JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5915–5923
0021-9193/90/105915-09$02.00/0

Vol. 172, No. 10

Molecular Comparison of a Nonhemolytic and a Hemolytic Phospholipase C from *Pseudomonas aeruginosa*

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Received 27 February 1990/Accepted 12 July 1990

Pseudomonas aeruginosa **produces two secreted phospholipase C (PLC) enzymes. The expression of both PLCs is regulated by P_i. One of the PLCs is hemolytic, and one is nonhemolytic. Low-stringency hybridization studies suggested that the genes encoding these two PLCs shared DNA homology. This information was used to clone *plcN*, the gene encoding the 77-kilodalton nonhemolytic PLC, PLC-N. A fragment of *plcN* was used to mutate the chromosomal copy of *plcN* by the generation of a gene interruption mutation. This mutant produces 55% less total PLC activity than the wild type, confirming the successful cloning of *plcN*. *plcN* was sequenced and encodes a protein which is 40% identical to the hemolytic PLC (PLC-H). The majority of the homology lies within the NH_2 two-thirds of the proteins, while the remaining third of the amino acid sequence of the two proteins shows very little homology. Both PLCs hydrolyze phosphatidylcholine; however, each enzyme has a distinct substrate specificity. PLC-H hydrolyzes sphingomyelin in addition to phosphatidylcholine, whereas PLC-N is active on phosphatidylserine as well as phosphatidylcholine. These studies suggest structure-function relationships between PLC activity and hemolysis.**

Pseudomonas aeruginosa is an important opportunistic pathogen. One of the highest incidences of *P. aeruginosa* infections occurs in the lungs of patients with cystic fibrosis. Several secreted proteins of this organism may contribute to pathogenesis in the lungs (18), including a hemolytic phospholipase C (PLC-H). One of the substrates for PLC-H is phosphatidylcholine, which is hydrolyzed to release phosphorylcholine and diacylglycerol (6). Substrate specificity studies have shown that PLC-H preferentially hydrolyzes phospholipids containing quaternary ammonium groups, which are found primarily in eucaryotic membranes and lung surfactant (e.g., phosphatidylcholine), but has little activity toward phospholipids found in the procaryotic membrane (e.g., phosphatidylethanolamine) (2).

The structural gene encoding PLC-H (*plcS*) has been cloned and sequenced (4, 19, 26, 34). *plcS* is part of the three-gene *plcSR* operon, which is regulated by P_i at the level of transcription (26, 29). The gene product of *plcS* is an 82.6-kilodalton (kDa) protein containing a 38-amino-acid signal peptide which, when cleaved, yields a secreted 78.2-kDa mature hemolysin. Downstream of *plcS* are two in-phase overlapping genes, *plcR1* and *plcR2* (26, 29). The function of the *plcR* gene products is not known, but they may play a role in regulating or activating PLC-H (36; M. Vasil and A. Vasil, unpublished observations).

To study the function of PLC-H, we constructed insertion and deletion mutations in *plcS* (24, 25). These mutations were recombined into the *P. aeruginosa* chromosome in place of the wild-type allele, resulting in nonhemolytic *plcS* mutant strains which were isogenic with the wild type at all other loci. Characterization of these mutants led to the discovery of an additional PLC produced by *P. aeruginosa* because culture supernatants of the *plcS* deletion mutant hydrolyze phosphatidylcholine (24, 25). The hydrolysis of phosphatidylcholine by supernatants produced by the *plcS* mutant is reduced by 50 to 70% in comparison with the activity of supernatants from the wild-type strain (24). This PLC, PLC-N, is secreted, and its synthesis is regulated by P_i. However, in contrast to PLC-H, PLC-N is nonhemolytic for human or sheep erythrocytes.

This report describes the characterization of PLC-N, including cloning and sequencing of the structural gene. The substrate specificity of PLC-N was investigated, and a *P. aeruginosa* PLC-N mutant was constructed. The amino acid sequences of PLC-N and PLC-H were compared, and structure-function relationships between hemolysis and PLC activity were suggested.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and antibiotics. *Escherichia coli* cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) or M9 minimal medium (21) at 37°C. Peptone medium (1% peptone, 1% NaCl, 1% glycerol) (14) was used for production of PLC from *P. aeruginosa* with or without the addition of 10 mM P_i at 32°C. *Pseudomonas* isolation agar (Difco) supplemented with the appropriate antibiotics was used to select for *P. aeruginosa* in mating experiments. Antibiotics were used in the following concentrations: for *E. coli*, carbenicillin at 100 mg/liter and tetracycline at 20 mg/liter; for *P. aeruginosa*, tetracycline at 200 mg/liter.

