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Primary Structures of the Wild-Type and Mutant Alleles Encoding the Phosphatidylglycerophosphate Synthase of *Escherichia coli*

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The nucleotide sequence of the *Escherichia coli pgsA* gene, encoding phosphatidylglycerophosphate synthase, is revised to code for an enzyme of 182 amino acid residues, instead of the 216 of a previous work (A. S. Gopalakrishnan, Y.-C. Chen, M. Temkin, and W. Dowhan, J. Biol. Chem. 261:1329–1338, 1986). The revised structure now explains the properties of the enzyme. Three *pgsA* mutants of different phenotypes were also analyzed: *pgsA3*, *pgsA36*, and *pgsA10* have single-base replacements in codons 60 (Thr→Pro), 1 (ATG→ATA), and 92 (Thr→Ile), respectively.

In *Escherichia coli*, phosphatidylglycerophosphate (PGP) synthase, encoded by *pgsA*, catalyzes the committed step in the acidic-phospholipid biosynthesis and is essential for cell growth. In view of the potential importance of these lipids in various cellular functions, it is important to understand the biosynthetic regulation and properties of this enzyme on a molecular basis. It is also desirable to know the molecular structures of mutant *pgsA* alleles that are especially useful in studying the metabolic regulation and biological functions of acidic phospholipids. However, none of the primary structures of mutant *pgsA* alleles has been elucidated (for a recent review, see reference 32).

Gopalakrishnan and coworkers reported the nucleotide sequence of the wild-type *pgsA* gene (10), but it indicated curious features of the gene product: (i) a spontaneous insertion of insertion element IS*1* (10, 26) and a shortening by *Hin*cII digestion (12), both of which must have drastically altered the carboxyl terminus of the gene product, do not affect the enzymatic activity (32), and (ii) the deduced molecular mass of the protein (24 kDa) is not consistent with that actually observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for purified PGP synthase preparations (19 kDa). In addition, the translation initiation site was not unequivocally assigned, because of the blocked amino terminus of the gene product and the apparent absence of a Shine-Dalgarno sequence. We report here the result of a careful reexamination of the primary structure of the wild-type *pgsA* gene which clearly requires revisions of the previously reported one (10). We also describe the structures of three mutant *pgsA* alleles.

E. coli K-12 strains and plasmids used in this study are listed in Table 1. Cells were grown in LB medium (20) at 37°C unless otherwise stated. Recombinant DNA techniques were conducted essentially as described previously (31). The *pgsA* loci of the genomic DNAs were amplified by PCR with *Taq* DNA polymerase (30) and subcloned into M13 vectors mp18 and mp19 (31), and single-stranded DNAs were subjected to sequencing. The chain termination method was performed with fluorescent M13 universal primers (Yuki Gosei Kogyo, Tokyo, Japan) and primers shown in Fig. 1 by using T7 DNA polymerase in an automated laser fluorescent DNA sequencer (model DSQ-1; Shimadzu, Kyoto, Japan). 7-Deaza-dATP and 7-deaza-dGTP (Pharmacia) were employed to prevent formation of intrastrand secondary structures. PGP synthase was assayed in sonically ruptured cells by measuring the CDP-diacylglycerol-dependent incorporation of *sn*-[2-^{3}H]glycerol-3-phosphate in the presence of Triton X-100 as described previously (25). β-Galactosidase was assayed by the method of Tesfa-Selase and Drabble (35) in cells in the exponential growth phase. Its specific activity is defined as nanomoles of 2-nitrophenol formed per minute per milliliter culture at 100 Klett units (approximately 8×10^8 cells per ml).

Primary structure of the wild-type *pgsA* locus. The chromosomal *pgsA* loci of five *E. coli* K-12 strains, W3110, JE5512, YA5512, SD136, and AD10, as well as the *pgsA* locus of pPG1-L, the recombinant plasmid used for sequencing in the previous work (10), were carefully sequenced. Both DNA strands of each sample covering nucleotides 1 to 764 of Fig. 1 were subjected to analysis. Sequencing was repeatedly carried out for independently isolated subclones; for instance, regions around nucleotides 453 and 609 were examined 58 and 43 times in all, respectively. Except for the presence of one displacement in each *pgsA* mutant strain as described later, all samples consistently and clearly gave a sequence which differed in two points from that reported by Gopalakrishnan et al. (10). In Fig. 1, the nucleotide sequence obtained here and the deduced amino acid sequence for PGP synthase are shown, together with the relevant sites of restriction cleavage and primer oligonucleotides used in this work. The C of position 453 was T and the T of position 609 was missing in the previous work. The former discrepancy does not influence the protein structure, but the latter corresponds to a radical difference: our sequence predicts a gene product of 182 amino acid residues, instead of 216 of the previous one, and lacks the unique carboxyl-terminal region unusually enriched with basic residues.

