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Multiple Genes for Membrane-Bound Phosphatases in *Escherichia coli* and Their Action on Phospholipid Precursors

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We have devised a coupled radiochemical assay for detecting phosphatidylglycerolphosphate (PGP) phosphatase activity in *Escherichia coli* colonies immobilized on filter paper. There appeared to be at least two enzymes capable of dephosphorylating PGP, as judged by the characterization of mutations in two genes designated *pgpA* and *pgpB*. The former is located near min 10 and is cotransducible with *proC* and *dnaZ*. The latter is situated near min 28 and is closely linked to *cysB*. The available mutant alleles of *pgpA* reduced the specific activity of PGP phosphatase in crude extracts by about 30%, but they had no effect on phosphatidic acid (or lysophosphatidic acid) phosphatase. Mutants altered in the *pgpB* locus inactivated most of the residual PGP phosphatase activity present in single-step *pgpA* mutants, and the level of phosphatidic acid phosphatase was also reduced 20-fold. The available mutations in *pgpA* and *pgpB* elevated the cellular PGP pool by 10- to 50-fold. The maximal PGP levels never exceeded 5%, and these strains were not conditionally lethal. The simplest interpretation of our findings is that there are at least two membrane-associated phosphatases in *E. coli*, both distinct from alkaline phosphatase. The *pgpA* gene product is specific for PGP, whereas the *pgpB* gene product also acts on phosphatidic acid and lysophosphatidic acid.

The membranes of *Escherichia coli* contain several phosphatases capable of attacking phosphorylated intermediates involved in lipid bilayer assembly (6, 25). Although the biochemistry of these enzymes has received very little attention, it is certain that they are distinct from the well-characterized, periplasmic alkaline phosphatase (6, 25).

The membrane-associated phosphatases of *E. coli* catalyze the following reactions: (i) dephosphorylation of phosphatidylglycerolphosphate (PGP), which is an obligatory intermediate (6, 11, 14, 22) in the synthesis of phosphatidylglycerol (a major membrane lipid); (ii) dephosphorylation of phosphatidic acid, a precursor common to all phospholipids (2, 25); and (iii) dephosphorylation of lysophosphatidic acid (2, 25). The interrelationship of these enzymes is unclear, since they have not been purified to homogeneity (6, 22, 25). Further, the biological significance of phosphatidic acid and lysophosphatidic acid phosphatases is obscure, since *E. coli* (in contrast to higher eucaryotes) does not utilize diglyceride directly for the synthesis of phosphatidylethanolamine (12, 22).

To explore these problems, we have devised a biochemical screening assay for detecting PGP phosphatase activity in *E. coli* colonies immobilized on filter paper (3, 20). We have isolated and characterized a series of mutants defective in this enzyme. Our results indicate that there are at least two PGP phosphatases in *E. coli*, one of which also dephosphorylates phosphatidic acid (and possibly lysophosphatidic acid). These mutants offer a powerful new approach for elucidating the biological functions of the lipid-specific phosphatases present in the *E. coli* envelope.

MATERIALS AND METHODS

Materials. $^{32}P_i$, [γ-^{32}P]ATP, and *sn*-[*U*-^{14}C]glycerol-3-phosphate were products of New England Nuclear, Boston, Mass. Triton X-100 was obtained from Research Products International Corp., Elk Grove Village, Ill. Glycerolkinase from *Candida mycoderma* (Sigma Chemical Co., St. Louis, Mo.) was used to generate *sn*-glycerol-3-^{32}P from glycerol and [γ-^{32}P]ATP (4). The *sn*-glycerol-3-^{32}P was purified by ion-exchange chromatography (4) on Bio-Rad AGl-X8 (200 to 400 mesh) in the formate form. Next, it was converted to phosphatidylglycerol-[^{32}P]phosphate (PG-^{32}P] by incubation in the presence of CDP-diglyceride and highly purified PGP synthase from *E. coli* (11). Before its use in PGP phosphatase assays, the PG-^{32}P was further purified by DEAE-cellulose (Whatman DE52) chromatography (23) in chloroform-methanol-water (2:3:1, vol/vol) by step elution with increasing amounts of ammonium acetate. The peak fractions eluting at a salt concentration of 40 to 50 mM were pooled, partitioned under acidic conditions to remove salts (18), dried under N_2, and stored at