Isolation and manipulation of DNA and Southern blot hybridization. Conditions for DNA purification and manipulation for cloning were as described previously (21). Restriction endonucleases and DNA-modifying enzymes were used as indicated by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Genomic DNA from *P. aeruginosa* was isolated by a modification of the method of Marmur (13, 35). Southern blot hybridization was as previously described (31, 35). Low-stringency hybridization conditions were 25% (vol/vol) formamide, 1 M NaCl, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 100 μg of heat-denatured salmon sperm DNA per ml at 42°C overnight. Low-stringency washes were performed in 5×

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype or phenotype	Reference
E. coli		
DH5-α	F⁻ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*) *U169* ϕ80d*lacZ*Δ*M15*	11
S17-1	*thi pro hsdR hsdM*⁺ *recA* integrated RP4-2-Tc^r::Mu Kn^r::Tn7	30
BL21(DE3)	F⁻ *hsdS* (r_B^- m_B^-) *ompT gal* λ lysogen containing T7 RNA polymerase under control of the *lacUV5* promoter	32
P. aeruginosa		
PAO1	Prototroph, *chl-3*	12
PLC SR	Δ*plcSR*::Tc^r	25
PLC N-C1	PAO1 with pSUP/Hinc420 integrated	This study
PLC N-C2	PAO1 with pSUP/Hinc444 integrated	This study
Plasmids		
pSUP203	Ap^r/Cb^r Cm^r Tc^r *mob*	30
pUC/PLC-N	Ap^r/Cb^r pUC18 8-kb *Eco*RI-*Bam*HI PLC-N	This study
pGEM1/PLC-N	Ap^r/Cb^r pGEM1 3-kb *Cla*I-*Bam*HI PLC-N	This study
pGEM2/PLC-N	Ap^r/Cb^r pGEM2 3-kb *Cla*I-*Bam*HI PLC-N	This study
pGEM2/PLC-H	Ap^r/Cb^r pGEM2 6.1-kb *Bam*HI PLC-H	26; this study
pSUP/Hinc420	Ap^r/Cb^r Tc^r pSUP203/0.420 kb *Hin*cII of *plcN*	This study
pSUP/Hinc444	Ap^r/Cb^r Tc^r pSUP203/0.444 kb *Hin*cII of *plcN*	This study

SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0])–0.1% SDS for 1 h at 55°C.

Enzyme assays. PLC activity was measured by using the method of Kurioka and Matsuda (15), in which the hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC; Sigma) is monitored.

Sphingomyelinase activity was measured as described previously (7) by the hydrolysis of *N*-omegatrinitrophenylaminolaurylsphingosylphosphorylcholine (TNPAL-sphingomyelin; Sigma). Hydrolysis of phosphatidylserine by cloned PLC enzymes produced in *E. coli* was performed as described by Berka and Vasil (2). Release of soluble phosphorus was measured spectrophotometrically by the method of Chen et al. (3). Assays were performed with either supernatants from *P. aeruginosa* cultures or cell lysates from 1 ml of *E. coli* cultures.

T7 RNA polymerase-directed expression of cloned proteins. The system for selective expression of genes cloned downstream of a T7 promoter was developed by F. W. Studier (32). pGEM plasmids (Promega-Biotec, Madison, Wis.) containing cloned genes downstream of the vector T7 promoter were transformed in *E. coli* BL21(DE3). T7 RNA polymerase expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Bethesda Research Laboratories, Inc.) when the culture had reached an A_{590} of 0.6. Then, 30 min later, rifampin was added to 200 μg/ml and the cells were incubated for 30 min longer. L-[^{35}S]methionine–L-[^{35}S]cysteine (Tran^{35}S-label; ICN Radiochemicals Inc., Irvine, Calif.) was added at 0.15 μCi/ml of culture, and samples (500 μl) were taken 30 min later. The cells were suspended in sample buffer, boiled, and run on SDS–10% polyacrylamide gels (16). The gel was fixed in 10% acetic acid–30% methanol for 1 h, placed in distilled H_2O for 30 min, soaked in 0.5 M sodium salicylate–5% glycerol for 1 h, dried, and used to expose X-ray film. Samples for enzyme assays were obtained by freezing.

