minimal medium plus glucose. These PE contents were not significantly different from that of UB1005 grown under the same conditions (81·2%). Comparison with data given in Table 2 shows that the proportion of PE is lower in nutrient broth than in minimal medium for UB1005, DC7 and DC11. Moreover, all strains become more solvent-resistant in richer media. One interpretation of these results is that the correlation between phospho-

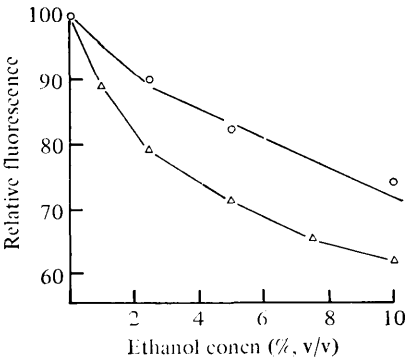

Fig. 1. Disruption by ethanol of membranes of strain UB1005 (○) and mutant DC7 (△). The disruption of purified envelope material from stationary phase bacteria grown in nutrient broth was monitored by the use of the fluorescent probe molecule ANS. The fluorescence of samples with ANS but without ethanol was assigned a value of 100.

lipid content and solvent resistance is common both to mutants and different growth media. Alternatively, these differences may merely reflect a labelling artifact resulting from the more rapid turnover of PG in cells growing faster in rich media (Cronan & Vagelos, 1972). Strain DC11 grew at the same rate as UB1005, in both nutrient broth and minimal medium. Strain DC7 grew more slowly than UB1005, but only in minimal medium. However, the solvent-resistant revertants of DC7 with wild-type phospholipid compositions retained the slow growth characteristic of DC7 in minimal medium. Thus, the changes in lipid composition in mutants DC7 and DC11 are not due to altered growth rates.

Lipopolysaccharide analysis. In envelope fractions the ratios of ketodeoxyoctonate (KDO) to protein and the total carbohydrate contents were the same for UB1005 and both mutants. The ratios of the major carbohydrate components of the LPS (approx. 2Glc: 1Gal:2Hep:1·5KDO:0·5GlcN) were also the same. Rhamnose, a minor component of the LPS in some *E. coli* K12 lines (Nikaido *et al.*, 1965) was absent from both the parent and mutant strains. The fatty acids of the LPS contained mainly myristate ($C_{14:0}$) and β-hydroxy-myristate with small amounts of another component, possibly a C_{16} hydroxy acid, and traces of acids characteristic of phospholipids. Separate samples from identical strains contained between 30 and 50% $C_{14:0}$. This poor reproducibility was probably due to some loss of the more volatile $C_{14:0}$ methyl ester and dehydrative breakdown of the hydroxy acids (Rooney *et al.*, 1972). Within these limits, no differences were observed between the parent and any mutant.

Envelope protein composition. Envelopes were prepared and tube gels were run to separate the constituent proteins as described in Methods. No significant differences were observed between UB1005 and DC7 or DC11.

Perturbation of membrane structure by ethanol. The fluorescent probe anilino-napthalene sulphonate (ANS) fluoresces very poorly in aqueous media. In a hydrophobic environment, such as cell membranes, the fluorescence yield greatly increases (see Chance *et al.*, 1971). On addition of ethanol to envelope material containing ANS, fluorescence decreases as the membrane is disrupted (Lenaz *et al.*, 1976). The envelopes of DC7 (solvent-sensitive) were more sensitive to disruption by ethanol than those of the wild-type, UB1005 (Fig. 1). Solvent-resistant mutants showed the opposite effect (data not shown).

Genetic mapping

The alcohol-sensitive mutation in DC7 was mapped using response to solvents as an unselected marker. A Lac$^-$ derivative of DC7 (designated DC76) was isolated by treating

Table 3. *Cotransduction experiments*

P1 donor	Recipient	Marker selected	Donor marker*/ Colonies scored	Cotransduction (%)
DC7	PA3306	*nadB*$^+$	121/233	51·9
DC7	PA3306	*purI*$^+$	40/200	20·0
DC7	H680	*tyrA*$^+$	74/137	54·0
DC7	KA197	*pheA*$^+$	108/200	54·0
DC9	PA3306	*nadB*$^+$	46/100	46·0
DC9	PA3306	*purI*$^+$	10/100	10·0
DC9	H680	*tyrA*$^+$	47/100	47·0
DC9	KA197	*pheA*$^+$	45/100	45·0
DC11	PA3306	*nadB*$^+$	61/100	61·0
DC11	H680	*tyrA*$^+$	32/70	45·0
DC11	KA197	*pheA*$^+$	17/53	32·1
OS2101	PA3306	*nadB*$^+$	15/132	11·3
OS2101	PA3306	*purI*$^+$	0/48	0
OS2101	H680	*tyrA*$^+$	19/64	29·7
OS2101	KA197	*pheA*$^+$	60/160	37·5

* The donor marker was sensitivity to *tert*-butanol for DC7 and DC9, sensitivity to furazolidone plus sulphanilamide for DC11, and temperature sensitivity for OS2101.

