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# Two Interacting Mutations Causing Temperature-Sensitive Phosphatidylglycerol Synthesis in *Escherichia coli* Membranes

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A conditionally lethal mutant of *Escherichia coli* lacking phosphatidylglycerol in vivo at 42°C has been previously isolated by two-stage mutagenesis (M. Nishijima and C. R. H. Raetz, J. Biol. Chem. **254**:7837-7844, 1979). In the first step (designated *pgsA444*) the phosphatidylglycerophosphate synthetase is partially inactivated, but the resulting strain continues to make about two-thirds of the normal level of phosphatidylglycerol and is not temperature sensitive. The second lesion, termed *pgsB1*, causes temperature-sensitive growth and phosphatidylglycerol synthesis in strains harboring *pgsA444*. The *pgsA* locus appears to be the structural gene for the synthetase and maps near min 42. In the present study we mapped the *pgsB1* mutation and characterized its interaction with *pgsA444* by genetic and biochemical methods. Unexpectedly, *pgsB1* was not a second lesion in the *pgsA* structural gene, but rather mapped at a distinct site near minute 4. P1 *vir*-mediated contransduction suggested the gene order *pantonA-dapD-pgsB-dnaE* (clockwise). Independent evidence for the genetic mapping was provided by the identification of two hybrid ColE1 plasmids (pLC26-43 and pLC34-20, L. Clarke and J. Carbon, Cell **9**:91-99, 1976) which both carry $pgsB^+$ and $dnaE^+$. Introduction of either the $pgsA^+$ or the $pgsB^+$ gene (via episomes, hybrid plasmids, or P1 *vir* transduction) suppressed the temperature sensitivity of the double mutant (*pgsA444 pgsB1*) and restored normal levels of phosphatidylglycerol at 42°C. In addition, strains with the $pgsA^+$ *pgsB1* genotype produced a novel lipid (X) at all temperatures, whereas the double mutant (*pgsA444 pgsB1*) contained two unusual lipids (X and Y) after 3 h at 42°C. Both X and Y are precursors of lipopolysaccharide, and introduction of $pgsB^+$ into the double mutant caused the disappearance of X and Y. Although the biochemical basis of the *pgsB1* lesion is unknown, its existence suggests a previously unrecognized link between lipopolysaccharide and phosphatidylglycerol syntheses in *E. coli*.

Isolation of *Escherichia coli* mutants defective in phospholipid biosynthesis affords a powerful approach to elucidating the roles of individual phospholipid species in biological membranes (5, 28, 34). The identification of *E. coli* genes responsible for membrane lipid synthesis also provides a basis for studies of regulatory mechanisms and gene expression. In the past 5 years, several *E. coli* mutants defective in the formation of specific phospholipids, including phosphatidylserine (21-23, 26, 27), phosphatidylethanolamine (9, 10), phosphatidylglycerol (20), and cardiolipin (25), have been isolated. Such strains permit certain modifications of polar head group composition, although the effects of these changes on membrane functions have not been evaluated very thoroughly. Other manipulations of membrane phospholipids are possible in acyltransferase (1), diglyceride kinase (30, 31), and CDP-diglyceride synthetase (7) mutants and are reviewed elsewhere (28). In addition, recently developed techniques of molecular cloning have been applied to the phospholipid genes, permitting specific overproductions of certain lipid biosynthetic enzymes (11, 13, 28, 35) which are usually present in very small amounts in *E. coli*.

We have previously reported the isolation and biochemical characterization of a temperature-sensitive mutant of *E. coli* lacking phosphatidylglycerol at 42°C (20). This strain (11-2) has been isolated by introducing a second mutation (*pgsB1*) into a parent already partially defective in the *pgsA* locus, which appears to be the structural gene for phosphatidylglycerophosphate synthetase. In the present study, we demonstrate that this second mutation (*psgB1*) is closely linked to the *dapD* locus, near min 4 on

the *E. coli* chromosome, far from the *pgsA* locus, near min 42. This is shown by Hfr mating and P1 *vir*-mediated cotransduction experiments and, further, by the identification of two hybrid ColE1 plasmids (pLC26-43 and pLC34-20) from the Clarke and Carbon collection (3) which harbor $pgsB^+$ as well as $dnaE^+$ (near min 4). We also document the unusual interaction of the *pgsA444* and *pgsB1* mutations by genetic and biochemical methods and present evidence that the *pgsB* gene plays an additional role in lipopolysaccharide formation.

## MATERIALS AND METHODS

**Materials.** $^{32}P_i$, *sn*-[*U*-$^{14}$C]glycerol-3-phosphate, and Triton X-100 were products of New England Nuclear Corp., Boston, Mass. Yeast extract, tryptone, and agars were obtained from Difco Laboratories, Detroit, Mich. CDP-diglyceride was prepared as described previously (26).

**Bacterial strains, bacteriophage stocks, and growth media.** Bacterial strains used in this study are shown in Table 1. The isolations of strains R477-4441 (*pgsA444*) and 11-2 (*pgsA444 pgsB1*) have been described elsewhere (20). Most of the other strains either were from the Coli Genetic Stock Center (Yale University, New Haven, Conn.) or were constructed as indicated. Preparation of bacteriophages P1 *vir* and $\phi$80 lysates and P1 *vir* transduction were performed by the methods described by Miller (17).