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

Strain	Relevant markers	Source/reference
8	HfrC *glpD3 glpR2 phoA8 pgpA*$^+$ *pgpB*$^+$ *tonA22*	10
E12	*pgpA2* derivative of strain 8	This work
E13	*pgpA3* derivative of strain 8	This work
E20	*pgpA2 pgpB20* derivative of E12	This work
E26	*pgpA2 pgpB26* derivative of E12	This work
E27	*pgpA2 pgpB27* derivative of E12	This work
E28	*pgpA2 pgpB28* derivative of E12	This work
X478	F$^-$ *leuB6 proC32 purE42 trpE38 lysA23 metE70 rpsL109*	CGSC[a]
AX727	F$^-$ *dnaZ2016*	CGSC[a]
W3747	F'13 *lac*$^+$ *purE*$^+$/*metB1 relA1*	CGSC[a]
CSH28	F' *lac*$^+$ *proA,B*$^+$/*trp pyrF his rpsL* Δ(*lac-pro*)	Cold Spring Harbor Laboratory
CSH28.6	F$^-$ derivative of CSH28 by acridine curing	This work
CB64	F$^-$ *trp-75 cysB93*	CGSC
RL88	F$^-$ *his-68 tyrA2* Δ(*attφ80-cysB*)	CGSC
DM800	F$^-$ Δ(*top-cysB*)	CGSC
TI63	*purE*$^+$ *dnaZ2016* transductant of X478 (AX727 donor)	This work
TI70	*proC*$^+$ *phoA8* transductant of X478 (strain 8 donor)	This work
TI71	*proC*$^+$ *phoA8 pgpA2* transductant of X478 (E12 donor)	This work
TI72	*trp*$^+$ *pgpB26 rpsL109* recombinant of mating E26 and TI71; also *phoA8 pgpA2*	This work
TI73	*purE*$^+$ *lac*$^+$*rpsL109* recombinant of mating strain 8 and TI72; also *phoA8 pgpB26 pgpA*$^+$	This work
TI61	*cysB93 trp*::Tn*10* transductant of TI71	This work

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

−80°C. CDP-diglyceride was synthesized as reported earlier (23), except that phosphatidic acid derived from egg lecithin was used. [^{32}P]phosphatidic acid was obtained by treating ^{32}P-labeled phospholipid from *E. coli* with cabbage phospholipase D (13), which was purchased from Sigma Chemical Co. Radioactive phosphatidic acid was also purified by DEAE-cellulose chromatography.

Bacterial strains, bacteriophage stocks, and growth media. Strains used in this study are listed in Table 1. Methods used for strain construction or manipulations of genetic markers are also indicated in Table 1 or in the text. Cultures were usually grown on LB broth (17) at the temperatures indicated. Routine methods for P1 *vir* transduction or mating were essentially the same as those described by Miller (17). Selection of transductants or recombinants was performed on minimal A agar plates (17).

In vitro assay method I, PGP phosphatase. The in vitro assay used to measure PGP phosphatase was essentially the same as that described by Chang and Kennedy (6). Briefly, the reaction mixture contained 100 μM PG-^{32}P (10^3 to 10^4 cpm/nmol), 50 mM Tris-hydrochloride (pH 7.4), 0.12% Triton X-100, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, and about 60 μg of crude extract protein. The final volume was 100 μl, and the reaction was allowed to proceed for 20 min in a 1.5-ml Eppendorf tube at 42°C. In assay method I the dephosphorylation of PG-^{32}P was measured by following the release of trichloroacetic acid-soluble $^{32}P_i$. This was done by adding 0.5 ml of 10% trichloroacetic acid to each reaction mixture, along with 0.1 ml of 5 mM Na_2HPO_4 and 0.1 ml of 2% bovine serum albumin as carriers. The tubes were chilled and centrifuged, and 0.7 ml of each supernatant was removed. These were extracted with $CHCl_3$ to remove residual phospholipid (6). Finally, 0.2 ml of each aqueous phase was placed in 10 ml of Patterson-Green fluid (19) with 0.5 ml of methanol and counted in a scintillation spectrometer.

In vitro assay method II, PGP phosphatase. Method II was used for time course experiments. Samples were incubated as described in method I. At each time indicated, a 10-μl portion was withdrawn from each mixture and spotted on trichloroacetic acid-impregnated filter paper (8). This was washed on a Büchner funnel with several portions of 2% trichloroacetic acid, and the radioactivity remaining in each spot was analyzed by scintillation spectrophotometry. Although method II was technically simpler than method I, it was not as sensitive for the detection of slow reaction rates.

In vitro assay method III, phosphatidic acid phosphatase. The dephosphorylation of phosphatidic acid was measured after 60 min at 42°C under the same conditions as PGP phosphatase (method I), except that 50 μM [^{32}P]phosphatidic acid (10^2 to 10^3 cpm/nmol) was substituted for the 100 μM PG-^{32}P. The reaction was monitored by following $^{32}P_i$ formation as in method I.

In vitro assay method IV, phosphatidic acid phosphatase. Conditions for method IV were the same as described under method III, except that the dephosphorylation was assayed by determining the remaining [^{32}P]phosphatidic acid, using the trichloroacetic acid-impregnated filter procedure (see method II).

Colony autoradiography for detection of mutants defective in PGP phosphatase. In all cases a coupled assay was used to provide a rapid (though indirect) measure of PGP phosphatase. Colonies derived from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated stock

were grown on LB agar at 30°C and transferred to filter paper, and then the cells were rendered permeable by freezing and desiccation-induced lysis (3). However, KF and 2,4-dinitrophenol (3, 20) were omitted throughout all steps, since these inhibit PGP phosphatase (6). Next, the filters were incubated with CDP-diglyceride and *sn*-glycerol-3-^{32}P (Table 2). Since PGP synthetase and PGP phosphatase are present simultaneously in the cell extracts, this results in the concurrent synthesis and dephosphorylation of PG-^{32}P. In both of the assay conditions used (see Table 2), the concentrations of the reagents were poised so that the level of PG-^{32}P around each wild-type colony lysate was low at the end of the assay. Consequently, when the filters were fixed with 10% trichloroacetic acid, washed with 2% trichloroacetic acid on a Büchner funnel, and analyzed by autoradiography, wild-type colonies appeared as faint spots, whereas mutants defective in phosphatase activity were dark spots. Furthermore, the appearance of the PGP phosphatase mutants could be simulated in wild-type colonies under both of the conditions described in Table 2 by inclusion of 5 mM $HgCl_2$ during the incubation, which completely inhibits PGP phosphatase but not PGP synthetase (4, 6).

