Identification of a New Phospholipase C Activity by Analysis of an Insertional Mutation in the Hemolytic Phospholipase C Structural Gene of *Pseudomonas aeruginosa*

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The phospholipase C (PLC) gene of *Pseudomonas aeruginosa* encodes a heat-labile secreted hemolysin which is part of a P_i-regulated operon. The structural gene for PLC, *plcS*, was mutated in vitro by insertion of a tetracycline resistance gene cartridge. Gene replacement techniques were used to introduce the mutated *plcS* gene into the *P. aeruginosa* chromosome in place of the wild-type gene. The precise replacement of wild-type sequences by mutant sequences was confirmed by Southern hybridization. The mutant strain, designated PLC S, is nonhemolytic and lacks a 78-kilodalton protein corresponding to the size of the wild-type PLC. However, there is an additional phospholipase activity present in PLC S capable of hydrolyzing *p*-nitrophenylphosphorylcholine, a synthetic PLC substrate, and phosphatidylcholine. This enzymatic activity is not a result of a truncated product produced from the mutated *plcS* gene. The phospholipase activity of PLC S was identified as a nonhemolytic PLC.

Pseudomonas aeruginosa is an important opportunistic pathogen that is a common cause of nosocomial infections. Lung infections with this pathogen are associated with high mortality rates in cystic fibrosis patients. Among the numerous extracellular products produced by *P. aeruginosa* which may contribute to pathogenesis, including toxins, proteases, and exopolysaccharides, are two hemolysins (13, 21). One of these hemolysins is a heat-stable glycolipid. The other hemolysin is a heat-labile phospholipase C (PLC) which catalyzes the hydrolysis of phosphatidylcholine, yielding phosphorylcholine and diacylglycerol (7). PLC may play an important role in the pathogenesis of lung infections (13) and may also be an important virulence determinant in other types of infections.

The PLC hemolysin produced by *P. aeruginosa* is synthesized maximally during the late log and early stationary phases of growth in low-phosphate media, but PLC synthesis is repressed in high-phosphate media (13). PLC may enhance colonization of lung tissue by providing essential nutrients from the hydrolysis of phospholipids in surfactant (13), which is composed primarily of phosphatidylcholine. Substrate specificity studies have shown that PLC preferentially hydrolyzes phospholipids containing quaternary ammonium groups, which are found primarily in eucaryotic membranes (e.g., phosphatidylcholine) but has little activity towards phospholipids found in the procaryotic membrane (4).

The structural gene encoding PLC (*plcS*) has been cloned and sequenced (14, 22, 18). *plcS* is part of a multigene operon which is regulated by P_i at the level of transcription (18). The gene product of *plcS* is an 82.6-kilodalton (kDa) protein. This protein contains a 4.4-kDa putative signal peptide which may be cleaved from the native PLC to yield the secreted 78.2-kDa mature hemolysin (18). Downstream of *plcS* are two in-phase overlapping genes designated *plcR1* and *plcR2* (19). When expressed in *Escherichia coli*, a cytoplasmic form and a secreted form of the *plcR* gene products are observed (19). The function of the products of the *plcR* genes

is not known. However, preliminary studies indicate that *plcR* products may negatively regulate the synthesis of PLC and other P_i-regulated proteins in high-P_i media (R. M. Ostroff and M. L. Vasil, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D111, p. 90). To investigate the role of the PLC operon in pathogenesis and to better understand its regulation, an insertional mutation in *plcS* was constructed. Gene replacement techniques were used to introduce the mutated copy of the PLC operon into the chromosome in place of the wild-type operon in *P. aeruginosa* strain PAO1. The construction and analysis of the *plcS* mutant strain are described in this study.

MATERIALS AND METHODS

Materials. Phospholipids, *p*-nitrophenylphosphate, and *p*-nitrophenylphosphorylcholine (NPPC) were obtained from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories, Bethesda, Md., and used as described by the supplier.

Bacterial strains and plasmids. The bacterial strains and plasmids used in these studies are listed in Table 1.