The cells were generally grown at either 30 or 42°C on LB broth (17), which contains 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter. For experiments in which recombinants of genetic matings or P1 *vir* transductants were selected, the bacterial colonies were grown on 1.5% agar containing either LB broth or minimal A salts and 0.2% glucose (17). The following chemicals were included, if required: L-amino acids at 40 $\mu$g/ml, galactose at 0.2%, thiamine and pantothenate at 5 $\mu$g/ml, DL-diaminopimelic acid at 50 $\mu$g/ml, and streptomycin at 100 $\mu$g/ml.

**Preparation of plasmid DNA and transformation.** DNAs of plasmids pSC101 and pPG1 were prepared according to standard procedures which involve plasmid amplification by chloramphenicol treatment (4). The DNAs were used to transform $CaCl_2$-treated cells of strains 11-2 and MN7 according to the method of Mandel and Higa (16). Transformants were selected on LB plates containing tetracycline (10 $\mu$g/ml).

**Identification of strains in the Clarke and Carbon collection carrying the $pgsB^+$ gene on hybrid ColE1 plasmids.** Identification of strains in the Clarke and Carbon collection carrying the $pgsB^+$ gene on hybrid ColE1 plasmids was done by methods essentially the same as those used for the *pss* gene (29) except that the cells were allowed to mate for 3 to 6 h before selecting simultaneously for the desired temperature-resistant, streptomycin-resistant, and colicin E1 toxin-resistant macrocolonies.

**Enzymatic assays.** Phosphatidylglycerophosphate synthetase was assayed as described previously (26). Protein concentration was determined by the method of Lowry et al. (15). The rapid autoradiographic colony screening assay for phosphatidylglycerophosphate synthetase has been published elsewhere (26).

**Extraction and fractionation of lipids.** Phospholipids and X and Y were extracted under acidic conditions as described earlier (20). Separation of chloroform-soluble substances was accomplished by two-dimensional thin-layer chromatography using glass-backed plates coated with a 250-$\mu$m layer of Silica Gel 60 (E. Merck AG, Darmstadt, Federal Republic of Germany) (20). (Silica Gel 60 was incorrectly designated "Silica Gel G" previously [20].) To quantitate the radioactivity in each spot, the silica gel was scraped off (19) and analyzed by liquid scintillation counting, using Patterson-Green fluid (24).

## RESULTS

**Anomalous phenotypic and genetic behavior of strain 11-2.** Since the temperature-sensitive phenotype and the phosphatidylglycerol defect of 11-2 were suppressed on LB broth by introduction of F'150 (14), an episome known to carry the $pgsA^+$ gene near min 42, we initially suspected that the second-step mutation in 11-2 (designated *pgsB1*) represented an additional lesion within the *pgsA* gene. To demonstrate this, we prepared a P1 *vir* lysate on 11-2 ($eda^+$ *pgsA*) in order to move the putative doubly mutated *pgsA* gene into a fresh recipient organism (R477, *eda* $pgsA^+$). About 3% of the $eda^+$ transductants generated in this manner were enzymatically defective in phosphatidylglycerophosphate synthetase, compatible with previous mapping of *pgsA* (20). However, none of these $eda^+$ *pgsA* transductants were temperature sensitive for growth (data not shown). Thus, the second-step lesion of 11-2 was not a second mutation within the *pgsA* locus, but rather a lesion at a new site, separable from *pgsA* by P1 *vir* transduction.

Since it was furthermore impossible to demonstrate any cotransduction of the *pgsB1* mutation with *his, supD, eda,* or *pps* (data not shown), all of which map around min 40 to 45, gradient-of-transmission experiments (17) were performed by mating 11-2 with HfrH and HfrC (Table 1). Analysis of the resulting recombinants revealed a high frequency of cotransfer (data not shown) of *leu* and *pgsB,* indicating that the *pgsB1* mutation was located between the origins of HfrH and HfrC (min 1 to 10), far from *pgsA* and min 42.

**Mapping of the *pgsB* locus by P1 *vir* transduction.** Further mapping of *pgsB* was carried out by a series of P1 *vir*-mediated transduction experiments. First, cells of 11-2 (*pgsA444 pgsB1 tonA thr-1 leu-6 his-4*) were infected with bacteriophage P1 *vir* grown on T1GP ($pgsA^+$ $pgsB^+$ $tonA^+$ $thr^+$ $leu^+$ $his^+$), and temperature-resistant transductants selected on