It should be noted that the incubation conditions designated "A" in Table 2 are similar to those used routinely to assay PGP synthetase (4), whereas the "B" conditions are optimal for PGP phosphatase in crude extracts of *E. coli* (6).

Miscellaneous procedures. Lipids were labeled with $^{32}P_i$ and extracted under acid conditions as described earlier (18). Two-dimensional thin-layer chromatography was performed on 250-μm Silica Gel 60 glass-backed plates (E. Merck AG, Darmstadt, West Germany), using $CHCl_3$-methanol-water-acetic acid (25:15:4:2, vol/vol) in the first dimension and $CHCl_3$-methanol-acetic acid (13:5:2, vol/vol) in the second. Sample radioactivity was determined by liquid scintillation counting in Patterson-Green fluid (19), and protein was estimated by the method of Lowry et al. (16).

RESULTS

Autoradiographic detection of mutants defective in PG-^{32}P dephosphorylation and identification of the *pgpA* gene. Cells of *E. coli* strain 8 (*pgpA*$^+$ *pgpB*$^+$ *phoA*) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (17), and colonies were grown at 30°C on LB agar plates. After 20 h, filter paper copies were prepared (3) and assayed under the conditions designated "A" in Table 2. Thirty-two candidates (dark spots) were identified among 25,000 colonies. These were retrieved from the master plates and reassayed by colony autoradiography. Fifteen strains from this group, such as E12 or E13 (Table 1), consistently showed a significant defect in PG-^{32}P dephosphorylation.

Wild-type and several *pgpA* mutants were grown to late log phase on LB broth, and extracts were prepared by sonic oscillation. These were tested for defects in PG-^{32}P phosphatase, using a coupled assay (Fig. 1, legend).

TABLE 2. Conditions for detection of mutations in genes *pgpA* and *pgpB* by colony autoradiography

Component	Concn	
	"A" condition for *pgpA*[a]	"B" condition for *pgpB*[a]
sn-Glycerol-3-^{32}P[b]	5 μM	50 μM
CDP-diglyceride	100 μM	100 μM
Tris-hydrochloride	250 mM, pH 8	50 mM, pH 7.4
Triton X-100	0.2%	0.12%
$MgCl_2$	30 mM	2 mM
EDTA		1 mM
2-Mercaptoethanol	5 mM	5 mM
Sodium azide	20 mM	20 mM
$Na_2HA_5O_4$	1 mM	1 mM

[a] The "A" assay was carried out for 75 min at 42°C. The "B" assay was performed for 60 min at 40°C.

[b] Specific activity: 10^5 cpm/nmol in "A"; 10^4 cpm/nmol in "B."

Figure 1A shows the results with parental strain 8. There was transient PG-^{32}P accumulation when extracts of strain 8 were incubated with 0.1 mM CDP-diglyceride and 5 μM *sn*-glycerol-3-^{32}P, but the small amount of PG-^{32}P observed after 20 min disappeared again by 60 min due to PGP phosphatase activity. Inclusion of Hg^{2+} (a nonspecific phosphatase inhibitor) resulted in the complete conversion of all *sn*-glycerol-3-^{32}P to PG-^{32}P (Fig.1A), simulating a PGP phosphatase mutant. In all cases the formation of acid-precipitable PG-^{32}P required the presence of CDP-diglyceride during the incubation.

Figure 1B shows the behavior of strain E12 (*pgpA2*) under comparable conditions. In this case there is complete conversion of *sn*-glycerol-3-^{32}P to PG-^{32}P whether or not Hg^{2+} is present. This behavior indicates that PGP phosphatase is absent (or altered) in strain E12 under the conditions used and is the best criterion for a mutation in the *pgpA* gene. It should be noted that the assay conditions in the experiment of Fig. 1 are optimized for PGP synthetase and not for PGP phosphatase.

Mapping of the *pgpA* gene. Since the *pgpA* lesion was easily identified by colony autoradiography, the location of the *pgpA* gene on the *E. coli* chromosome was determined. Gradient transmission experiments (17) with different Hfr strains indicated that *pgpA* was located between min 6 and 13 (data not shown). This was confirmed by P1 *vir* transduction analysis, which demonstrated significant cotransfer of *pgpA* with *proC* and *dnaZ* (Table 3). Therefore, *pgpA* maps between these two sites at min 10. However, the exact gene order with respect to several markers (such as *tsx*, *lon*, and *acrA*) (1) between *purC* and *dnaZ* has not yet been established. There was no cotransduction of *pgpA* and *proA*

