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Unbalanced Membrane Phospholipid Compositions Affect Transcriptional Expression of Certain Regulatory Genes in *Escherichia coli*

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The amount of porin protein OmpF in the outer membrane of *Escherichia coli* was reduced to one-third by the *pgsA3* mutation that diminishes the amount of phosphatidylglycerol and cardiolipin in the membrane, whereas a *cls* (cardiolipin synthase) null mutation had no effect. Osmoregulation of OmpF was functional in the *pgsA3* mutant. As assessed by the β-galactosidase activities of *lacZ* fusions, the *ompF* expression was not reduced at the transcriptional level but was reduced about threefold at the posttranscriptional level by *pgsA3*. This reduction was mostly restored by a *micF* null mutation, and the *micF* RNA that inhibits the *ompF* mRNA translation was present 1.3 to 1.4 times more in the *pgsA3* mutant, as assayed by RNase protection and Northern blot analyses. Elevation of the level of *micF* RNA was not restricted to acidic-phospholipid deficiency: OmpF was hardly detected and *micF* RNA was present 2.7 to 2.8 times more in a *pssA* null mutant that lacked phosphatidylethanolamine. Other common phenotypes of *pgsA3* and *pssA* null mutants, reduced rates of cell growth and phospholipid synthesis, were not the cause of *micF* activation. Salicylate, which activates *micF* expression and inhibits cell motility, did not repress the flagellar master operon. These results imply that an unbalanced phospholipid composition, rather than a decrease or increase in the amount of specific phospholipid species, induces a phospholipid-specific stress signal to which certain regulatory genes respond positively or negatively according to their intrinsic mechanisms.

To understand the biological roles of individual membrane phospholipids, molecular genetic approaches to correlate mutationally modified lipid compositions with altered membrane functions have been successfully used with *Escherichia coli* (for a review, see reference 51). Recently, we found that flagellin formation is seriously impaired and the cells become nonmotile as a result of the *pgsA3* mutation but not of a *cls* null mutation (37). Tomura et al. independently made a similar observation with a *pgsA3* mutant (57). The *pgsA3* mutants harbor a missense mutation (Thr-60→Pro) in phosphatidylglycerophosphate synthase (EC 2.7.8.5) (59) that catalyzes the committed step in the biosynthetic pathway for acidic phospholipids, and these mutants therefore contain extremely low levels of otherwise abundant phosphatidylglycerol and cardiolipin (33). Null *cls* mutations cause loss of cardiolipin synthase, resulting in extremely low levels of cardiolipin (36). Subsequently, we found that the *pgsA3* mutation represses the transcriptional expression of the flagellar master operon (*flhD-flhC*) and that an upstream region of the operon is required for this repression (25). However, the precise localization of the region that is responsive to *pgsA3* has not been known, since the length of the cloned fragment was limited because of the limitation of the sequence data at the time of the study (8). Later, the flagellar master operon of *Salmonella typhimurium* was reported to be similarly repressed by *pgsA3* in *E. coli*, although the length of the region fused to *lacZ* was not described (35). In flagellar formation and chemotaxis in *E. coli*, more than 40 genes, organized into a regulatory hierarchy, are involved: expression of the master operon is essential for the function of all other genes (for a review, see reference 29). In this connection, it is important to note that Shi et al. showed that the flagellar master operon was repressed by *pssA* null and *psd-2* mutations (49). In their *pssA* mutants, the *pssA* gene (formerly *pss*), coding for phosphatidylserine synthase (EC 2.7.8.8), was insertion inactivated, resulting in the loss of phosphatidylethanolamine (12), whereas in the *psd-2* mutant, phosphatidylethanolamine is partially replaced with otherwise the minor component phosphatidylserine because of a defect in phosphatidylserine decarboxylase (EC 4.1.1.65) (20).

It is interesting that the phospholipid mutations profoundly affect not only various membrane functions but also a central and apparently nonmembranous cellular event, the transcriptional expression of the flagellar master operon. However, the molecular mechanisms underlying the phenomena have not been known, and an important point arises: the key feature that actually repressed the operon is not known. The membrane phospholipid compositions caused by these mutations are radically different: in *pgsA3* mutants, two major acidic phospholipids, phosphatidylglycerol and cardiolipin, are deficient, forming unusual membranes with the zwitterionic phosphatidylethanolamine as the sole major phospholipid (33), whereas phosphatidylethanolamine is missing and phospholipids are all acidic in null *pssA* mutants (12, 46). It is therefore unlikely that a specific phospholipid species, such as phosphatidylglycerol, is required for normal flagellation, as speculated previously (35, 57).