Media and antibiotics. *E. coli* cultures were grown in brain heart infusion broth (Difco Laboratories) at 37°C. Tris minimal medium (20) or peptone medium (1% peptone, 1% NaCl) was used for production of PLC from *P. aeruginosa* with or without the addition of 10 mM P_i. Blood agar (5% sheep erythrocytes) (Pasco Labs, Wheatridge, Colo.) was used to screen hemolytic activity. Pseudomonas isolation agar (Difco) supplemented with the appropriate antibiotics was used to select for *P. aeruginosa* in the mating experiments. Antibiotics were used in the following concentrations (milligrams per liter): for *E. coli*, carbenicillin (200), tetracycline (20), and trimethoprim (200); for *P. aeruginosa*, carbenicillin (1,000), tetracycline (200), and trimethoprim (1,000).

Transfer of plasmids from *E. coli* to *P. aeruginosa*. *E. coli* plasmid donor strains were grown in brain heart infusion broth with appropriate antibiotics overnight at 37°C. *P. aeruginosa* strains were grown at 43°C with aeration over-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source/ reference
Bacterial strain		
E. coli HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13* *ara-14 proA2* *lacY1 galK2* *rpsL20* (Sm^r) *xyl-5 mtl-1* *supE44* λ^-	A. L. Taylor (15)
P. aeruginosa PAO1	Prototroph *chl-3*	B. W. Holloway (9)
P. aeruginosa PLC S	*plcS::tetAR* deriva- tive of PAO1	This study
Plasmid		
ColE1::Tn5-*132*	Tc	R. Gill (3)
pGV26	Ap/Cb Hly$^+$	22
pGM78	Ap/Cb Hly::Tc	This study
pME301	Ap/Cb Tc Km Tra Rep(Ts) IncP-1	D. Haas (10)
pR751	Tp IncP-1	R. J. Meyer (16)
pRR1	Tp Hly::Tc	This study

[a] The genotype symbols have been described by Bachmann (2). Sm, Ap/Cb, Tc, and Tp refer to resistance to streptomycin, ampicillin/carbenicillin, tetracycline, and trimethoprim, respectively. Hly refers to the hemolytic or PLC phenotype of the plasmid.

night and then incubated for 3 to 5 h without aeration at 43°C. A 3-ml amount of each culture was filtered onto a 0.45-μm Nalgene filter (diameter, 5.5 cm). The filters were placed on brain heart infusion broth plates at 30°C overnight. Cells were suspended in 5 ml of phosphate-buffered saline (80 mM NaCl, 43 mM Na₂HPO₄, 11 mM KH₂PO₄), diluted, and plated on pseudomonas isolation agar with the appropriate antibiotics.

Isolation and manipulation of DNA and Southern blot hybridization. Genomic DNAs of *P. aeruginosa* strains were prepared and digested as previously described (23). Southern blot hybridization and high-stringency washes were performed as previously described (23). Conditions for DNA purification and manipulation for cloning were as described before (15).

Preparation of concentrated culture supernatants. Two-liter flasks were washed with chromic acid and then extensively rinsed with triple-distilled water to remove any trace of phosphate. Starter cultures were prepared from overnight cultures of *P. aeruginosa* strains grown in brain heart infusion broth. The cells were harvested by centrifugation and washed three times with Tris minimal medium before use as an inoculum. A 500-ml portion of Tris minimal medium with or without the addition of 10 mM P_i was inoculated to an A_{540} of 0.05. Cultures were grown overnight at 32°C. The cells were removed by centrifugation. Proteins in the supernatants were precipitated with ammonium sulfate to 70% saturation. The material was centrifuged at 15,000 × g for 30 min. The precipitated proteins from 500 ml of supernatant were dissolved in 5 ml of 10 mM Tris hydrochloride buffer (pH 7.2) and dialyzed against the same buffer overnight at 4°C. Samples used for hemolytic assays were dialyzed against phosphate-buffered saline overnight at 4°C. Protein concentrations for concentrated culture supernatants were determined by the method of Bradford (5), using a commercially prepared reagent (Bio-Rad Laboratories, Richmond, Calif.).