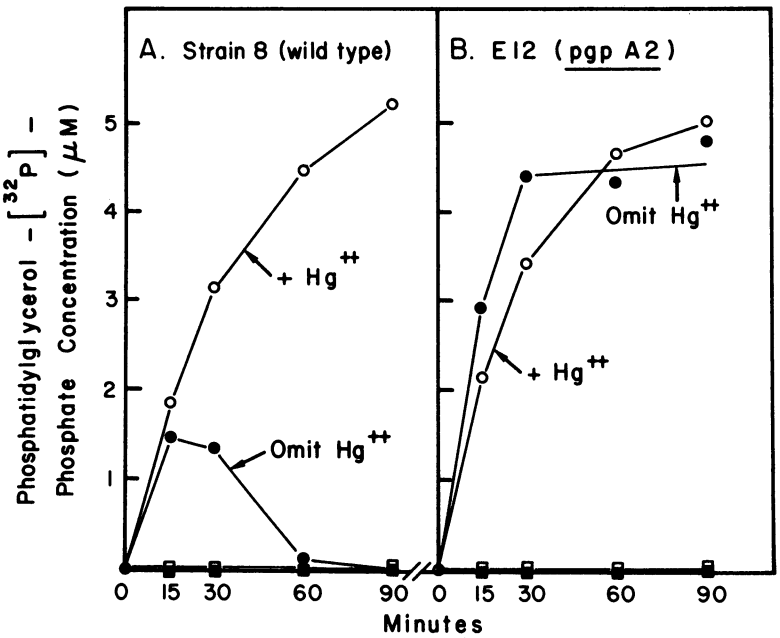

FIG. 1. Coupled synthesis and dephosphorylation of PG-^{32}P in crude extracts of a wild-type and a *pgpA2* mutant strain. Cells were grown at 30°C on LB broth to late log phase (absorbance at 600 nm = 1), harvested by rapid centrifugation at 2°C, and washed once with 0.02 M Tris-hydrochloride (pH 7.5) containing 10 mM 2-mercaptoethanol. After resuspension at about 3 mg of protein per ml, cells were disrupted by sonic oscillation, and extracts were stored at 0°C. Formation of PG-^{32}P and its concurrent dephosphorylation were measured under conditions the same as those used to assay PGP synthetase (6, 20) except that 5 μM *sn*-glycerol-3-^{32}P (10^5 cpm/nmol) was used as the radioactive substrate. Other components of the assay included 0.1 mM CDP-diglyceride, 0.25 M Tris-hydrochloride (pH 8), 30 mM $MgCl_2$, 0.2% Triton X-100, and 5 mM 2-mercaptoethanol. Further addition of 5 mM $HgCl_2$ to the reaction inhibited dephosphorylation completely, but had relatively little effect on PG-^{32}P synthesis. In all cases, the reaction was initiated by the addition of 60 μg of crude sonicate protein, yielding a final volume of 100 μl. The mixture was incubated at 42°C. At the times indicated, 10-μl portions were withdrawn and spotted on filter paper impregnated with trichloroacetic acid (8) and allowed to air dry. The filters were washed on a Büchner funnel with 2% trichloroacetic acid, and the acid-precipitable counts representing PG-^{32}P were determined with a liquid scintillation spectrometer. Over 90% of the radioactive material on the filter was PG-^{32}P. The results are expressed as the actual concentration of PG-^{32}P present in the reaction mixture. (A) Time course of PG-^{32}P accumulation in an extract of strain 8 (wild type). In the presence of Hg^{2+}, all of the *sn*-glycerol-3-^{32}P is converted to acid-precipitable lipid; in the absence of Hg^{2+} there is transient PG-^{32}P accumulation, but this material disappears again after 60 min because of phosphatase activity. (B) A similar experiment but with the mutant strain E12 (*pgpA2*), which was isolated by colony autoradiography. In this case, the accumulation of PG-^{32}P is stable whether or not Hg^{2+} is present. As expected, omission of CDP-diglyceride in the reaction mixture results in the complete elimination of PG-^{32}P formation, independent of the presence (□) or absence (■) of Hg^{2+}.

(min 6) or *purE* (min 12). F'13, which carries the region around min 10 (1, 15), restored activity to strains harboring the *pgpA* mutation.

Quantitation of residual PGP phosphatase specific activity in *pgpA* mutants. The conditions of colony autoradiography and the coupled in vitro assay (Fig. 1) used to detect the *pgpA* lesion were optimized for PGP synthetase and not for PGP phosphatase. To quantitate the residual specific activity of PGP phosphatase in strain E12 (*pgpA2*), we scaled down the assay of Chang and Kennedy (6) in which 100 μM PG-^{32}P is added directly as the substrate. There is only a 20% reduction in PGP phosphatase specific ac-

TABLE 3. Mapping of the *pgpA* gene by P1 *vir* transduction[a]

Expt	P1 donor × recipient	Selected gene	Unselected gene	Cotransduction frequency (%)
A	E12 × TI63	*proC*$^+$	*pgpA2*	15
		dnaZ$^+$	*pgpA2*	2
B	E13 × TI63	*proC*$^+$	*pgpA3*	14
		dnaZ$^+$	*pgpA3*	5

[a] In each transduction at least 100 transductant colonies were picked up, purified, and assayed by colony autoradiography for the *pgpA* lesion.