One of the cellular defects caused by the *pgsA3* mutation was a significant decrease in the level of OmpF protein in the outer membrane (37). OmpF is a major porin protein, encoded by *ompF*, and its formation is regulated both by a two-component regulatory system, comprised of EnvZ and OmpR, at the transcriptional level and by the antisense RNA, *micF* RNA, at the translational level (for reviews, see references 13, 17, and 41). In the present work, we analyzed this phenomenon and found that *micF* expression was elevated not only in *pgsA3* mutants

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TABLE 1. *E. coli* strains, plasmids, and phages used in this study

Strain, plasmid or phage	Relevant characteristics	Source or reference
Strains		
P90C	Δ(*lac-pro*)	32
ST001	P90C *lpp-2 uvrC279*::Tn*10*	This study
ST002	ST001 *pgsA3*	This study
ST003	ST001 Δ*micF1*::*kan*	This study
ST004	ST002 Δ*micF1*::*kan*	This study
ST010	ST001 λST01 lysogen (*ompF* operon fusion)	This study
ST020	ST002 λST01 lysogen (*ompF* operon fusion)	This study
ST030	ST001 λST03 lysogen (*ompC* operon fusion)	This study
ST040	ST002 λST03 lysogen (*ompC* operon fusion)	This study
ST100	ST001 λST02 lysogen (*ompF* protein fusion)	This study
ST200	ST002 λST02 lysogen (*ompF* protein fusion)	This study
ST300	ST003 λST02 lysogen (*ompF* protein fusion)	This study
ST400	ST004 λST02 lysogen (*ompF* protein fusion)	This study
FS401	P90C *lpp-2 uvrC279*::Tn*10* λ lysogen (*flhD* operon fusion)	K. Busujima
FS501	FS401 *pgsA3*	K. Busujima
AT141	Δ*ompB* (*envZ-ompR*)::*kan*	34
SM3001	Δ*micF1*::*kan*	30
W3110	Prototroph	Laboratory stock
GN10	W3110 Δ*pssA10*::*cam*	46
GN80	*cds-8*	19
GN85	GN80 cds^+	19
GN80L	GN80 *lacZ*::Tn*5*	This study
GN85L	GN85 *lacZ*::Tn*5*	This study
RK4784	Δ*ompC*	21
RK4786	*ompF*::Tn*5*	21
Plasmids		
pGEM-3Zf(−)	Ap^r; T7 RNA polymerase initiation site	Promega
pRS414	Ap^r; '*lacZ* protein fusion vector derived from pBR322	54
pRS415	Ap^r; $lacZ^+$ operon fusion vector derived from pBR322	54
pRS551	Ap^r Km^r; $lacZ^+$ operon fusion vector derived from pBR322	54
pRS1274	Ap^r; $lacZ^+$ operon fusion vector derived from pBR322	54
pST01	pRS415 Φ(*ompF'-lacZ*$^+$)*1*	This study
pST02	pRS414 Φ(*ompF'-'lacZ*)*1*(Hyb)	This study
pST03	pRS551 Φ(*ompC'-lacZ*$^+$)*1*	This study
pST04	pRS1274 Φ(*micF'-lacZ*$^+$)*1*	This study
pST05	pRS1274 harboring $micF^+$	This study
pST06	pGEM-3Zf(−) harboring *micF* (−35 to +69)	This study
Phages		
$P1_{vir}$		H. Ikeda
λRS88	Fusion vector	54
λST01	λRS88 harboring pST01	This study
λST02	λRS88 harboring pST02	This study
λST03	λRS88 harboring pST03	This study

but also in a *pssA* null mutant in which the *pssA* gene was replaced with a drug marker and thus phosphatidylethanolamine was completely absent (46). Among many environmental parameters that affect porin regulation and flagellation, high temperatures, high osmolarity, and ethanol were previously shown to affect these two cellular events simultaneously and reversely, all activating *micF* and inhibiting *flhD* (5, 50). In addition to these, an aromatic weak acid, salicylate, affects these adverse conditions, activating *micF* expression (45) and impairing cell motility (27), although the effect on the *flhD* expression has not been studied. In this respect, the *pgsA3* and *pssA* mutations might also be considered to fall into the same category of stress signals, raising another important question about how the same mutations affect one gene positively and the other negatively.