To confirm the presence of an extra T after 608-G, a series of reading frame analyses by a protein fusion technique was performed. The *pgsA* locus of strain W3110 was amplified by PCR, and three fragments (nucleotides 1 to 592 by digestion with *Bgl*II and *Ssp*I, nucleotides 1 to 600 by digestion with *Bgl*II and *Aor*51HI, and nucleotides 1 to 621 by digestion with *Bgl*II and *Bcl*I) were prepared. They were inserted into protein fusion vectors pRS552, pRS577, and pRS591. Junction regions of all constructs were sequenced to confirm the insertion directions and structures. As illustrated in Fig. 2, digestion of pRS552 with *Bam*HI, pRS577 with *Bam*HI, and pRS591 with *Sma*I left 6, 22, and 15 nucleotides in front of codon 9 of *lacZ*,

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
W3110	F⁻ IN(*rrnD-rrnE*)*1*	2
JE5512	Hfr *man-1 pps*	14
YA5512	JE5512 *pgsA3 uvrC279*::Tn*10*	1
SD136	*pgsA36 pssA1 cls-1 his-68 rpsL118 lpp-12*	21
SD336	SD136 *pssA*⁺ *his*⁺	This work
AD10	*pgsA10*	26, 28
XL1-Blue	*recA1 hsdR17 lac*/F' (*lacZ*Δ*M15*::Tn*10*)	5
Plasmids		
pPG1-L	pSC101 harboring the F150-derived *pgsA* locus with IS*1* insertion	26
pRS552	'*lacZ* protein fusion vector	34
pRS577	'*lacZ* protein fusion vector	34
pRS591	'*lacZ* protein fusion vector	34

respectively. The β-galactosidase activities in strain XL1-Blue (*lac*) harboring these fusion plasmids (Fig. 2) clearly showed that the *pgsA* reading frame continues only in the presence of 609-T.

The present analysis also indicated that there is another reading frame actually expressed at a very low but still significant level. It starts somewhere between the *Ssp*I and *Bcl*I sites, most probably using nucleotides 608 to 610 (GUG in the mRNA) as the initiation codon. Assuming that the cellular level of PGP synthase is 1,400 molecules per cell (19) and that the observed β-galactosidase activities are proportional to the levels of chromosomal expression, the product of this reading frame may be present at about 7 molecules per cell.

The new sequence (Fig. 1) explains several properties of the gene product, PGP synthase, previously described by Dowhan and coworkers (10, 12, 13): (i) the deduced molecular mass (20.7 kDa) agrees well with that actually obtained; (ii) the amino acid composition deduced from the new sequence is closer than that from the previous one to the actual composition; and (iii) the sites of IS*1* insertion and *Hin*cII cleavage are outside the *pgsA* coding region, explaining the unchanged PGP synthase activities of the gene products upon these manipulations (10, 12).

Dowhan has examined the discrepancy between the previously reported data and our new data for wild-type *pgsA* by sequencing again the two regions in question of pPG1, the *pgsA*-carrying plasmid used in the previous sequencing work (10, 26), and is now in complete agreement with our sequence (6).