TABLE 4. Specific activities of PGP phosphatase and phosphatidic acid phosphatase in various mutants

Strain (genotype)	Sp act (nmol/min per mg of protein)	
	PGP phosphatase	Phosphatidic acid phosphatase
Strain 8 (*pgpA*$^+$ *pgpB*$^+$)	5.7	0.54
E12 (*pgpA2 pgpB*$^+$)	4.4	0.54
E20 (*pgpA2 pgpB20*)	0.6	0.01
E26 (*pgpA2 pgpB26*)	0.6	0.02
E27 (*pgpA2 pgpB27*)	0.5	0.01
E28 (*pgpA2 pgpB28*)	0.7	0.01

tivity under these conditions (Table 4). A more detailed time course of PG-^{32}P hydrolysis comparing the parent (strain 8) and a *pgpA* mutant (E12) is shown in Fig. 2A. Table 4 and Fig. 2B demonstrate that the *pgpA* mutation causes no detectable alteration of phosphatidic acid phosphatase.

Isolation of second-step mutants with low levels of PGP phosphatase and phosphatidic acid phosphatase. The very substantial amount of PGP phosphatase remaining in strains harboring *pgpA* lesions suggested the existence of multiple phosphatases. As an approach to this problem we subjected strain E12 (*pgpA2*) to a second cycle of chemical mutagenesis (as above) and analyzed colonies grown on LB agar by filter paper autoradiography, using the conditions designated "B" in Table 2. These conditions are optimal for PGP phosphatase and mask the *pgpA* lesion. However, sufficient synthesis of PG-^{32}P from CDP-diglyceride and *sn*-glycerol-3-^{32}P occurs to permit a search for dark spots by colony autoradiography. About 40,000 colonies derived from mutagen-treated E12 cells were assayed by the "B" method (Table 2), and 15 candidates appearing somewhat darker than the surrounding colonies were identified. Four of them (E20, E26, E27, and E28) did indeed have very low levels of PGP phosphatase (10 to 15% of wild type) when quantitated by method I (Table 4, Fig. 2A). Also, the presence of the *pgpB* mutation lowered the specific activity of phosphatidic acid phosphatase by 20 to 40-fold relative to strain 8 or E12 (Table 4, Fig. 2B). The latter trait is the best criterion for mutations defective in the *pgpB* gene.

Genetic mapping of the *pgpB* locus. The chro-

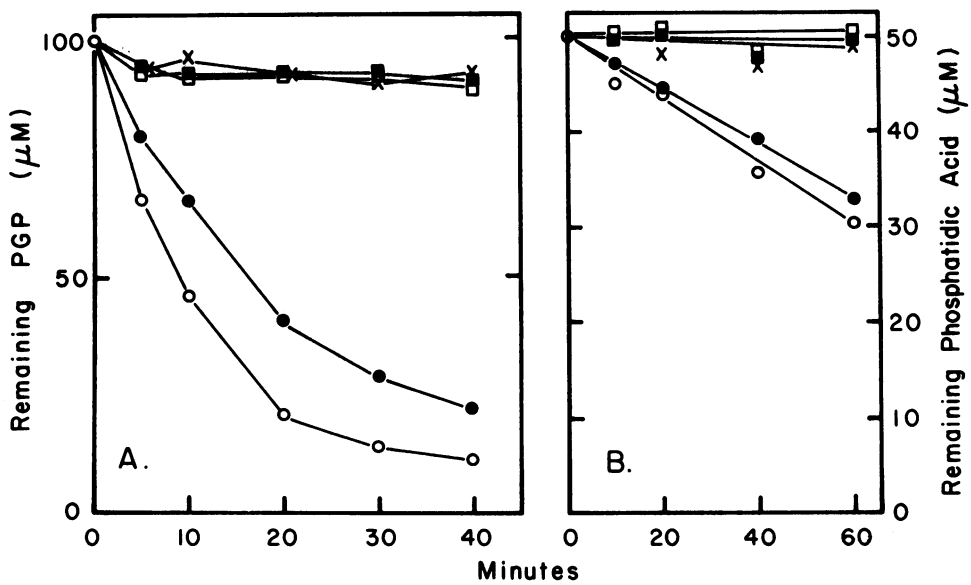

FIG. 2. Time course of hydrolysis of PG-^{32}P and [^{32}P]phosphatidic acid in extracts from a wild type, a single-step *pgpA* mutant, and a two-step *pgpA pgpB* mutant. (A) Hydrolysis of PGP, as determined by assay method II. Under these conditions the PGP phosphatase reaction proceeded at an optimal rate, and the starting concentration of substrate was 100 μM. (Note that in Fig. 1 the conditions have been optimized for PGP synthetase). (B) Hydrolysis of phosphatidic acid, as determined by assay method IV. In (B) the conditions were similar to those of (A), expect that 50 μM phosphatidic acid was present at the start of the reaction instead of PGP. In all cases 60 μg of crude cell extract protein was used in a final volume of 100 μl, allowing a direct comparison of the reaction rates. Symbols: ○, wild-type strain 8; ●, E12 (*pgpA2*); □, E20 (*pgpA2 pgpB20*); ■, E26 (*pgpA2 pgpB26*); ×, strain 8 plus 5 mM $HgCl_2$.