We describe here the observation that the level of the OmpF protein was decreased by phospholipid mutations through the activation of *micF* transcription and discuss, by combining the results on the regulation of the flagellar master operon, the causal relationships between the phospholipid mutations and transcriptional expression of the genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains, plasmids, and phages used in this study are listed in Table 1. New strains were constructed by P1 phage transduction (31), and their genotypes were verified by determining drug resistance, enzyme activities, and phospholipid compositions, whenever applicable. The *lpp-2* allele was used to suppress the lethal nature of the *pgsA3* allele (7).

ompF-operon fusion plasmid pST01 and *ompF*-protein fusion plasmid pST02 were derivatives of pRS415 and pRS414 and contained sequences from nucleotides −666 to +10 and −666 to +135 of the *ompF* gene (6, 38), respectively (taking the transcription start site as +1). *ompC*-operon fusion plasmid pST03, derived from pRS551, contained nucleotides −396 to +22 of the *ompC* gene (38), and *micF*-operon fusion plasmid pST04, derived from pRS1274, contained nucleotides −274 to +70 of the 4.5S *micF* gene (11, 38). Strains FS401 and FS501, provided by Kouji Busujima prior to publication, were lysogenic for a λ phage that carried an operon fusion of *lacZ* with a *flhD* upstream sequence that was derived from strain C600 and covered nucleotides −305 to +290, taking the

TABLE 2. Major outer membrane proteins in *pgsA3* and null *micF* mutants

Strain	Genotype	Sucrose concn (%)	Relative amt of[a]:			OmpF/OmpC ratio
			OmpA	OmpF	OmpC	
ST001	Wild type	0	100	47	77	0.61
		15	100	<1[b]	220	
ST002	*pgsA3*	0	100	15	61	0.25
		15	100	<1[b]	190	
ST003	Δ*micF*	0	100	63	102	0.62
ST004	*pgsA3* Δ*micF*	0	100	42	90	0.47

[a] Values are means of duplicate measurements for the cells grown to the mid-exponential phase at 37°C in medium A with or without sucrose supplementation and expressed relative to that of OmpA for each sample; the amounts of the OmpA protein did not vary more than 7% among the samples examined.
[b] Below the detection limit.

putative transcription start site of Shin and Park (53) as +1. Chromosomal fusion strains were constructed by the method of Simons et al. (54). To avoid the lethal overexpression of membrane proteins, strain AT141 (Δ*ompB*) was transformed with plasmid pST01, pST02, or pST03 and used as the host for growth of the λRS88 phage vector. Plasmid pST06 was constructed by inserting the *micF* gene (covering nucleotides −35 to +69) into pGEM-3Zf(−) (Promega) (42) to form, upon digestion with *Bam*HI, RNA complementary to the *micF* RNA by runoff transcription.

Media and bacterial growth. Broth media Luria-Bertani (LB) medium containing 0.5% NaCl (31), NBY (52), medium A (24), and synthetic media H56 (19) and minimal A (31) were used with the following supplements when required (per liter): 50 mg of thymine, 50 mg of ampicillin, 25 mg of kanamycin, 10 mg of chloramphenicol, and/or 10 mg of tetracycline hydrochloride. Motility agar plates were described previously (28). To examine the growth-rate-dependent OmpF contents, 0.2% glucose in minimal A was replaced with 0.4% D-glucose, D-mannitol, or D-mannose or 0.6% D-ribose. For strain GN10 (Δ*pssA10*::*cam*), the broth media were supplemented with 50 mM $MgCl_2$ (46). The cells were grown at 37°C with constant shaking, and growth was monitored by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 54 filter). One Klett unit corresponded to approximately 5×10^6 cells per ml.

DNA manipulations. Recombinant DNA techniques were conducted essentially as described previously (47). Restriction endonuclease digestion, filling of cohesive ends, and ligation were performed with enzymes from Takara Shuzo (Kyoto, Japan), Nippon Gene (Tokyo, Japan), and New England BioLabs (Beverly, Mass.) under conditions recommended by the suppliers. Genomic DNAs were amplified by PCR with *Taq* DNA polymerase and synthetic primers (25, 59).