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

| Strain | Relevant properties | Source or reference |
|---|---|---|
| HfrH | Hfr (see reference 14); also designated Hfr 3000 | CGSC[a] |
| HfrC | Hfr (see reference 14); also designated KL226 | CGSC |
| KL16 | Hfr (see reference 14) | CGSC |
| R477 | F⁻ *rpsL136 his-4 leu-6 thr-1 tonA eda pgsA⁺ pgsB⁺* | 7, 20 |
| T1GP | F⁻ *ilv met cls lacI* | 25 |
| AT982 | Hfr *dapD4 thi-1 relA1* λ⁻ (point of origin: P045 of HfrKL16) | CGSC |
| YA139 | Hfr *pan-6 thi-1 relA1* λ⁻ (point of origin: P01 of HfrH) | CGSC |
| R477-4441 | F⁻ *rpsL136 his-4 leu-6 thr-1 pgsA444 nalA tonA* | 20 |
| R477-4441P[b] | F⁻ *rpsL136 his-4 pgsA444 nalA pan-6 tonA⁺* | This work |
| 11-2 | F⁻ *rpsL136 his-4 leu-6 thr-1 pgsA444 pgsB1 nalA tonA gal lac* | 20 |
| MN1[c] | *pan⁺ pgsB⁺* transductant of R477-4441P (11-2 donor) | This work |
| MN7[c] | *pan⁺ pgsB1* transductant of R477-4441P (11-2 donor) | This work |
| MN7-D[c,d] | *pgsB⁺ dapD4* transductant of MN7 (AT982 donor) | This work |
| MN7-A[c] | *pgsB⁺* transductant of MN7 (AT982 donor) | This work |
| MN7-B[c] | *pgsA⁺* transductant of MN7 (GL60 donor) | This work |
| GL60 | Hfr *thi-1 relA1 cds-6* | 7 |
| JC1553 | F'150/*argG6 metB1 his-1 leu-6 recA1 rpsL104* | CGSC |
| JA200(pLC26-43) | F⁺/*ΔtrpE thr leu recA*/ColE1[*dnaE⁺ pgsB⁺*] | 3 |
| JA200(pLC34-20) | F⁺/*ΔtrpE thr leu recA*/ColE1[*dnaE⁺ pgsB⁺*] | 3 |
| R477-10*(pSC101) | F⁺/*pgsA10 recA*/pSC101[*Tc*] | W. Dowhan |
| R477-10*(pPG1) | F⁺/*pgsA10 recA*/pPG1[*Tc pgsA⁺ uvrC⁺*] | W. Dowhan |

[a] CGSC, Coli Genetic Stock Center.

[b] R477-4441P (*pgsA444 rpsL136 pan-6 leu⁺*) was obtained by mating YA139 (HfrH *leu⁺ pan-6*) with R477-4441 (F⁻ *pgsA444 leu-6 pan⁺ rpsL136*) for 10 min and identifying pantothenate-requiring strains among the selected *leu⁺ rpsL136* recombinants.

[c] Among the temperature-resistant transductants (mostly *pgsB⁺*), strains requiring diaminopimelate were recovered with high frequency.

[d] MN7-D (*pgsA444 pgsB⁺ dapD4*) was constructed by using a P1 *vir* lysate prepared on AT982 (*pgsB⁺ dapD4*) to infect MN7 (*pgsA444 pgsB1 dapD⁺*).

minimal A agar were examined for the unselected markers. The *tonA* and temperature resistance (*pgsB⁺*) loci were 77% (53/69) contransducible, whereas *thr, leu,* and *his* were not cotransducible with *pgsB* (Table 2, experiment A). In other crosses with different P1 *vir* donors, it was also found that temperature resistance (*pgsB⁺*) was cotransducible with *pan* (28%, 18/64) (Table 2, experiment B) and *dapD* (71%, 12/17) (Table 2, experiment C), which are known to be closely linked to *tonA*.

**Orientation of the *pgsB* gene with respect to the *pan, tonA,* and *dapD* loci.** Three-factor transduction crosses were carried out to determine the orientation of the *pgsB* mutation with respect to *pan, tonA,* and *dapD*. Experiment 1 in Table 3 shows the cross in which a P1 *vir* lysate prepared on the strain YA139 (*pan-6, tonA⁺, pgsB⁺*) was used to transduce 11-2 (*tonA pgsB1*) to *pgsB⁺* (temperature resistance). The inheritance of φ80 sensitivity (75%) among *pgsB⁺* transductants was greater than the inheritance of the (*pan-6*) trait (28%), showing that the *pgsB* gene was nearer to the *tonA* gene than to *pan*. In addition, all of *pgsB⁺ pan-6* transductants were also φ80 sensitive; thus, the transducing fragments which covered the *pgsB* and *pan* markers also included the *tonA* locus. The inferred gene order was therefore *pan-tonA-pgsB* (clockwise direction).

Another three-factor transduction cross was done with strain MN7 (*pgsB1 pgsA444 tonA*) as the recipient and strain AT982 (*dapD4*) as the donor (Table 3, experiment 2). The selected marker again was *pgsB⁺*. In this case, the inheritance of φ80 sensitivity (71%) was similar to that of the *dapD4* trait (78%). This result was consistent with the previously published finding that *dapD* is 90% cotransducible with *tonA* (2). The least frequent recombinant class was *dapD⁺ tonA⁺*, suggesting that the gene order was *tonA-dapD-pgsB*. The overall inferred gene order was therefore *pan-tonA-dapD-pgsB* (clockwise) (Fig. 1).