Hemolytic titers. Serial twofold dilutions of concentrated

culture supernatants previously dialyzed against phosphate-buffered saline were added to 1% sheep erythrocytes. Hemolytic activity was estimated visually by determination of release of hemoglobin after 24 h of incubation at 37°C.

Enzyme assays. PLC was measured by NPPC hydrolysis as previously described (12) or by the hydrolysis of phospholipids and the release of P_i (11). The conditions for hydrolysis of phospholipids were as previously described (4). The release of P_i was measured spectrophotometrically by the method of Chen et al. (6).

RESULTS

Construction of an insertional mutation in *plcS*. Plasmid pGV26 contains the cloned PLC operon within a 6.1-kilobase (kb) *Bam*HI DNA fragment. This plasmid was identified from a *P. aeruginosa Bam*HI genomic library by the hemolytic phenotype it conferred to *E. coli* HB101 (22). The insertion of a tetracycline resistance (Tc^r) cartridge into *plcS* is shown in Fig. 1. A 2.7-kb Tc^r cartridge containing the *tetAR* locus from Tn*10* was isolated from Tn5-*132* by digesting ColE1::Tn5-*132* with *Bgl*II. The 5' protruding ends were filled in with Klenow fragment of DNA polymerase I and blunt end ligated into the single *Stu*I site of pGV26, which is in the *plcS* gene 504 nucleotides downstream from the starting codon. The resulting construct, pGM78, is nonhemolytic in *E. coli*.

The insertionally mutagenized PLC operon was used to construct a mutant *P. aeruginosa* strain isogenic with the wild-type strain, except at the *plcS* locus. pGM78 was digested with *Bam*HI, and the 8.9-kb fragment containing *plc::tetAR* was isolated and cloned into the *Bgl*II site of pR751, resulting in pRR1 (Fig. 1). pR751 is a large, low-copy-number vector plasmid of the IncP-1 group. It is self-transmissible and capable of replicating in *P. aeruginosa*. pRR1 was mobilized into the prototrophic strain PAO1. PAO1 containing pRR1 was grown without antibiotic selection for 146 h, and then cultures were diluted and plated on blood agar. Ten thousand colonies were examined for a nonhemolytic phenotype. The nonhemolytic phenotype is the result of inactivation of *plcS* through homologous recombination between sequences on pRR1 and the PAO1 chromosome on either side of the Tc^r insertion. Nonhemolytic Tc^r clones were isolated at a frequency of 0.1%. Southern hybridization data confirmed that the wild-type *plcS* gene had been replaced by a single copy of the insertionally mutated *plcS* sequences from pRR1 (data not shown). However, pRR1 was still present in these clones.

To eliminate pRR1, pME301 was mobilized into PAO1 *plc::tetAR*(pRR1). pME301 is a plasmid which is a member of the same incompatibility group as pRR1, encodes Cb^r, and is temperature sensitive for replication. By selection of Cb^r, pRR1 was replaced by pME301. PAO1 *plc::tetAR* was cured of pME301 by raising the temperature of the culture to 43°C, which is the nonpermissive temperature for pME301 replication. Tc^r, Cb^s colonies were isolated. The resulting plasmid-free strain, designated PLC S, contains a chromosomal insertional mutation in *plcS* but is isogenic with PAO1 at all other loci. PLC S is tetracycline resistant and nonhemolytic. It has no activity on egg yolk agar (lecithin agar), indicating that it is not capable of hydrolyzing lecithin (phosphatidylcholine) in this medium to release diacylglycerol (7).