Outer membrane fractions and electrophoresis. The outer membrane protein fraction was prepared by disrupting cells in 10 mM HEPES buffer (pH 7.4) by a French press and pelleting by centrifugation at $36,000 \times g$ for 1 h, as described previously (48). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the presence of 8 M urea (43). Protein bands were stained with Coomassie blue R250 and quantitated by using a model AE-6920M densitograph (Atto, Tokyo, Japan). OmpF and OmpC were identified by comparing with the protein profiles of strains RK4786 (*ompF*::Tn*5*) and RK4784 (Δ*ompC*), respectively.

Phospholipid analysis. Lipids were extracted essentially by the method of Ames (3) and separated by two-dimensional thin-layer chromatography on silica gel plates as described previously (52). For quantitative analysis, cells were labeled with [^{32}P]orthophosphate for at least five generations during exponential growth at 37°C. The radioactivity of each spot on two-dimensional chromatograms was measured with a bio-imaging analyzer (BAS 1000 MacBAS; Fuji Film, Tokyo, Japan). For rough estimation of the phospholipid composition, lipids were separated by one-dimensional silica gel thin-layer chromatography, visualized by spraying Dittmer-Lester reagent (14), and analyzed as described previously (52).

RNA analysis. For RNA extraction, cells were grown in medium A (strains ST001 and ST002), LB medium supplemented with 50 mM $MgCl_2$ (strains W3110 and GN10), or H56 medium (strains GN80 and GN85) at 37°C to the midexponential phase and transferred to a lysis buffer (2% SDS, 16 mM EDTA, and 20 mM NaCl in diethylpyrocarbonate-treated water). RNA samples were prepared as described previously (58). Transcriptional expression of the chromosomal *micF* gene was analyzed by an RNase protection assay specified by Ambion (2). A ^{32}P-labeled RNA probe complementary to a part of *micF* RNA was synthesized with pST06 as the template for runoff transcription by using the MAXIscript kit (2). [α-^{32}P]ATP was purchased from Institute of Isotopes Co., Budapest, Hungary. The *micF* RNA levels in cells carrying multicopy pST05 (*micF*$^+$) were determined by Northern blot analysis. Samples containing equal quantities (20 to 25 μg) of total RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6% formaldehyde and 20 mM morpholinopropanesulfonate (pH 7.4) followed by transfer to a nylon membrane (Hybond-N+; Amersham, Little Chalfont, England). Hybridization was performed for 20 h at 55°C with the 5′ ^{32}P-labeled synthetic 54-mer oligodeoxynucleotide (5×10^7 cpm) complementary to the central portion (nucleotides 11 to 64) of the 4.5S *micF* RNA. [γ-^{32}P]ATP was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.). The radioactivity of each band on the filter was measured with a bio-imaging analyzer.

Assays. β-Galactosidase activity was measured by the method of Tesfa-Selase and Drabble (55), and specific activity was defined as units (nanomoles of 2-nitrophenol formed per minute) per milliliter of culture at 40 Klett units. Total phosphorus contents were measured by the method of Bartlett (9). Protein was assayed by the method of Peterson (40) with crystalline bovine serum albumin as the standard. Osmolalities of culture media (in milliosmoles per kilogram) were measured in a microosmometer (type 13 DR; Roebling Co., Berlin, Germany). Swarms were examined after incubation of the cells on the motility plates supplemented with or without salicylate for 9 h at 35°C.

RESULTS

Effect of the *pgsA3* mutation on the *ompF* expression. The outer membrane fractions of *E. coli* ST001 (*pgsA*$^+$) and ST002 (*pgsA3*) were examined by urea-SDS-polyacrylamide gel electrophoresis and densitometry in the absence of sucrose (Table 2). The OmpF level in the mutant was one-third of that in the isogenic wild-type strain, confirming our preliminary observation made by using different genetic backgrounds and the cell fractionation procedure (37). The OmpC level was reduced to a lesser extent. Phosphatidylglycerophosphate synthase activity and acidic-phospholipid contents in *pgsA3* mutants are less than 1% of those in the wild-type cells (33). OmpF was hardly detected in both strains when the culture osmolality was raised from 136 (medium A) to 580 mOsm/kg by adding 15% sucrose to the medium (Table 2), suggesting that the osmoregulation of *ompF* was functional under acidic-phospholipid deficiency. In contrast, a null *cls* mutation that causes the loss of cardiolipin synthase, resulting in an extremely low level of cardiolipin (36), did not have a significant effect on the porin contents (data not shown).