**Construction of temperature-sensitive strains by introduction of the *pgsB1* gene into single-step mutants harboring *pgsA444*.** To verify that the *pgsB1* mutation interacts with the *pgsA444* mutation to cause the temperature sensitivity for growth and the defective phosphatidylglycerol synthesis at 42°C, we tried to find temperature-sensitive strains among *pan⁺* and *dapD⁺* transductants which were obtained, respectively, by the trans-

TABLE 2. *Mapping of the pgsB gene by P1 vir transduction*[a]

| Expt | P1 donor × recipient | Unselected marker | No. of transductants examined | Cotransduction frequency (%) |
|---|---|---|---|---|
| A | T1GP × 11-2 | *his*$^+$ | 630 | 0 |
| | | *leu*$^+$ | 600 | 0 |
| | | *thr*$^+$ | 310 | 0 |
| | | *tonA*$^+$ | 69 | 77 |
| B | YA139 × 11-2 | *pan-6* | 64 | 28 |
| C | AT928 × 11-2 | *dapD4* | 17 | 71 |

[a] In each transduction shown, a P1 *vir* lysate was prepared on the indicated donor strains by standard methods (17). These lysates were used to transduce strain 11-2 (*pgsA444 pgsB1*) to the temperature-resistant phenotype (either *pgsB*$^+$ or *pgsA*$^+$) on a minimal agar plate (see text). The selected temperature-resistant (*pgsB*$^+$) transductants were tested for unselected markers directly (*his*$^+$, *leu*$^+$, and *thr*$^+$) or after a repurification (*tonA*$^+$, *pan*, and *dapD*). In all cases, temperature-resistant transductants having the *pgsB*$^+$ *pgsA444* genotype predominated and represented 90 to 97% of the total. In each cross, however, 3 to 10% of the temperature-resistant transductants had the *pgsA*$^+$ *pgsB1* genotype, as judged by colony autoradiography (data not shown). Restoration of temperature resistance of 11-2 could be achieved by introduction of either the *pgsB*$^+$ or the *pgsA*$^+$ gene (see text), although the frequency of the *pgsB*$^+$ transductants was consistently 10-fold higher.

duction of R477-4441P (*pgsA444 pan-6*) and MN7-D (*pgsA444 dapD4*) with P1 lysates prepared on 11-2. After the *pan*$^+$ transductants (grown at 30°C) were purified by two cycles of restreaking, they were tested for growth at 42°C. Of 100 *pan*$^+$ transductants examined, 7 colonies were found to be temperature sensitive, a somewhat lower cotransduction frequency than that for the reverse cross shown in Table 2. All of the temperature-sensitive strains generated by this approach also exhibited decreased amounts of phosphatidylglycerol after 3 h of growth at 42° (1 to 2% of total phospholipid) (see below).

These results demonstrate that the temperature-sensitive phenotype, along with the decreased phosphatidylglycerol content, is cotransducible with the *pan* gene provided the recipient also harbors *pgsA444*. One of these temperature-sensitive transductants, MN7 *pgsA444 pgsB1 pan*$^+$, and its isogenic relative, MN1 *pgsA444 pgsB*$^+$ *pan*$^+$, have been studied further with regard to biochemical properties as described below.

When MN7-D (*pgsA444 dapD4*) was transduced to *dapD*$^+$ with a P1 *vir* lysate made on 11-2 (*pgsA444 pgsB1 dapD*$^+$), about 70% of the *dapD*$^+$ colonies were temperature sensitive. This is in good agreement with the reverse transduction shown in Table 2.

**Introduction of plasmids carrying either the *pgsA*$^+$ or the *pgsB*$^+$ gene into *pgsA444 pgsB1* double mutants.** Use of two sets of hybrid plasmids carrying either the *pgsA*$^+$ or the *pgsB*$^+$ gene provided additional evidence for the mapping of the *pgsB* locus and the interaction of the *pgsA444* and *pgsB1* mutations.

Among the 2,000 strains of the Clarke and Carbon collection (3) carrying hybrid ColE1

TABLE 3. *Three-factor analyses of pan, tonA, dapD, and pgsB*[a]

| Expt | P1 donor × recipient | Unselected markers | No. of cotransductants |
|---|---|---|---|
| 1 | YA139 × 11-2 | *pan*$^+$ *tonA* | 16 |
| | | *pan*$^+$ *tonA*$^+$ | 30 |
| | | *pan-6 tonA* | 0 |
| | | *pan-6 tonA*$^+$ | 18 |
| 2 | AT982 × MN7 | *dapD4 tonA*$^+$ | 34 |
| | | *dapD4 tonA* | 4 |
| | | *dapD*$^+$ *tonA* | 10 |
| | | *dapD*$^+$ *tonA*$^+$ | 1 |

[a] P1 *vir* lysates of YA139 (*pgsB*$^+$ *pan-6 tonA*$^+$) and AT982 (*pgsB*$^+$ *tonA*$^+$ *dapD4*) were used to transduce 11-2 (*pgsB1 pan*$^+$ *tonA*) or MN7 (*pgsB1 dapD*$^+$ *tonA*) to the temperature-resistant (TR) state. After purification, the selected *pgsB*$^+$ (temperature-resistant) transductants were scored for the unselected markers. The less common temperature-resistant colonies with the *pgsA*$^+$ *pgsB1* genotype (see Table 2, footnote) were not counted in this cross.