Analysis of PLC S genomic DNA by Southern hybridization to PLC probes. Genomic DNAs from PAO1 and PLC S were purified, digested with several restriction enzymes, and hybridized with the 6.1-kb *Bam*HI-*Bam*HI PLC probe in a
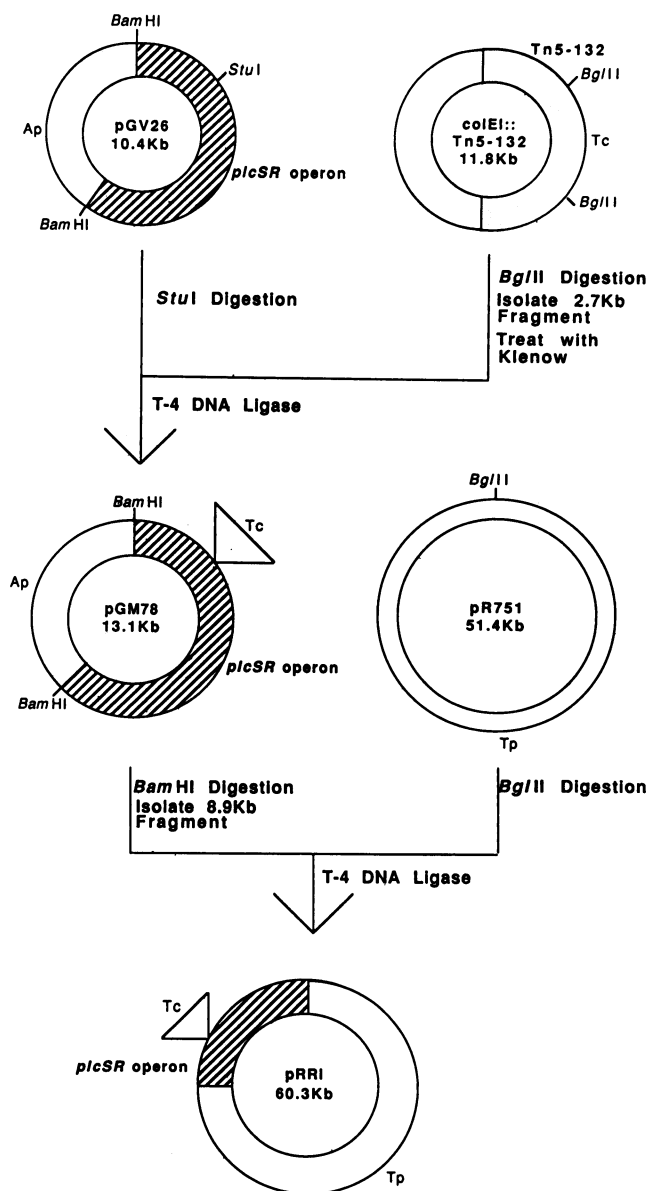
FIG. 1. Construction of pRR1 by insertion of the 2.7-kb Tc fragment into the *Stu*I site of *plcS*. See text for details.
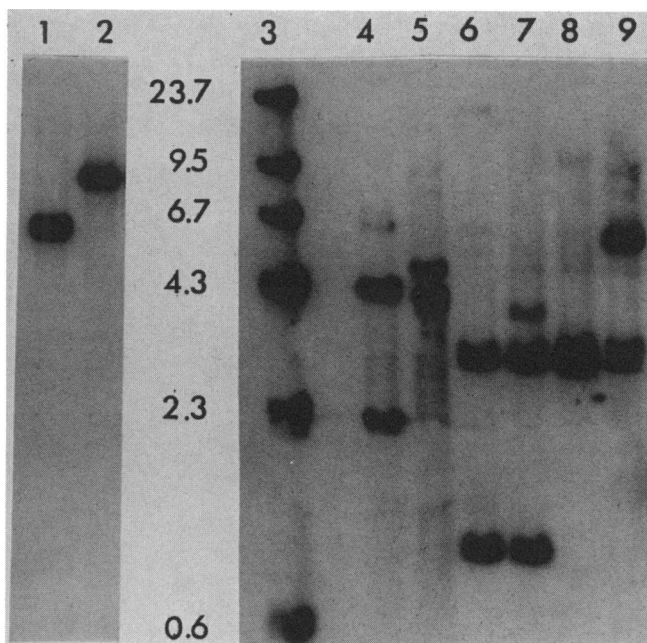

FIG. 2. Southern blot of PAO1 and PLC S hybridized with a 6.1-kb *Bam*HI PLC probe. Lane 1, PAO1 digested with *Bam*HI; lane 2, PLC S digested with *Bam*HI; lane 3, lambda DNA digested with *Hin*dIII and the 3' recessed ends filled in by using [α-^{32}P]CTP; lane 4, PAO1 digested with *Bam*HI and *Xho*I; lane 5, PLC S digested with *Bam*HI and *Xho*I; lane 6, PAO1 digested with *Bam*HI and *Sma*I; lane 7, PLC S digested with *Bam*HI and *Sma*I; lane 8, PAO1 digested with *Bam*HI and *Pst*I; lane 9, PLC S digested with *Bam*HI and *Pst*I.