To reveal the molecular mechanism of the decrease in porin contents by the *pgsA3* mutation, both the transcriptional expression and translational expression of porin genes were examined with operon and protein fusions to the *lacZ* reporter, respectively, by measuring the β-galactosidase activity. The transcriptional expression of *ompF*, as well as of *ompC*, was not reduced but, rather, was elevated slightly by *pgsA3*, under conditions of either low or high osmolarity (Table 3), whereas the translation of *ompF* in the *pgsA3* mutant was one-third of that in the wild-type strain (Table 4). Addition of 10% sucrose to the medium resulted in a fivefold reduction of the β-galactosidase activities in both strains (140 ± 10 in ST001 and 60 ± 10 in ST002). Table 4 also shows that this reduction was in most part restored by introducing a *micF* null mutation. The *micF* null mutation similarly restored the decrease in the level of OmpF in the outer membrane as a result of *pgsA3* (Table 2), suggesting that the reduction in the level of OmpF was mainly if not entirely due to the elevated expression of the *micF* gene. Figure 1A demonstrates that this was the case. The amount of

TABLE 3. Transcriptional expression of the *ompF* and *ompC* genes in *pgsA3* mutants

Strain	Operon fusion	*pgsA*	β-Galactosidase sp act[a] with sucrose concn (%) of:	
			0	10
ST010	*ompF-lacZ*	Wild type	200 ± 48	120 ± 30
ST020	*ompF-lacZ*	*pgsA3*	250 ± 13	130 ± 6
ST030	*ompC-lacZ*	Wild type	140 ± 8	170 ± 20
ST040	*ompC-lacZ*	*pgsA3*	160 ± 30	230 ± 50

[a] Cells were grown at 37°C in medium A to 40 Klett units. Values are means and standard deviations of six and three independent measurements for *ompF* and *ompC* fusion strains, respectively.

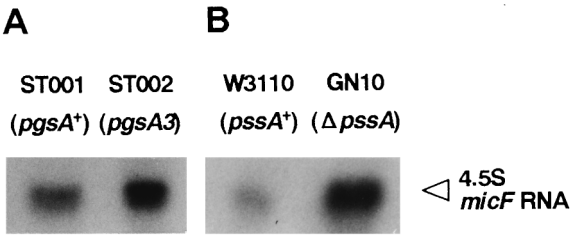

FIG. 1. Effect of phospholipid mutations on the expression of the chromosomal *micF* gene. *micF* RNA levels were determined by an RNase protection assay as described in Materials and Methods. (A) Effect of the *pgsA3* mutation. (B) Effect of the null *pssA* mutation.

4.5S *micF* RNA, as assayed by RNase protection, was 1.3 times larger in ST002 (*pgsA3*) than in ST001 (*pgsA*$^+$). Essentially the same result was obtained by Northern blot analysis with cells harboring a multicopy plasmid containing the *micF* locus: the amount of 4.5S *micF* RNA was 1.4 times larger in ST002/pST05 than that in ST001/pST05.

Effect of the null *pssA* mutation on *ompF* expression. Since the *pgsA3* mutation, but not a null *cls* mutation, affected the OmpF level in the outer membrane, the effect of another type of phospholipid mutation, a *pssA* null allele that causes the complete loss of phosphatidylethanolamine, was examined. As shown in Table 5, OmpF in strain GN10 (Δ*pssA10*::*cam*) was reduced to a negligible level under conditions of either low (NBY) or high (LB medium) osmolarity. GN10 did not grow well and aggregated in Mg^{2+}-containing medium A. The amount of *micF* RNA in GN10 was 2.8 times larger than that in W3110, as assessed by the RNase protection assay (Fig. 1B). Northern blot analysis of the cells harboring pST05 (*micF*$^+$) gave practically the same result: GN10/pST05 had 2.7 times more *micF* RNA than did W3110/pST05. Therefore, the *pssA* null mutation exerted an enhancing effect similar to, but much more significant than, that of the *pgsA3* mutation on the *micF* expression, despite a radical difference in the phospholipid polar head group composition caused by the two mutations. This suggested that the *micF* activation was caused by a factor common to these two types of phospholipid mutants not by the increase or decrease of specific phospholipid species.