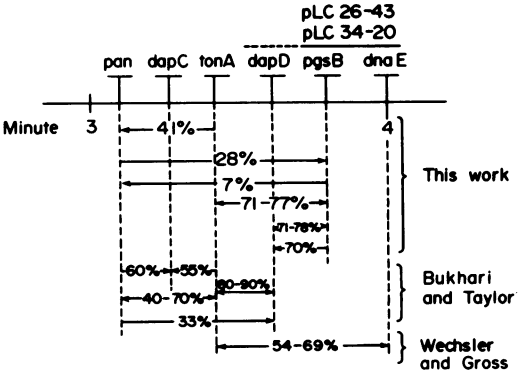


FIG. 1. *Location of the pgsB gene near min 4. This figure summarizes the transduction frequencies of Tables 2 and 3, as well as related data described by other workers (Bukhari and Taylor [2] and Wechsler and Gross [36]. The position of pgsB relative to dnaE is not firmly established.*

plasmids, we have found two hybrid plasmids that restore normal growth to either 11-2 or MN7 at 44°C on LB broth agar. Fortuitously, these plasmids (pLC26-43 and pLC34-20) have been shown by others to carry *dnaE*$^+$ (3). Because the *dnaE* locus is known to be close to the *tonA* gene (54 to 53% cotransduction [36]), the finding that pLC26-43 and pLC34-20 carry *pgsB*$^+$ in addition to *dnaE*$^+$ provided independent evidence for the genetic mapping of the *pgsB* locus discussed above. The cotransduction frequency of *pgsB* with *tonA* was higher than that of *dnaE* with *tonA*, suggesting that the gene order might be *tonA-dapD-pgsB-dnaE*.

The Clarke and Carbon collection does not contain hybrid plasmids bearing *pgsA*$^+$, but the *pgsA*$^+$ gene has recently been cloned from a restriction digest of F'150 on plasmid pSC101 (A. Ohta and W. Dowhan, Int. Congr. Biochem. Abstr. 11:376, 1979). To demonstrate that the *pgsA*$^+$ gene was also able to suppress the temperature sensitivity to the double mutant, we introduced the hybrid plasmid carrying *pgsA*$^+$ (pPG1, Table 1) into either strain 11-2 or strain MN7. The tetracycline-resistant transformants carrying the pPG1 plasmid [11-2(pPG1) and MN7(pPG1)] were able to grow at 44°C on LB broth agar, whereas those carrying the original vector, pSC101 [designated 11-2(pSC101) and MN7(pSC101)] remained temperature sensitive (data not shown). This indicated that the *pgsA*$^+$ gene also suppresses the phenotype of the double mutant.

**Biochemical characterization of constructed strains carrying *pgsA444*, *pgsB1*, or both mutations.** As noted above, we reconstructed isogenic strains with respect to the *pgsA* and *pgsB* mutations by P1 *vir* transduction (Table 1). These included MN1 (*pgsA444 pgsB*$^+$), MN7 (*pgsA444 pgsB1*), and MN7-B (*pgsA*$^+$ *pgsB1*), which have the same background except for the regions around the *pgsA* and *pgsB* loci. We also constructed two derivatives of MN7 (see above), namely, MN7(pPG1) and MN7(pLC26-43), which harbor pSC101[*pgsA*$^+$] and ColE1[*pgsB*$^+$] hybrids, respectively. The phosphatidylglycerophosphate synthetase-specific activities (Table 4) and lipid compositions (Table 5) of these strains were compared to examine the function of the *pgsB* gene and the nature of the interaction between the *pgsA* and *pgsB* mutations.

When extracts were made from cells of MN1 and MN7 grown on LB broth at 30°C, both extracts showed almost the identical specific enzyme activities, which were about 30% of wild-type (i.e., R477) activity and comparable to those of R477-4441 and 11-2 (20). In contrast, when extracts were prepared from cells of MN7 shifted to 42°C, the specific activity of the synthetase gradually decayed (data not shown). These results (using the isogenic strains) indicated that the double mutation, *pgsA444 pgsB1*, makes temperature sensitive the net synthesis of phosphatidylglycerophosphate synthetase, as has also been observed in the original isolate, 11-2 (20). MN7(pLC26-43) possessed the same level of the enzyme activity as single-step *pgsA444* strains, whereas MN7-B regained the wild-type (*pgsA*$^+$) enzyme activity. MN7(pPG1) showed about a fourfold higher specific activity than that of the wild type, since the hybrid plasmids were maintained in multiple copies. These results implied that the *pgsB1* mutation does not affect the synthesis of phosphatidylglycerophosphate synthetase coded by the *pgsA*$^+$ gene, but only senses the *pgsA444* allele. The thermal stability of the synthetase in the strains shown in Table 4 was consistent with the previous observation that the enzyme of *pgsA*$^+$ wild-type strains is relatively stable to preincubation at 70°C, whereas that of *pgsA444* strains is completely inactivated by the same treatment (20).

As shown in Table 5, MN7 (*pgsA444 pgsB1*) was strikingly deficient in phosphatidylglycerol content at 42°C, indicating that the *pgsB1* mutation, in participation with *pgsA444* mutation, was responsible for the deficiency of phosphatidylglycerol. When either the *pgsA*$^+$ gene on P1 *vir* phage or the pPG1 plasmid or the *pgsB*$^+$ gene on P1 *vir* phage or the pLC26-43 plasmid was introduced into MN7, all such derivatives of MN7 regained relatively high levels of phospha-

TABLE 4. *Phosphatidylglycerophosphate synthetase-specific activity in extracts prepared from a pgsA444 pgsB1 double mutant and its various derivatives*

| Strain | Relevant genotype | Sp act at 30°C (U/mg)[a] with: | |
|---|---|---|---|
| | | No incubation | Preincubation at 70°C for 20 min |
| R477 | *pgsA*$^+$ *pgsB*$^+$ | 0.98 | 0.92 |
| MN7 | *pgsA444 pgsB1* | 0.32 | 0.03 |
| MN1 | *pgsA444 pgsB*$^+$ | 0.34 | 0.02 |
| MN7-B | *pgsA*$^+$ *pgsB1* | 1.07 | 0.78 |
| MN7(pPG1) | *pgsA444 pgsB1*(pSC101[*pgsA*$^+$]) | 4.04 | 2.78 |
| MN7(pLC26-43) | *pgsA444 pgsB1*(ColE1[*pgsB*$^+$]) | 0.37 | 0.02 |

[a] All cultures were grown to late log phase on LB broth at 30°C, and extracts were assayed at 30°C (26). Preincubation of extracts was carried out at 70°C for 20 min; 1 U = 1 nmol of phospholipid synthesized per min.