Primary structures of mutant *pgsA* alleles. The chromosomal *pgsA* loci of three *pgsA* mutants, YA5512 (*pgsA3*), SD136 (*pgsA36*), and AD10 (*pgsA10*), were similarly amplified by PCR and sequenced. All alleles had single-base replacements as indicated in Fig. 1.

pgsA3, the most powerful mutation to cause acidic-phospholipid deficiency (1, 21, 32), has a C in place of the wild-type

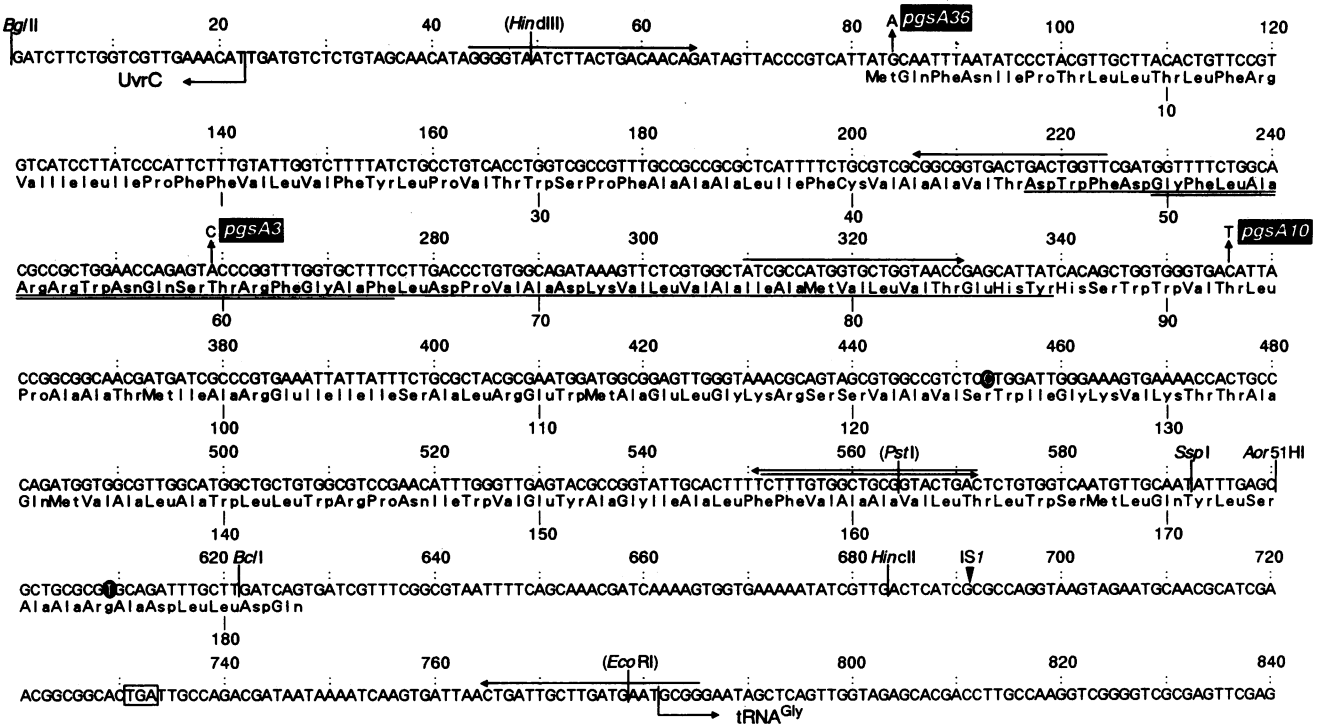

FIG. 1. Revised DNA sequence and derived protein sequence of the *E. coli pgsA* locus. Nucleotide replacements in the *pgsA36*, *pgsA3*, and *pgsA10* mutations are also included. The sequence beyond position 764 is taken from reference 10. Two nucleotides (positions 453 and 609) different from the previously reported sequence (10) are shown in reversed letters. The boxed TGA (nucleotides 731 to 733) is the termination codon assigned in the previous sequence. Relevant restriction cleavage sites and the site of IS*1* insertion in pPG1-L are indicated. Horizontal arrows placed above the sequence represent the fluorescent oligonucleotide primers used in both PCR amplification and sequencing. Restriction sites shown in parentheses were created in the primers. A domain in the gene product with local homology to other phospholipid enzymes is underlined, and the hydrophilic subdomain in it is doubly underlined.