Effects of phenotypes common to the *pgsA3* and *pssA* null mutants on the *micF* expression. We asked if the key feature that actually elevated the *micF* transcription was not the alteration of phospholipid composition but some other phenotypes common to the *pgsA3* and the *pssA* null mutants. Possible reduction of the total phospholipid content relative to that in the membrane protein was first examined, since the total phospholipid contents were significantly lower in *pssA* null mutants (12, 46) and the biosynthetic rates for all major phospholipids were reduced in a *pgsA3* mutant (46). Unexpectedly, the total phospholipid content in strain ST002 (*pgsA3*) was not low but, instead, slightly higher than that of the isogenic wild type, ST001: there was 440 nmol of lipid-phosphorus per mg of membrane protein in ST002 grown in medium A in the midexponential phase and 360 nmol/mg in the late exponential phase, whereas the values for ST001 were 400 and 340 nmol/mg, respectively (means of four independent determinations). We obtained essentially the same ratios when comparing the total radioactivities of the lipid fractions uniformly labeled with ^{32}P (1.25 times more radioactivity from ST002 than from ST001). These results suggested that the variation of the total phospholipid contents was not directly related to the expression of *micF*.

Possible effects of the reduced rates of phospholipid synthesis were next examined by using a *cds-8* mutant in which CDP-diacylglycerol synthetase is alkali labile and thus the biosynthetic rate for the total phospholipids decreases rapidly upon a pH shift from 6 to 8.5 (19). We confirmed these characteristics of the mutation with our constructs (data not shown). At 30 min after the pH shift from 6 to 8.5 in H56 medium, the β-galactosidase activity of strain GN80L (*cds-8 lacZ*::Tn*5*) harboring the *micF′-lacZ*$^+$ operon fusion plasmid, pST04, was 97% of that of GN85L (*cds*$^+$ *lacZ*::Tn*5*) harboring the same plasmid. The amount of *micF* RNA in GN80 harboring pST05 (*micF*$^+$) was 94% of that in GN85 with the same plasmid 30 min after the pH shift, as assessed by Northern blot analysis. Therefore, it is unlikely that the reduced rate of total phospholipid synthesis is related to the *micF* expression.

Since both the *pgsA3* and null *pssA* mutations lower the cell growth rates (12, 33, 46), we examined whether the reduction of growth rate, independent of phospholipid alteration, affected the porin protein contents. Table 6 shows that the various carbon sources yielded different growth rates and different porin contents in the outer membrane. The amount of OmpF, relative to that of OmpA, increased when the growth rate decreased, whereas that of OmpC varied. The molecular mechanism for these changes is unknown, but it is clear that the reduction of growth rate does not reduce the amount of OmpF formed.

Effects of the *pgsA3* mutation and salicylate on the flagellar master operon. We have performed additional experiments to

TABLE 4. Translational expression of the *ompF* gene in the *pgsA3* and *micF* null mutants

Strain	Protein fusion	*pgsA*	*micF*	β-Galactosidase sp act[a]	Ratio of sp act for *pgsA3*/*pgsA*$^+$
ST100	*ompF-lacZ*	Wild type	Wild type	750 ± 26	
ST200	*ompF-lacZ*	*pgsA3*	Wild type	230 ± 27	0.3
ST300	*ompF-lacZ*	Wild type	Null	750 ± 10	
ST400	*ompF-lacZ*	*pgsA3*	Null	680 ± 60	0.9

[a] Values represent the means of four independent measurements with standard deviations for the cells grown in medium A at 37°C.

TABLE 5. Major outer membrane proteins in a null *pssA* mutant[a]

Strain	*pssA*	Basal medium	Relative amt[b] of:		
			OmpA	OmpF	OmpC
W3110	Wild type	NBY	100	96	195
GN10	Δ*pssA10*::*cam*	NBY	100	<1[c]	225
W3110	Wild type	LB	100	8	216
GN10	Δ*pssA10*::*cam*	LB	100	<1[c]	243

[a] Cells were grown at 37°C to the midexponential phase either in NBY medium (140 mOsm/kg) or in LB medium (260 mOsm/kg), both of which were supplemented with 50 mM $MgCl_2$.
[b] Values are means of duplicate measurements and are shown relative to those of OmpA.
[c] Below the detection limit.

facilitate the understanding of the nature of the putative signal generated by the phospholipid mutations. Table 7 shows the effect of salicylate, as well as the *pgsA3* mutation, on the transcriptional expression of the *flhD* gene. In this lysogenized λ, the *flhD* upstream region fused to *lacZ* was 230 bp longer than that in the previously used fusion plasmids (25) and therefore contained the entire and intact sequence responsive to the *pgsA3* mutation (23), unlike the previously reported Mu-mediated chromosomal fusion strains (25, 26) that are now known to contain an insertion sequence near the putative promoter site (23). Without salicylate, the *pgsA3* mutation repressed the *flhD* expression 40-fold and the cells became completely nonmotile. The repression was 6- and 14-fold more severe than that observed with the Mu-mediated fusion strains and with the fusion plasmids harboring shorter *flhD* regions, respectively, confirming the previously observed repression of the flagellar master operon qualitatively but not quantitatively.