Southern hybridization (Fig. 2). The Southern blots demonstrate the addition of the 2.7-kb Tc^r fragment into the *Stu*I site of PAO1. For example, lane 1 is a *Bam*HI genomic digestion of PAO1, in which a 6.1-kb fragment hybridizes to the probe. Lane 2 is a *Bam*HI genomic digestion of PLC S, and the size of the hybridizing fragment has increased to 8.8 kb. The hybridization patterns of PAO1 and PLC S in lanes 4 through 9 are consistent with the insertion of 2.7 kb into *plcS* at the *Stu*I site. PAO1 and PLC S genomic DNAs were also reacted with a probe consisting of the Tc^r *Bgl*II fragment in a Southern hybridization (data not shown). PAO1 did not react with the probe, while PLC S genomic DNA hybridization patterns confirmed the insertion of the Tc^r cartridge into the *Stu*I site of *plcS*. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of concentrated culture supernatants from PAO1 and PLC S grown under low-P$_i$ conditions revealed that a 78-kDa protein corresponding to

the size of the mature hemolytic PLC of *P. aeruginosa* is present in the PAO1 sample but absent from the PLC S sample (data not shown).

Analysis of PAO1 and PLC S culture supernatants. PLC S has no detectable hemolytic activity toward sheep erythrocytes (Table 2). The hemolytic titer of the PAO1 culture supernatant is increased 16-fold in low phosphate over the titer of the supernatant from growth in a high-P$_i$ medium.

An unexpected characteristic of PLC S is that it retains PLC activity at a level only slightly less than that of PAO1, as measured by the hydrolysis of the synthetic substrate NPPC (Table 2). This activity is P$_i$ regulated and found extracellularly. It is also produced by PLC S at the same time during growth as the wild-type PLC activity (Fig. 3). PLC activity can be detected in PAO1 and PLC S culture supernatants after 4 h of growth, which corresponds to late logarithmic growth. This activity rises steadily through the early stationary phase of the culture.

Analysis of the phospholipase activity of PLC S. To confirm

TABLE 2. Hemolytic and PLC activity of PAO1 and PLC S^a

Strain	Hemolytic titer^b		PLC^c	
	+P$_i$	−P$_i$	+P$_i$	−P$_i$
PAO1	128	2,048	10.0	47.5
PLC S	<2	<2	5.3	33.0

^{a} Cultures were grown in Tris minimal medium with or without the addition of 10 mM P$_i$.

^{b} Hemolytic titers are expressed as the reciprocal of the highest dilution yielding the release of hemoglobin from sheep erythrocytes.

^{c} PLC activity is expressed as hydrolysis of the synthetic substrate NPPC in units per milliliter of concentrated culture supernatant.
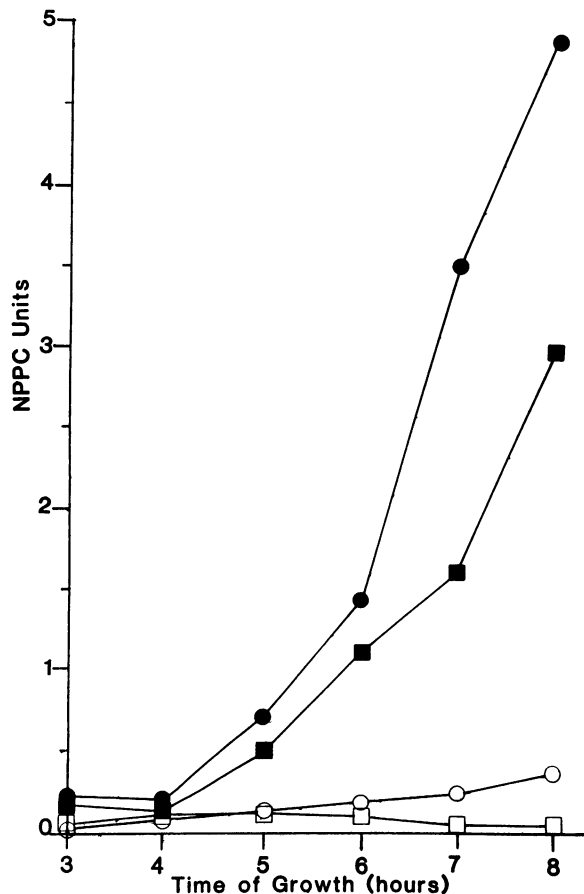
FIG. 3. Time course of NPPC activity of unconcentrated culture supernatants during growth. Symbols: ●, PAO1, low P_i; ○, PAO1, high P_i; ■, PLC S, low P_i; □, PLC S, high P_i.