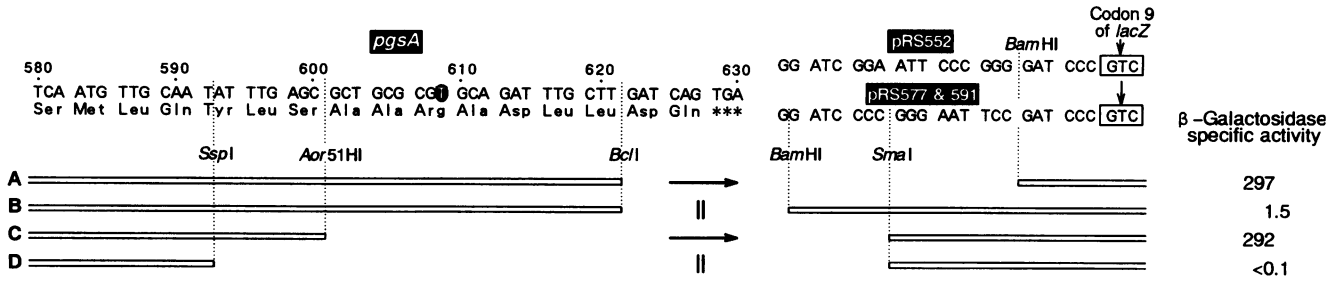

FIG. 2. Structures of the junction regions and the β-galactosidase activities of *pgsA'-'lacZ* protein fusion plasmids. Only the sequences of relevant regions are shown. β-Galactosidase was assayed for strain XL1-Blue harboring these fusion plasmids, and the specific activity is shown to the right of each construct. The central arrows and vertical double lines indicate the in-frame and out-of-frame ligations of the reading frame for PGP synthase and that of *lacZ*, respectively. Construct A is in frame, as is construct C, only if the insertion of a T after 608-G is correct, whereas the construct B would be in frame if the previous sequence lacking 609-T were correct. The reading frames relative to *lacZ* were the same in constructs B and D.

259-A, changing amino acid residue 60 from the wild-type Thr to Pro. This is located in a hydrophilic subdomain, being possibly exposed to the cytoplasm (residues 50 to 65, doubly underlined in Fig. 1). It is contained in a conserved domain (residues 46 to 86, underlined in Fig. 1), bearing local homology to some other phospholipid enzymes; the phosphatidylserine synthase (residues 127 to 167) (18, 23), phosphatidylinositol synthase (residues 56 to 96) (22), diacylglycerol cholinephosphotransferase (residues 111 to 150) (15), and ethanolaminephosphotransferase (residues 96 to 138) (16) of the yeast *Saccharomyces cerevisiae* and the phosphatidylserine synthase of *Bacillus subtilis* (residues 42 to 82) (27). These regions are likely to correspond to the reaction mechanisms, rather than mere affinities to substrates, of the enzymes, since the substrates for these synthases are different from those for phosphotransferases. Further, no significant sequence homology is found with the *E. coli* phosphatidylserine synthase which adopts a Ping-Pong reaction mechanism (19, 29), in contrast to the ordered sequential Bi-Bi mechanisms assumed for these synthases (3, 7, 8, 13, 29). Therefore, severe impairments of PGP synthase activities both in vivo (acidic-phospholipid synthesis) and in vitro (CDP-diacylglycerol-dependent incorporation of labeled *sn*-glycerol-3-phosphate in the presence of a detergent) by the *pgsA3* mutation (1, 21) are quite reasonable.

The *pgsA36* mutation (21) was identified to be an alteration of the initiation codon AUG to AUA. It is known that translation starting at AUA is temperature sensitive and very inefficient (about 1% of that with AUG at 37°C) (33), forming the wild-type gene product at a reduced rate. The PGP synthase activities in strain SD336 grown at 21, 30, 37, and 42°C were, however, essentially the same (16 ± 5 pmol of [2-^{3}H]glycerol-3-phosphate incorporated per min per mg of protein at 30°C, in contrast to 2,030 ± 200 for a *pgsA*$^+$ strain), suggesting that the translation does not start at the AUA codon. This must be related to the apparent absence of a Shine-Dalgarno sequence in the *pgsA* gene. The actual translation start site in the *pgsA36* mutant remains to be elucidated. Nonetheless, the defective PGP synthase activity in *pgsA36* mutants clearly indicated that translation starts at codon 1 of Fig. 1 in wild-type strains, as assumed by Gopalakrishnan et al. (10).