Salicylate at 5 mM impaired cell motility, as reported previously (27), but did not repress the *flhD* transcription. The growth rate of FS401 was 70% of that without the drug. Salicylate at higher concentrations severely inhibited cell growth, making the analysis difficult. Under these conditions, salicylate stimulated the β-galactosidase activity in both the wild-type and *pgsA3* cells. Although the site of action of salicylate to make cells nonmotile and the reason for the observed stimulation of *flhD* expression remain to be elucidated, it was clear that the putative stress signal generated by this drug is not the same as those generated by phospholipid mutations.

DISCUSSION

The present study showed that both the acidic-phospholipid deficiency caused by the *pgsA3* mutation and the lack of phosphatidylethanolamine due to the *pssA* null mutation lower the OmpF level in the outer membrane and, at the same time, increase the level of *micF* RNA. Although the increase caused by the *pgsA3* mutation was small (1.3- and 1.4-fold for the chromosomal and plasmid-encoded *micF* RNA, respectively), it must have been responsible for the OmpF reduction, since the null *micF* mutation mostly restored both the OmpF level (Table 2) and the translational expression of the *ompF* gene (Table 4) in *pgsA3* mutants. In fact, small extents of *micF* activation are known to strongly inhibit the *ompF* translation, resulting in drastic decreases in the level of OmpF protein; they were two- to threefold increases by *soxR101* and *soxQ1* mutations (10), twofold increase by an *lrp* mutation (16), and three- to fourfold increase in cells treated with salicylate (45). The only exception is a direct and moderate amplification of the *micF* gene: about sixfold amplification of *micF* by cloning into a pSC101-derived plasmid did not result in the inhibition of *ompF* translation (1). To explain this, *micF* RNA has been suggested to need a cognate protein, possibly the 80-kDa protein isolated by Andersen and Delihas (4), for destabilization of *ompF* mRNA (13). It is therefore possible that the reduction of OmpF takes place only by a concerted mechanism involving the induction of such a protein factor and that the phospholipid mutations, as well as those referred to above, generate signals to which the regulatory mechanisms of both the *micF* gene and a gene responsible for the putative protein factor respond simultaneously.

Even under the serious acidic-phospholipid deficiency in *pgsA3* mutants, osmoregulation of OmpF was well if not fully functional and the *ompF* transcription was not inhibited (Table 3), suggesting that the two-component system, EnvZ-OmpR, was not impaired by *pgsA3*. This was rather surprising, since the EnvZ protein, embedded in the membrane, is known to be sensitive to local anesthetics that perturb the membrane structure (39, 44) and is easily inactivated by replacing certain amino acid residues in the region in contact with membrane phospholipids (56).

The phospholipid alterations that activated the *micF* transcription in the present work were also known to affect the transcription of the flagellar master operon, thus abolishing the flagellation of the cell (25, 37, 49). In the present study, we confirmed the repression by *pgsA3* by using an upstream region of the operon that contained the entire and intact sequence responsive to the mutation. It should be noted that the two phospholipid mutations affect the transcriptional expressions of the two genes in opposite directions, i.e., downward to *flhD* and upward to *micF*, and that the lipid modification in opposite directions, i.e., an increase in the zwitterionic phosphatidylethanolamine level and a decrease in acidic phospholipid levels by *pgsA3* and vice versa by null *pssA*, causes the same responses of the two genes. These would imply that a more direct cause common to the two phospholipid mutants, other than the alteration of phospholipid polar head group compositions, was responsible for the altered gene expressions. Attempts to find such a cause were, however, unsuccessful: reduction of the growth rate by poor carbon sources and reduction of the total phospholipid biogenesis by introducing the *cds-8* mutation did not result in an enhanced *micF* expression. Null *pssA* mutations reduced the total phospholipid contents, but instead, the *pgsA3* mutation increased the ratio phospholipid to protein in the membrane, despite the extremely impaired biosynthesis not only of acidic phospholipids (100-fold) but also of phosphatidylethanolamine (10-fold) (46), and hence the decrease in the total phospholipid content was not common to the two phospholipid mutants. The apparent discrepancy between the biosynthetic rates and the contents of phospholipids in *pgsA3* mutants indicated the possibility that phospholipid biosynthesis proceeds only in a limited span within the wild-type cell cycle and the extent of the biosynthesis is determined by the