TABLE 5. Mapping of the *pgpB* gene by P1 *vir* transduction[a]

Expt	P1 donor × recipient	Selected gene	Unselected gene	No. unselected/ no. selected	Cotransduction frequency (%)
A	E26 × CSH28.6	*pyrF*$^+$	*pgsB26*	10/32	31
		pyrF$^+$	*trp*$^+$	9/32	28
		trp$^+$	*pgsB26*	8/24	33
		trp$^+$	*pyrF*$^+$	7/24	26
B	E26 × CB64	*cysB*$^+$	*pgpB26*	23/32	72
C	E20 × CSH28.6	*trp*$^+$	*pgpB20*	11/32	34
D	TI72 × TI61	*cysB*$^+$	*pgpB26*[b]	30/32	94
		cysB$^+$	*trp*$^+$ Tc^s	16/32	50

[a] In all cases the *pgpB* gene was scored by growing a late-log culture of each transductant on LB broth, preparing a cell extract, and assaying directly for phosphatidic acid phosphatase.

[b] Both phosphatidic acid and PGP phosphatase activities were tested.

mosomal location of the *pgpB* mutation (as defined by absent phosphatidic acid and low PGP phosphatase) was determined by conjugation and P1 *vir* transduction. Initially, strain E26 (HfrC *pgpA2 pgpB26*) was allowed to mate with X478 (F$^-$ *leuB6 trpE38 lysA23 metE70 rpsL109*), and various prototrophic, streptomycin-resistant recombinants were selected on minimal A agar plates. Due to the relative uncertainty of scoring the *pgpB* mutation by colony autoradiography (see above), about 20 recombinants of each class were grown to late log phase in LB broth, and cell extracts were assayed directly for the presence or absence of phosphatidic acid phosphatase as the unselected marker. Preliminary results of this kind (not shown) indicated a high degree of linkage between *trp* and *pgpB*.

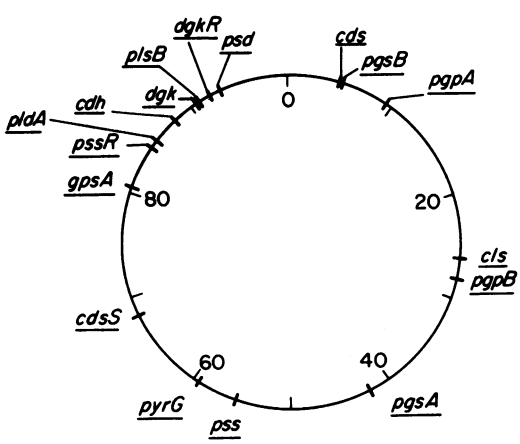

FIG. 3. Locations of *pgpA* and *pgpB* in relation to other phospholipid synthesis genes on the chromosome of *E. coli*.

TABLE 6. Evidence for two PGP phosphatases in *E. coli* and their relationship to phosphatidic acid phosphatase

Strain (genotype)[a]	Sp act (nmol/min/mg of protein)	
	PGP phosphatase	Phosphatidic acid phosphatase
TI70 (*pgpA*$^+$ *pgpB*$^+$)	2.8	0.27
TI71 (*pgpA2 pgpB*$^+$)	1.8	0.27
TI72 (*pgpA2 pgpB26*)	0.4	0.01
TI73 (*pgpA*$^+$ *pgpB26*)	2.1	0.01

[a] The strains were constructed as follows: TI70 (*phoA8 pgpA*$^+$ *pgpB*$^+$) by P1 *vir*-mediated transfer of the *phoA8* gene from strain 8 into X478 (Table 1); TI71 (*phoA8 pgpA2 pgpB*$^+$) by P1 *vir* transduction from E12 to X478 (Table 1); TI72 (*phoA8 pgpA2 pgpB26*) by mating TI71 with E26 (HfrC *pgpA2 pgpB26*) and selecting *trp*$^+$ *pgpB26 rpsL109* recombinants; and TI73 (*phoA8 pgpA*$^+$ *pgpB26*) by a mating of strain 8 (HfrC *phoA8 pgpA*$^+$ *lac*$^+$ *purE*$^+$) with TI72 and selecting *purE*$^+$ *lac*$^+$ *pgpA*$^+$ *rpsL109* recombinants.

The localization of *pgpB* near min 28 was verified by a detailed P1 *vir* transduction analysis (Table 5), which revealed over 90% cotransduction of *pgpB* and *cysB* and about 25 to 35% cotransduction between *trp* and *pgpB*. Since the phosphatidic acid and PGP phosphatases were both unaffected in strain RL88 with a chromosomal deletion (Table 1) of the *trp* to *cysB* region and in strain DM800 with a deletion of *top* to *cysB*, it is likely that the clockwise gene order near min 28 is *trp-cysB-pgpB-pyrF*. In all transductions (Table 5) the *pgpB* mutations were analyzed as the unselected genes by assaying extracts of fresh overnight cultures from 10 to 30 transductant colonies for phosphatidic acid phosphatase. However, in transduction experiment D (Table 5), both PGP and phosphatidic acid phosphatases were measured. It was not possible to separate the defects in these two activities by this method.