TABLE 5. *Phospholipid composition and contents of X and Y in a pgsA444 pgsB1 double mutant and its derivatives*[a]

| Expt. | Strain (plasmid) | Chromosomal genotype | Lipid composition (%)[b] | | | | X and Y content (%)[c] | |
|---|---|---|---|---|---|---|---|---|
| | | | PE | PG | CL | Other | X | Y |
| 1 | R477 | *pgsA*$^+$ *pgsB*$^+$ | 77.7 | 20.2 | 1.2 | 1.0 | — | — |
| | MN1 | *pgsA444 pgsB*$^+$ | 84.7 | 13.0 | 0.8 | 1.5 | — | — |
| | MN7 | *pgsA444 pgsB1* | 85.0 | 2.3 | 1.2 | 1.5 | 6.6 | — |
| | MN7-A | *pgsA444 pgsB*$^+$ | 82.9 | 15.2 | 0.6 | 1.3 | — | — |
| | MN7-B | *pgsA*$^+$ *pgsB1* | 75.2 | 22.2 | 1.8 | 0.8 | 3.4 | <0.1 |
| | MN7(pPG1) | See Table 1 | 72.5 | 25.8 | 0.7 | 1.0 | 3.8 | — |
| | MN7(pLC26-43) | See Table 1 | 84.4 | 14.0 | 0.4 | 1.2 | — | — |
| 2 | R477 | *pgsA*$^+$ *pgsB*$^+$ | 73.8 | 22.1 | 2.6 | 1.5 | — | — |
| | MN1 | *pgsA444 pgsB*$^+$ | 83.3 | 12.8 | 1.4 | 2.5 | — | — |
| | MN7 | *pgsA444 pgsB1* | 93.7 | 1.4 | 0.8 | 4.1 | 6.5 | 8.4 |
| | MN7-A | *pgsA444 pgsB*$^+$ | 83.3 | 13.3 | 1.3 | 2.1 | — | — |
| | MN7-B | *pgsA*$^+$ *pgsB1* | 72.4 | 24.2 | 2.7 | 0.7 | 5.1 | <0.1 |
| | MN7(pPG1) | See Table 1 | 58.0 | 38.2 | 2.9 | 0.9 | 5.6 | — |
| | MN7(pLC26-43) | See Table 1 | 83.7 | 13.8 | 0.9 | 1.6 | — | — |

[a] Cells were grown on LB broth and labeled uniformly for many generations with $^{32}P_i$ (2 μCi/ml). Lipids were then extracted and analyzed by two-dimensional thin-layer chromatography (see text). In experiment 1 cells were harvested in late log phase (absorbance at 600 nm of about 0.6) at 30°C. In experiment 2 the cells were shifted to 42°C when the absorbance at 600 nm had reached about 0.2, and then the radioactive cultures were incubated for 3 h more before the cells were harvested.

[b] Percentage of $^{32}P$ radioactivity in chloroform extracts, excluding X and Y. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

[c] Percentage of $^{32}P$ radioactivity in chloroform extracts, including phospholipids; —, not detectable.

tidylglycerol along with a temperature-resistant phenotype.

We have previously found two novel lipids, designated X and Y, in chloroform extracts of strain 11-2 grown at 42°C. Structural studies of those compounds have suggested that they are precursors of lipid A biosynthesis (M. Nishijima, Fed. Proc. **39**:1982, 1980). The contents of X and Y in MN7 and its derivatives grown at 30 and 42°C were analyzed (Table 5). The results clearly indicated that the accumulation of X was primarily associated with the *pgsB1* mutation. However, Y accumulated only when cells which carried both *pgsA444* and *pgsB1* mutations were cultured at 42°C.

Figure 2B shows the time course of continuous $^{32}P_i$ incorporation into X and Y in strain MN7 after a shift to 42°C; for comparison, continuous $^{32}P_i$ incorporation into phosphatidylglycerol and phosphatidylethanolamine in strains MN1 and MN7 is also shown in Fig. 2A. After shift to 42°C, accumulation of $^{32}P_i$ into X immediately increased for about 1 h, then the accumulation stopped, and there was a gradual decrease in its amount after about 1.5 h at 42°C. In contrast, $^{32}P$-labeled Y continued to increase, and the radioactivity in Y reached the same level as that in X after 3 h at 42°C. In contrast to the increased synthesis of X and Y, the net incorporation of $^{32}P_i$ into phosphatidylglycerol in MN7 was specifically inhibited at nonpermissive temperature (Fig. 2A), as in the case of its parental strain, 11-2 (20).