TABLE 6. Growth rates and porin protein contents[a]

Carbon source	Generation time (min)	Relative amt[b] of:		
		OmpA	OmpF	OmpC
D-Glucose	60	100	48	78
D-Mannitol	66	100	70	61
D-Mannose	132	100	105	105
D-Ribose	372	100	146	53

[a] Cells were grown at 37°C in minimal A medium containing the various carbon sources as described in Materials and Methods. Medium osmolalities did not vary appreciably (234 to 246 mOsm/kg).
[b] Values are relative to those for OmpA (taken as 100).

TABLE 7. Effects of the *pgsA3* mutation and salicylate on the flagellar master operon

Strain	Genotype	Salicylate concn (mM)[a]	β-Galactosidase sp act[b]	Motility[c] (mm)
FS401	*flhD*$^+$ *pgsA*$^+$ λ(*flhD′-lacZ*$^+$)	0	67.6	13
		5	84.4	4
FS501	*flhD*$^+$ *pgsA3* λ(*flhD′-lacZ*$^+$)	0	2.8	<1
		5	4.0	<1

[a] For *flhD* expression, cells were grown in medium A with or without supplementation by 5 mM sodium salicylate at 35°C to 40 Klett units.
[b] Values of duplicate assays did not vary more than 1%.
[c] Motility was evaluated by measuring the diameter of swarms formed after 9 h of incubation at 35°C.

membrane structure. More phosphatidylethanolamine molecules may be allowed in the membrane than acidic phospholipids that should repel each other. The results suggested that the abnormality of the phospholipid composition generates a signal to which certain genes respond by using their own regulatory mechanisms.

The phospholipid compositions of the *pgsA3*, *pssA*, and *psd* mutants but not the *cls* mutants differ in the ratio of zwitterionic to acidic phospholipids from that of the wild-type cells; this wild-type ratio is considered important for various membrane functions and is kept constant by a unique regulatory mechanism in the wild-type *E. coli* cell (46, 51). The *cls* mutations that do not affect the OmpF content and flagellation (37) reduce the cardiolipin content but do not change the amount of the total acidic phospholipids, phosphatidylglycerol plus cardiolipin (36), whereas the *psd-2* mutation that represses *flhD* (49) partially replaces zwitterionic phosphatidylethanolamine with acidic phosphatidylserine (20). It is therefore highly likely that the key feature of the phospholipid mutations that affect the transcriptional expressions of *micF* and *flhD* is the unbalanced ratio of zwitterionic to acidic phospholipids in the membrane.

In relation to the nature of the putative signals generated by the perturbation of the balanced phospholipid composition, we also observed transcription activation of the genes *sulA* (suppressor of SOS-induced filamentation [60]) and *sodA* (manganese-containing superoxide dismutase [15]) in *pgsA3* mutants, by assaying the β-galactosidase activities of the cells lysogenic for phage λ carrying *lacZ* operon fusions (22). Although *sodA* and *micF* are known to be induced by oxidative stress (10, 15), as by the *pgsA3* mutation, the stress signal generated by phospholipid mutations is not necessarily the same as that generated by oxidative stresses: nalidixic acid which possibly activates *sulA* through *recA* induction and *sodA* (15), has no effect on oxidative phosphorylation in *E. coli* (18), indicating that different signals are generated by different stimuli. This notion is supported by our finding that salicylate, which increases *micF* transcription (45) and impairs cell motility (27), did not affect the *flhD* transcription (Table 7). Several adverse conditions, such as high temperature, high osmolarity, and ethanol, have similar effects on both *micF* and *flhD* expression (5, 50). It may be physiologically advantageous for *E. coli* cells to shut down the costly formation of OmpF protein and flagellum under these conditions, and the regulatory regions of the *micF* and the flagellar master operon must have evolved to respond in this way. Further studies to reveal the molecular mechanism underlying these phenomena should facilitate the full understanding of the global regulatory networks in *E. coli* cells.

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