pgsA10 is one of the many *pgsA* mutations isolated by Raetz and coworkers by means of colony autoradiography (24, 28). In contrast to *pgsA3* and *pgsA36*, mutations of this group significantly lower the in vitro PGP synthase activities but do not appreciably affect acidic-phospholipid synthesis in vivo. The *pgsA10* allele has been shown to be a single-base replacement of nucleotide 356 from C to T, changing amino acid residue 92 from Thr to Ile (Fig. 1). This residue is located in one of the hydrophobic regions of the enzyme, possibly being embedded in the membrane. This possible topology suggests that the mutationally altered structure is protected in the membrane but not in a test tube containing a detergent. The possible membrane topologies of the *pgsA3* and *pgsA10* mutants may explain the different effects on the in vivo phospholipid synthesis: the former mutant is severely defective in both in vivo and in vitro PGP synthase activities, whereas all *pgsA* mutants so far isolated, except for *pgsA3* and *pgsA36*, are of the latter type, being deficient only in in vitro activities (24, 28). Although Jackson et al. attempted to explain the latter characteristics in terms of the catalytic excess of PGP synthase (17), it seems more plausible to assume that the moderately defective structures of integral membrane enzymes are easily protected in the membrane and do not express phenotypes in vivo. Other examples are the *plsB26* (4), *psd-2* (11), and *cds* (9) mutations. The isolation of the *pgsA36* mutant that is moderately defective in both in vivo and in vitro activities (21) supports this notion.

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REFERENCES

1. **Asai, Y., Y. Katayose, C. Hikita, A. Ohta, and I. Shibuya.** 1989. Suppression of the lethal effect of acidic-phospholipid deficiency by defective formation of the major outer membrane lipoprotein in *Escherichia coli*. J. Bacteriol. **171**:6867–6869.
2. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
3. **Bae-Lee, M. S., and G. M. Carman.** 1984. Phosphatidylserine synthesis in *Saccharomyces cerevisiae*. Purification and characterization of membrane-associated phosphatidylserine synthase. J. Biol. Chem. **259**:10857–10862.
4. **Bell, R. M.** 1975. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis. Properties of wild type and K_m defective *sn*-glycerol-3-phosphate acyltransferase activities. J. Biol. Chem. **250**:7147–7152.
5. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5**:376–379.