The apparent synthetic rates of phospholipids (Table 6) and of X (Fig. 2B, represented by bars) in MN7 were also measured at various times after a shift to 42°C. Interestingly, the synthetic rate of X was about fivefold higher at 42 than at 30°C, even immediately after the shift up. Thereafter, the ability to synthesize X decreased. Under these pulse-labeling conditions, synthesis of Y was negligible. The relative prominence of phosphatidic acid in these short-term pulse labelings was observed with all strains harboring the *pgsA444* mutation (Table 6).

## DISCUSSION

We have previously identified several strains defective in phosphatidylglycerophosphate synthetase (*pgsA*) by using rapid colony autoradiography (20). Our genetic and biochemical studies and, further, the recent cloning study of the *pgsA* gene by Ohta and Dowhan (Int. Congr. Biochem. Abstr. 11:376, 1979) suggest that *pgsA* may be a structural gene for the enzyme. Although many of the *pgsA* mutants contain less than 5% of the wild-type enzymatic activity, none are temperature sensitive for growth, and the phosphatidylglycerol content is reduced no more than twofold in comparison with the wild type (20). The temperature-sensitive strain 11-2

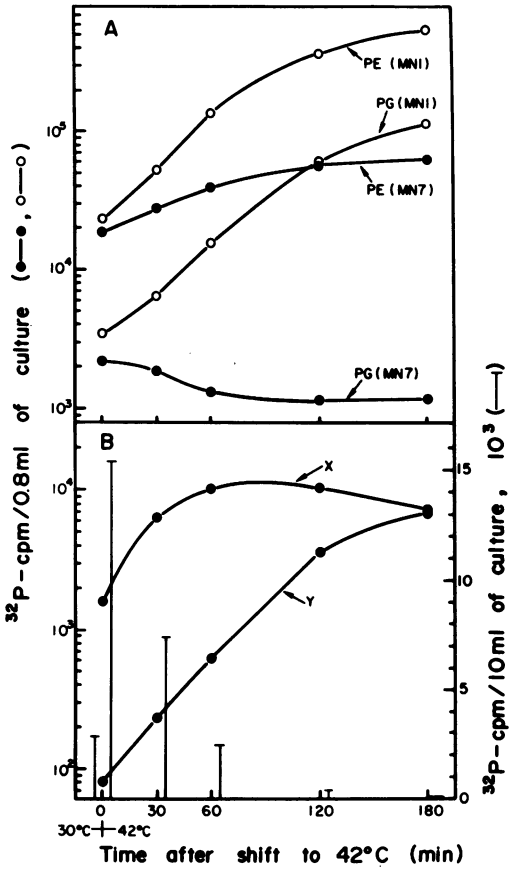


FIG. 2. *Time course of incorporation of $^{32}P_i$ into phosphatidylethanolamine (PE), phosphatidylglycerol (PG), X, and Y in MN1 (pgsA444) and MN7 (pgsA444 pgsB1) and the synthetic rates of X in MN7 after shifting the cells to 42°C. (A) Cells were uniformly labeled with $^{32}P_i$ at 30°C as described in Table 5, footnote a. At an optical density at 600 nm of about 0.1, cultures of each strain were shifted to 42°C (time zero). At appropriate intervals (see figure), a 0.8-ml portion of each culture was withdrawn, and the lipids were extracted directly by the methods of Ames (see reference 20) and analyzed as described in the text. (B) The data for the synthetic rates of X were obtained in the experiment shown in Table 6 and presented here by bars, separately.*

(*pgsA444 pgsB1*), defective in phosphatidylglycerol synthesis at 42°C, has been obtained by a second step mutagenesis, starting with one of the *pgsA* mutants (harboring *pgsA444*) already partially defective in the synthetase (20). Our initial expectation had been to induce a second mutation within *pgsA* gene, but unexpectedly the second mutation (*pgsB1*) in 11-2 was induced at a new locus, far removed from the *pgsA* gene.

Several lines of evidence in the present study strongly suggest that the *pgsB* gene is located near the *tonA* and *dapD* loci (Tables 2 and 3). Recently, Ganong et al. (7) have also identified the *cds* gene near *dapD*, which is responsible for phosphatidate cytidylyltransferase. Cotransduction frequencies of *pgsB* and *cds* with *dapD* are 70 to 80% and 80 to 90%, respectively (data not shown); however, although the pLC26-43 and pLC34-20 plasmids carry the *pgsB* gene, the *cds* gene is not present on these plasmids (unpublished data). These results do not eliminate the possibility that both *pgsB* and *cds* belong to the same cistron. Another gene (*envN*), which relates to cell envelope structure, has been identified near *tonA* by Egan and Russell, but the *envN* gene has not been cotransduced with *dapD* (6).