The chromosomal locations of *pgpA*, *pgpB*, and other genes of phospholipid bilayer synthesis of *E. coli* are shown in Fig. 3.

Properties of PGP and phosphatidic acid phosphatases in various recombinants. To further examine the relationship of the membrane-associated phosphatases, we constructed the recombinants shown in Table 6. These strains (all defective in alkaline phosphatase) were compared for their specific activities of PGP and

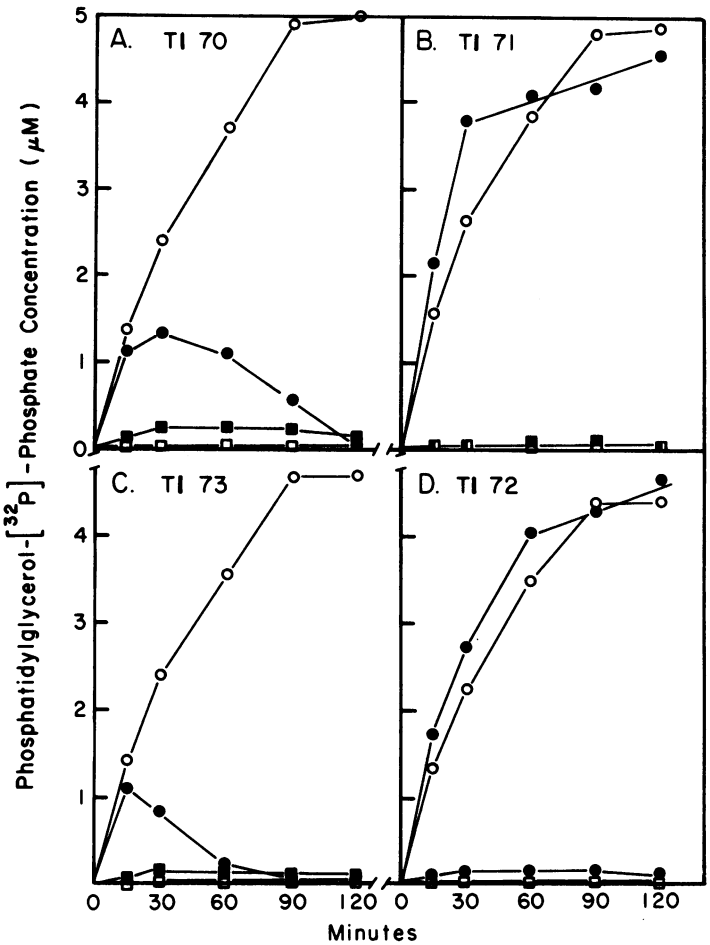

FIG. 4. Coupled assay demonstrating the presence of the *pgpA* gene in various recombinants. The construction of strains is described in Tables 1 and 6. Assays were performed by the methods described in the legend to Fig. 1. Symbols indicate that the assay was done as follows: ○, plus Hg^{2+}, plus CDP-diglyceride; ●, minus Hg^{2+}, plus CDP-diglyceride; □, plus Hg^{2+}, minus CDP-diglyceride; ■, minus Hg^{2+}, minus CDP-diglyceride. (A) Strain T170 (*phoA8 pgpA*$^+$ *pgpB*$^+$); (B) strain T171 (*phoA8 pgpA2 pgpB*$^+$); (C) strain T173 (*phoA8 pgpA*$^+$ *pgpB26*); (D) strain T172 (*phoA8 pgpA2 pgpB26*).

phosphatidic acid phosphatases in cell extracts (Table 6). As expected from the analysis of the original isolates (Table 4), strain TI71 (*pgpA2*) has about two-thirds the PGP phosphatase specific activity of the parent TI70, whereas the phosphatidic acid phosphatase is unaffected. TI71 also shows the expected *pgpA2* phenotype in the more sensitive coupled assay (cf. Fig. 4A and 4B). On the other hand, TI72 (*pgpA2 pgpB6*) has low levels of both enzymes (Table 6). Importantly, TI73 (*pgpA*$^+$ *pgpB6*) regains considerable PGP phosphatase activity and is not significantly different from wild type by the criteria of Fig. 4C and D, but the phosphatidic acid phosphatase levels remain 20- to 40-fold lower than wild type because of the *pgpB* lesion (Table 6).

These results strongly support the view that there are two PGP phosphatases in *E. coli*, one of which also functions as a phosphatidic acid phosphatase. Analysis of the dephosphorylation products generated in extracts of various strains confirmed that both the *pgpA* and *pgpB* enzymes liberated P_i from PG-^{32}P. Phosphatidylglycerol could be demonstrated as the other product if lipid labeled with [^{14}C]glycerol was used as the substrate. Further, the defect in phosphatidic acid phosphatase associated with the *pgpB* mutation was also verified by monitoring the release of 1,2-diglyceride from [^{14}C]-phosphatidic acid (not shown).