S supernatant hydrolyzes NPPC, apparently as a result of the nonhemolytic PLC activity. Both staining patterns are less intense in samples prepared from high-P_i cultures.

DISCUSSION

In this study we report the use of gene replacement techniques in the construction of a PLC mutant *P. aeruginosa* strain which is isogenic with the wild-type strain except at the *plcS* locus. The mutant strain, PLC S, does not secrete a 78-kDa protein corresponding to the parental PLC. PLC S is nonhemolytic and does not produce a lecithinase (PLC) reaction on egg yolk plates. However, PLC S is capable of cleaving the synthetic PLC substrate NPPC at 70% of the wild-type rate when grown under phosphate-limiting conditions. The rate of cleavage of NPPC by PLC S grown in P_i-sufficient conditions is 50% of the wild-type rate. In addition, the PLC activity of PLC S is P_i regulated and found extracellularly. The nonhemolytic PLC is also produced during the same stage of growth as the hemolytic PLC. This may not be an unexpected observation if one hypothesizes that a P_i regulon exists in *P. aeruginosa* which is similar to that of *E. coli*. Many P_i-regulated genes may be coordinately expressed under P_i starvation conditions.

The nonhemolytic PLC activity of PLC S is not the result of a truncated PLC synthesized from the mutated *plcS* gene. The existence of a second PLC in *P. aeruginosa* was confirmed through studies of a recently constructed PAO1 mutant in which the entire *plcS* gene and most of *plcR1* and *plcR2*, the downstream genes of the PLC operon, have been deleted. This deletion mutant retains PLC activity like the *plcS* mutant as measured by NPPC hydrolysis (R. M. Ostroff

the results obtained with the synthetic PLC substrate, the phospholipase activity of concentrated culture supernatants was assayed by hydrolysis of the natural phospholipid substrate (Fig. 4). The phospholipase activity from PLC S is only 30 to 50% of the level from PAO1, as measured by hydrolysis of phosphatidylcholine. The substrate specificity of the PLC S phospholipase is similar to that of PAO1. Both PAO1 and PLC S hydrolyze phosphatidylcholine but have little activity towards phosphatidylethanolamine.

To identify the type of phospholipase activity present in the PLC S supernatant, thin-layer chromatography with ^{3}H- and ^{14}C-labeled phosphatidylcholine was used. The reaction products after cleavage of phosphatidylcholine by both PAO1 and PLC S were diacylglycerol, phosphorylcholine, and choline (data not shown). The generation of diacylglycerol and phosphorylcholine from phosphatidylcholine confirms the existence of a PLC produced by PLC S. The choline is generated from release of P_i from phosphorylcholine by alkaline phosphatase in the concentrated culture supernatants. The hydrolysis of phosphatidylcholine by both strains was P_i regulated.