The *pgsB1* mutation by itself has been shown to induce the accumulation of some X (Table 5). We have recently determined its structure as a diphosphorylated, glucosamine-containing disaccharide, derivatized (on the average) with two amide-linked and two ester-linked 3-hydroxymyristic acid moieties, but lacking 2-keto-3-deoxyoctulosonic acid (KDO) (Nishijima, Fed. Proc. **39**:1982, 1980). This structural work suggests that X is a precursor of lipid A. Therefore, the *pgsB* gene may be involved somehow in lipid A biosynthesis. Compounds similar to X (but less acylated) have been found in mutants of *Salmonella typhimurium* defective in KDO-8-phosphate synthetase as described by Rick et al. (32) and Lehmann (12). The former authors have localized their mutation to approximately 57 min on the *Salmonella* genetic map. On the other hand, the *pgsB* gene is localized near 4 min on *E. coli* chromosome. These facts suggest, on the assumption of general similarities of gene locations between *E. coli* and *S. typhimurium*, that the *pgsB* gene is probably not related to KDO-8-phosphate synthetase. Further, there is no difference in CMP-KDO synthetase specific activity (8) between MN1 and MN7 grown at 42°C (unpublished data). Membrane-bound KDO transferase (18), which catalyzes transfer of the KDO moiety from CMP-KDO to an incomplete, KDO-deficient precursor of lipid A, is an interesting candidate for the function of the *pgsB* gene but this possibility has not yet been examined.

Although the *pgsB* gene is located at a distant position from the *pgsA* gene on the *E. coli* chromosome, the *pgsA444* and *pgsB1* lesions interact with each other. Both mutations are required for the expression of the following phenotypes at nonpermissive temperature: cessation of growth, decreased synthesis of phosphatidylglycerol, and accumulation of Y. Introduction of either $pgsA^+$ or $pgsB^+$ into the double

TABLE 6. *Ratio of newly synthesized phosphatidylglycerol to phosphatidylethanolamine in strain MN7 (pgsA444 pgsB1) after a shift to 42°C*[a]

| Labeling | | Cell density (optical density at 600 nm) | $^{32}P$ incorporation into lipid (cpm/10 ml of culture) | Lipid composition[b] | | | |
|---|---|---|---|---|---|---|---|
| Time (min) | Temp (°C) | | | % of total | | | PG/PE ratio |
| | | | | PE | PG | PA | |
| 0 | 30 | 0.080 | 29,505 | 74.7 | 10.2 | 15.1 | 0.14 |
| 0 | 42 | 0.080 | 38,477 | 82.6 | 11.0 | 6.3 | 0.13 |
| 30 | 42 | 0.175 | 45,210 | 86.2 | 4.7 | 9.1 | 0.054 |
| 60 | 42 | 0.237 | 34,284 | 85.0 | 3.3 | 11.7 | 0.039 |
| 120 | 42 | 0.325 | 8,425 | 87.0 | 1.2 | 11.8 | 0.014 |
| 180 | 42 | 0.382 | 1,058 | 85.1 | 0.4 | 14.5 | 0.005 |

[a] Cells grown in LB broth at 30°C to an optical density at 600 nm of 0.080 were shifted to 42°C (time zero). At each time point shown in the table, a 10-ml portion of the culture was withdrawn into a flask containing about 100 μCi of carrier-free $^{32}P_i$ and incubated at either 30 or 42°C. After 10 min, 1.1 ml of 5% (wt/vol) trichloroacetic acid solution was added to each radioactive culture, and cells were collected by centrifugation at 5,000 × *g* for 15 min. Lipids were analyzed as described in the text. The synthesis of X was also determined in this experiment (see Fig. 2).

[b] PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid.

mutant suppresses these pleiotropic phenotypes simultaneously. In this regard, Rick and Osborn (33) have found no remarkable abnormality of phospholipid composition in their *Salmonella* mutant defective in KDO synthesis described above. This is consistent with our finding that phosphatidylglycerol synthesis is not altered in the *pgsA*$^+$ *pgsB1* strains.

The content of X is about 1.5-fold higher at 42 than at 30°C in *pgsA*$^+$ *pgsB1* strains (Table 5), and furthermore, the response of increased accumulation of X is very immediate after a shift to 42°C in MN7 (*pgsA444 pgsB1*) (Figure 2). These results suggest that the *pgsB1* mutation may have a temperature-sensitive property. In contrast, the *pgsA444* mutation has no apparent temperature-sensitive phenotype. Therefore, the trigger for pleiotropic effects at 42°C in the double mutant may be ascribed to the *pgsB1* lesion. In contrast, Y builds up only when both *pgsA444* and *pgsB1* mutations are present and when phosphatidylglycerol levels are depleted after several hours at 42°C. Y has been shown to be similar to X, differing in having 1 mol extra of esterified palmitic acid in addition to the four hydroxymyristic acids found in X (Nishijima, Fed. Proc. **39**:1982, 1980). Furthermore, chase experimnts suggest that Y is derived from X (data not shown). Therefore, it is possible that Y is synthesized from X by an acylation reaction(s) which may be stimulated by the decreased synthesis or levels of phosphatidylglycerol. Alternatively, phosphatidylglycerol may be required for some later step in the processing of Y to mature lipopolysaccharide.

Finally, some possible explanations for the interaction between the *pgsA444* and *pgsB1* mutations deserve consideration. We have already shown that the accumulation of functional phosphatidylglycerophosphate synthetase is inhibited at the nonpermissive temperature in 11-2 (*pgsA444 pgsB1*) (20). This is also true in MN7 (data not shown). Thus, the partially defective enzyme determined by the *pgsA444* allele may not be processed or integrated into membranes which have a defect ascribed to the *pgsB1* mutation. Alternatively, the gene products of *pgsA*$^+$ and *pgsB*$^+$ may specifically interact in membranes, and those of *pgsA444* and *pgsB1* may not properly function when both mutations are present simultaneously.

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