Growth properties and phospholipid composition of *E. coli* strains harboring the *pgpA* and

TABLE 7. Phospholipid compositions of various mutants[a]

Strain	Composition (% of $CHCl_3$-soluble $^{32}P_i$)				
	PGP	PE	PG	CL	Unidentified
Strain 8	0.1	73.6	17.7	7.2	1.4
E12	1.3	71.5	14.5	11.0	1.7
E20	4.6	70.0	15.1	8.6	1.7
E26	5.4	66.7	18.1	8.3	1.5

[a] Compositions were determined on fresh overnight cultures grown on LB broth continuously with $^{32}P_i$ (10 μCi/ml) and two-dimensional thin-layer chromatography of the extracted phospholipids, as described in the text. Similar results were obtained with exponentially growing cells (not shown). PE, Phosphatidyl-ethanolamine; -PG, phosphatidylglycerol; CL, cardiolipin.

***pgpB* mutations.** The *pgpA* and *pgpB* alleles we have examined so far do not confer any obvious conditional lethality on strains harboring them. However, this does not eliminate the possibility that the *pgpA* and *pgpB* genes are essential, since the existing alleles are not deletions. Also, the measurable residual PGP phosphatase activity, which is present even in the double mutant, may be sufficient to permit adequate phosphatidylglycerol synthesis in vivo.

The phospholipid compositions of various PGP phosphatase mutants are shown in Table 7. The *pgpA* lesion caused a 5 to 10-fold increase in the steady-state level of PGP, as judged by two-dimensional thin-layer chromatography and deacylation analysis (5). The presence of both the *pgpA* and *pgpB* mutations causes a further 3- to 4-fold accumulation of this material, which amounts at most to about 5% of the total phospholipid. The expanded pool of PGP in strains such as E12 and E20 probably accelerates the rate of phosphatidylglycerol synthesis, compensating for the reduced levels of PGP phosphatase. Based on previous studies with other phospholipid mutants (7, 9), the accumulation of an intermediate such as PGP to 5% of the total membrane lipid is unlikely to have any serious physiological consequences.

DISCUSSION

As shown in previous work from this laboratory (7, 18, 20, 21, 24), enzyme-specific colony autoradiography greatly facilitates the isolation of biochemically defined mutants, especially when classical enrichment techniques are not available. Most of the existing phospholipid mutants (Fig. 3) have been obtained by colony autoradiography. However, until recently the applications of this method were restricted to biosynthetic enzymes which generate acid-precipitable radioactive products (7, 18, 20, 21, 24). The present study demonstrates that hydrolytic reactions (which usually yield acid-soluble substances) can also be analyzed by colony autoradiography, provided that a suitable coupled assay can be devised (i.e., CDP-diglyceride + *sn*-glycerol-3-$^{32}P \rightarrow$ PG-$^{32}P \rightarrow$ phosphatidylglycerol + $^{32}P_i$. Analogous two-step schemes could be envisaged for detection of mutants in phosphoprotein phosphatases, phospholipases, nucleases, or even proteases. In addition, mutants defective in PGP phosphatase (which yield dark spots by autoradiography) can be used as hosts for the molecular cloning of the *pgp* genes, since the same colony-screening assay can be used to pick out occasional nonradioactive variants that have regained the function of interest (T. Icho and C.R.H. Raetz, unpublished data).

The discovery of two genes for PGP phosphatase raises a number of interesting issues. The simplest interpretation of the data shown in Tables 4 and 6 is that there are two PGP phosphatases, one of which can also attack phosphatidic acid. Molecular cloning of the *pgp* genes (T. Icho and C.R.H. Raetz, unpublished data) supports this view, since multicopy plasmids carrying *pgpB* cause 10-fold overproduction of both activities, whereas cloning of the *pgpA* locus only increases the specific activity of PGP phosphatase. In any case, the availability of the mutants and the clones will greatly facilitate the biochemical analysis of these enzymes.

We have also examined strains harboring *pgp* mutations for the presence of lysophosphatidic acid phosphatase (data not shown). The *pgpA* lesions have no effect on this enzyme. The *pgpB* mutations lower the specific activity of lysophosphatidic acid phosphatase by about 50%, whereas phosphatidic acid phosphatase is reduced 20-fold (Table 4). Consequently, there may be a third enzyme in *E. coli* which acts preferentially on lysophosphatidic acid.

The putative functions of the multiple membrane-bound phosphatases deserve some consideration. It is not readily apparent why there should be two PGP phosphatases and why intermediates such as phosphatidic acid should be subject to hydrolysis. We favor the following explanation. Since the *pgpA* gene product has the highest catalytic specificity and exerts the greatest relative influence on cellular PGP levels, it probably functions as the primary biosynthetic enzyme. The *pgpB* gene product, on the other hand, is less specific. Perhaps its true function involves some other, as yet unidentified substrate, such as a lipopolysaccharide precursor or a phosphoprotein, or it could even act as a phosphotransferase under suitable conditions.

It is well established that the lipid biosynthetic enzymes are associated with the inner mem-

brane of *E. coli* (2, 22, 26). Although the experimental data have not been published, it has been suggested that phosphatidic acid phosphatase is mainly localized in the outer membrane (2). This raises the alternative possibility of topographically distinct pools of PGP or phosphatidic acid, requiring separate enzymes for their metabolism. Further characterization of both *pgp* mutations and gene products will resolve these possibilities.

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