cells with EMS and plating survivors on to lactose/McConkey agar (Difco). F42 *lac*$^+$ was introduced into DC76 by mating with NH4104, using nalidixic acid to counterselect against the donor. Strain DC76 (F42) was mated with the multiply marked recipient JC1552 and recombinants were selected at a variety of loci around the *E. coli* chromosome and then scored for response to *tert*-butanol. The results indicated significant cotransfer of solvent sensitivity with the *his* (44 min) and *argG* (68 min) loci. Next, DC76 (F42) was crossed with PA3306 and *nad*$^+$*purI*$^+$ recombinants were selected; 49/100 were solvent-sensitive. Since the gene for phosphatidyl serine synthetase (*pss*) lies very close to *nadB* (Raetz, 1976), we attempted cotransduction of our alcohol-sensitive mutation with several loci near *pss*. The DC7 mutation was cotransducible with *nadB*, *tyrA*, *pheA* and *purI* (Table 3). Using P1 grown on DC9 (an independently isolated solvent-sensitive mutant, similar biochemically to DC7), we found that its mutation mapped in the same place as that of DC7 (Table 3). Similarly, the lesion in DC11 also mapped between *nadB* and *pheA*, the transductants being tested on minimal agar containing furazolidone (0·05 μg ml^{-1}) and sulphanilamide (200 μg ml^{-1}) with 0·4% succinate as carbon source. Two transductants to *nadB*$^+$, one wild-type and the other solvent-sensitive, from the cross P1 (DC7) × PA3306 were purified by re-isolation of single colonies. The phospholipid composition of these strains was analysed after growth on glucose minimal medium containing [^{3}H]glycerol. The wild-type transductant had 67·2% PE and 32·8% PG plus CL, while the solvent-sensitive transductant had, respectively, 77·3% and 22·7%. Thus, the phospholipid differences observed in the PA3306 background appear to be greater than those between the original wild-type and mutant.

The cotransduction frequencies observed by other workers for various *pss* mutants are from 27 to 52% with *nadB*, 19 to 62% with *tyrA*, 10 to 44% with *pheA* and 9 to 24% with *purI* (Raetz, 1976; Ohta *et al.*, 1975). For comparison, we cotransduced the *pss* mutation in strain OS2101 of Ohta *et al.* (1975) with these loci in the same strains as we used to map DC7, DC9 and DC11. Our values for cotransduction of *pss* with *nadB* and *purI* are very low (Table 3). However, the *pss* recombinants derived from strain PA3306 were extremely unstable, indicating that the low cotransduction frequencies were due to strong selection against *pss* in this background. In contrast, our value for cotransduction with *pheA* (38%) is much higher than that of Raetz (1976) (10%). Since *pheA* lies on the side of *tyrA* nearer to *nadB* (Bachmann *et al.*, 1976) and hence, nearer to *pss*, one would expect cotransduction frequencies for *pss* to be higher with *pheA* than with *tyrA*, as we have found (see also

Ohta *et al.*, 1975). It seems possible that the *pheA* recipient used by Raetz (1976) may have been incapable of maintaining the *pss* mutation stably, as was our *nadB* recipient, PA3306.

DISCUSSION

The mutants described in this paper demonstrate that a change in sensitivity to organic solvents may be caused by an alteration in the proportions of the different phospholipid classes. Increasing the proportion of the zwitterionic PE at the expense of the anionic lipids correlates with increased susceptibility to solvents, while a decrease in PE content shows the opposite effect. This phenomenon is common to six independently isolated mutants of which DC7 and DC11 are representative, and reversion analysis shows that the phenotypic response to solvents and the phospholipid composition are genetically related. *Pseudomonas aeruginosa* PAO is solvent-sensitive compared with *E. coli* UB1005 (Clark, unpublished results), and Brian Booth of this laboratory has found a phospholipid composition approximating that of the solvent-sensitive mutants of *E. coli* described in this paper (i.e. 80% PE). Further, anaesthetics such as tetracaine which are known to decrease the zwitterionic and increase the anionic lipid species in animal cells (Brindley *et al.*, 1975) can, under certain conditions, protect *E. coli* against organic solvents (Clark, unpublished results). This observation is also consistent with a zwitterionic:anionic ratio mechanism for determining solvent resistance.

In the mutants DC7 and DC11, the only envelope alterations observed were in phospholipid composition. It is possible that these strains contain mutations affecting the activity of one of the enzymes of phospholipid metabolism. The two most likely candidates are phosphatidylserine synthetase and phosphatidylglycerophosphate synthetase (Cronan & Vagelos, 1972). The mutations in DC7, DC9 and DC11 all mapped between *nadB* and *pheA*, as does the *pss* locus affecting phosphatidylserine synthetase (Ohta *et al.*, 1975; Raetz, 1976). This rules out the possibility of alterations in phosphatidylglycerophosphate synthetase (which maps elsewhere) and strongly suggests that these mutants may have alterations in their phosphatidylserine synthetase activity. Further genetic analysis was prevented by the absence of good selective procedures for dealing with the solvent mutants and the high reversion frequency and leakiness of *pss* strains.

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