Another observation which suggests that *P. aeruginosa* produces a separate PLC from that encoded by *plcS* is the pattern of yellow staining by NPPC on a nondenaturing polyacrylamide gel of concentrated culture supernatants (data not shown). Two separate bands from the PAO1 supernatant hydrolyze NPPC. Only one band from the PLC
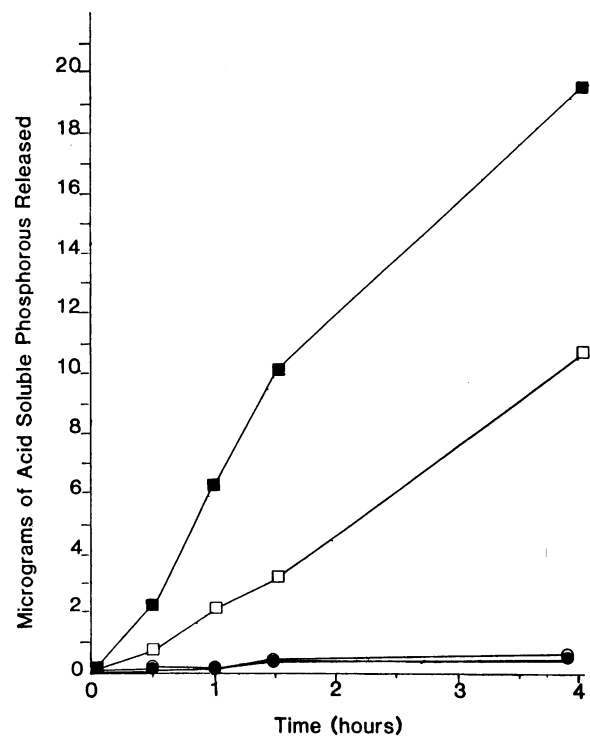

FIG. 4. Time course of hydrolysis of phosphatidylcholine and phosphatidylethanolamine by concentrated culture supernatants prepared from cultures grown in a low-P_i medium. Symbols: ■, PAO1, phosphatidylcholine; □, PLC S, phosphatidylcholine; ●, PAO1, phosphatidylethanolamine; ○, PLC S, phosphatidylethanolamine.

and M. L. Vasil, unpublished observation). The nonhemolytic PLC shares a similar substrate specificity with the hemolytic PLC encoded by *plcS*. Both enzymes hydrolyze phospholipids with quaternary ammonium groups, such as phosphatidylcholine, which is abundant in the eucaryotic cell membrane (1), and have little activity towards phosphatidylethanolamine, a phospholipid found primarily in the procaryotic membrane (1).

However, the PLC produced by PLC S hydrolyzes phosphatidylcholine at only 30 to 50% of the level of the parental strain. These results may explain why this PLC is nonhemolytic. Perhaps this second PLC does not efficiently attack phospholipids which are assembled into a membrane-type structure, but can easily cleave small molecules such as NPPC or solubilized phospholipids. The PLC produced by *Bacillus cereus* is nonhemolytic to intact erythrocytes (17). However, it can hydrolyze a broad spectrum of phospholipids in in vitro assays in which the phospholipids are detergent solubilized (17). Perhaps the second PLC of *P. aeruginosa* is nonhemolytic because the phospholipids of the eucaryotic membrane are not accessible to this enzyme. However, more careful investigation is required before this hypothesis can be confirmed.

The mutation in the *plcS* gene does not markedly affect the production of other extracellular P_i-regulated proteins examined thus far. The *plcS* insertional mutation contains a transcriptional terminator (8) and would be expected to produce polar effects on the downstream genes of the PLC operon, *plcR1* and *plcR2*, and reduce their expression. However, the function of the gene products of *plcR1* and *plcR2* is not known, and polar effects may occur which have not been identified.

The PLC S mutant strain will be useful in establishing the role of the hemolytic PLC in pathogenesis by comparing the virulence of PLC S to the wild-type strain in various animal models of infection. It is also possible to use gene replacement techniques to transfer the mutant *plcS* to other clinically relevant *P. aeruginosa* strains for use in pathogenesis studies. Additional molecular and biochemical studies of the two PLCs of *P. aeruginosa* will help in understanding the structure-function relationship of hemolytic versus nonhemolytic PLC activity. Finally, further investigation into the synthesis and regulation of the nonhemolytic PLC may answer some questions about the complex physiology of P_i regulation in *P. aeruginosa*.

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