6. **Dowhan, W. (University of Texas).** 1993. Personal communication.
7. **Dutt, A., and W. Dowhan.** 1985. Purification and characterization of a membrane-associated phosphatidylserine synthase from *Bacillus licheniformis*. Biochemistry **24:**1073–1079.
8. **Fischl, A. S., M. J. Homann, M. A. Poole, and G. M. Carman.** 1986. Phosphatidylinositol synthase from *Saccharomyces cerevisiae*. Reconstitution, characterization, and regulation of activity. J. Biol. Chem. **261:**3178–3183.
9. **Ganong, B. R., J. M. Leonard, and C. R. H. Raetz.** 1980. Phosphatidic acid accumulation in the membranes of *Escherichia coli* mutants defective in CDP-diglyceride synthetase. J. Biol. Chem. **255:**1623–1629.
10. **Gopalakrishnan, A. S., Y.-C. Chen, M. Temkin, and W. Dowhan.** 1986. Structure and expression of the gene locus encoding the phosphatidylglycerophosphate synthase of *Escherichia coli*. J. Biol. Chem. **261:**1329–1338.
11. **Hawrot, E., and E. P. Kennedy.** 1978. Phospholipid composition and membrane function in phosphatidylserine decarboxylase mutants of *Escherichia coli*. J. Biol. Chem. **253:**8213–8220.
12. **Heacock, P. N., and W. Dowhan.** 1987. Construction of a lethal mutation in the synthesis of the major acidic phospholipids of *Escherichia coli*. J. Biol. Chem. **262:**13044–13049.
13. **Hirabayashi, T., T. J. Larson, and W. Dowhan.** 1976. Membrane-associated phosphatidylglycerophosphate synthetase from *Escherichia coli*: purification by substrate affinity chromatography on cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol Sepharose. Biochemistry **15:**5205–5211.
14. **Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yasuda.** 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. USA **74:**1417–1420.
15. **Hjelmstad, R. H., and R. M. Bell.** 1990. The *sn*-diacylglycerol cholinephosphotransferase of *Saccharomyces cerevisiae*. Nucleotide sequence, transcriptional mapping, and gene product analysis of the *CPT1* gene. J. Biol. Chem. **265:**1755–1764.
16. **Hjelmstad, R. H., and R. M. Bell.** 1991. *sn*-1,2-Diacylglycerol choline- and ethanolaminephosphotransferases in *Saccharomyces cerevisiae*. Nucleotide sequence of the *EPT1* gene and comparison of the *CPT1* and *EPT1* gene products. J. Biol. Chem. **266:**5094–5103.
17. **Jackson, B. J., J. M. Gennity, and E. P. Kennedy.** 1986. Regulation of the balanced synthesis of membrane phospholipids. Experimental test of models for regulation in *Escherichia coli*. J. Biol. Chem. **261:**13464–13468.
18. **Kiyono, K., K. Miura, Y. Kushima, T. Hikiji, M. Fukushima, I. Shibuya, and A. Ohta.** 1987. Primary structure and product characterization of the *Saccharomyces cerevisiae CHO1* gene that encodes phosphatidylserine synthase. J. Biochem. (Tokyo) **102:**1089–1100.
19. **Larson, T. J., and W. Dowhan.** 1976. Ribosomal-associated phosphatidylserine synthetase from *Escherichia coli*: purification by substrate-specific elution from phosphocellulose using cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol. Biochemistry **15:**5212–5218.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Miyazaki, C., M. Kuroda, A. Ohta, and I. Shibuya.** 1985. Genetic manipulation of membrane phospholipid composition in *Escherichia coli*: *pgsA* mutants defective in phosphatidylglycerol synthesis. Proc. Natl. Acad. Sci. USA **82:**7530–7534.
22. **Nikawa, J., T. Kodaki, and S. Yamashita.** 1987. Primary structure and disruption of the phosphatidylinositol synthase gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **262:**4876–4881.
23. **Nikawa, J., Y. Tsukagoshi, T. Kodaki, and S. Yamashita.** 1987. Nucleotide sequence and characterization of the yeast *PSS* gene encoding phosphatidylserine synthase. Eur. J. Biochem. **167:**7–12.
24. **Nishijima, M., C. E. Bulawa, and C. R. H. Raetz.** 1981. Two interacting mutations causing temperature-sensitive phosphatidylglycerol synthesis in *Escherichia coli* membranes. J. Bacteriol. **145:**113–121.
25. **Nishino, T., E. Kitamura, H. Matsuzaki, S. Nishijima, K. Matsumoto, and I. Shibuya.** 1993. Flagellar formation depends on membrane acidic phospholipids in *Escherichia coli*. Biosci. Biotechnol. Biochem. **57:**1805–1808.
26. **Ohta, A., K. Waggoner, A. Radominska-Pyrek, and W. Dowhan.** 1981. Cloning of genes involved in membrane lipid synthesis: effects of amplification of phosphatidylglycerophosphate synthase in *Escherichia coli*. J. Bacteriol. **147:**552–562.
27. **Okada, M., I. Shibuya, and K. Matsumoto.** Unpublished data.
28. **Raetz, C. R. H.** 1975. Isolation of *Escherichia coli* mutants defective in enzymes of membrane lipid synthesis. Proc. Natl. Acad. Sci. USA **72:**2274–2278.
29. **Raetz, C. R. H., G. M. Carman, W. Dowhan, R.-T. Jiang, W. Waszkuc, W. Loffredo, and M.-D. Tsai.** 1987. Phospholipids chiral at phosphorus. Steric course of the reactions catalyzed by phosphatidylserine synthase from *Escherichia coli* and yeast. Biochemistry **26:**4022–4027.
30. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Shibuya, I.** 1992. Metabolic regulations and biological functions of phospholipids in *Escherichia coli*. Prog. Lipid Res. **31:**245–299.
33. **Shinedling, S., M. Gayle, D. Pribnow, and L. Gold.** 1987. Mutations affecting translation of the bacteriophage T4 *rIIB* gene cloned in *Escherichia coli*. Mol. Gen. Genet. **207:**224–232.
34. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
35. **Tesfa-Selase, F., and W. T. Drabble.** 1992. Regulation of the *gua* operon of *Escherichia coli* by the DnaA protein. Mol. Gen. Genet. **231:**256–264.