DNA sequencing. Both double-stranded and single-stranded DNAs were sequenced by the chain termination method of Sanger et al. (28) with a modified T7 DNA polymerase (Sequenase Kit; U.S. Biochemical Corp., Cleveland, Ohio). Double-stranded DNA was sequenced by using a primer complementary to either the SP6 or T7 promoter region of the pGEM vectors (Promega). Several oligonucleotide primers used in single-stranded template sequencing were generously provided by R. Berka, Genencor, Inc., South San Francisco, Calif. Primer annealing and DNA sequencing were performed as described in the Sequenase manual with deoxyadenosine 5′-α-[^{35}S]thiotriphosphate (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Single-stranded template DNA was isolated from the *E. coli* host JM107 (40) containing pGEM7Zf (Promega) vectors with *plcN* inserts by using an M13 helper phage as directed by the supplier (Promega). Double-stranded templates were either from deletion clones generated by using the Erase-A-Base system (Promega) or from subcloning restriction endonuclease fragments. Sequencing data were analyzed by using the IBI-Pustell Sequence Analysis Software (IBI, New Haven, Conn.). The coding region was examined for adherence to the codon usage bias of *P. aeruginosa* (39).

RESULTS

Cloning of PLC-N. Because PLC-H and PLC-N are both P_i-regulated PLC enzymes which have similar phospholipid substrate specificities, we proposed that their genes would also share sequence homology. A Southern hybridization was performed under low-stringency conditions (T_m −37°C) by using an internal 1.4-kilobase (kb) *Stu*I-*Pst*I probe from *plcS* (26) to test this hypothesis. Chromosomal DNA from strain PAO1 and the *plcSR* deletion mutant were tested for hybridization to this probe (Fig. 1). As expected, a 6.1-kb band from the PAO1 *Bam*HI genomic digestion hybridizes to the *plcS* probe (lane 3). Under low-stringency conditions a second band of about 12 kb is visible. This band also occurs in the *plcSR* mutant genomic sample (lane 6). Since this mutant is deleted for *plcS*, the structural gene for PLC-H, it was possible that *plcN*, the gene encoding PLC-N, was contained on this fragment. The hybridization data were used as a guide to clone *plcN*.

Further low-stringency hybridization studies identified an 8-kb *Eco*RI-*Bam*HI fragment in the *plcSR* mutant chromosome which hybridized to the *plcS* probe (data not shown). A 25-μg sample of the *plcSR* mutant chromosomal DNA was

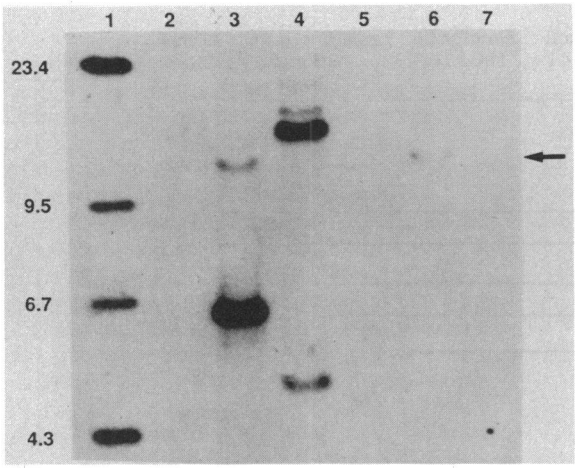

FIG. 1. Low-stringency Southern blot of chromosomal digests from PAO1 and PLC SR hybridized to the *Stu*I-*Pst*I *plcS* probe. Size standards are given in kilobases. Lanes: 1, lambda DNA, *Hin*dIII; 2, blank; 3, PAO1, *Bam*HI; 4, PAO1, *Xho*I; 5, blank; 6, PLC SR, *Bam*HI; 7, PLC SR *Xho*I.

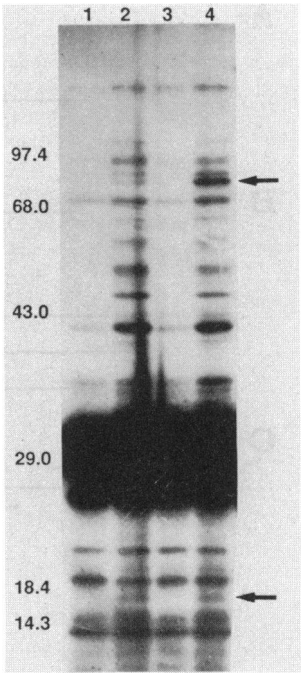

FIG. 2. Autoradiograph of SDS-PAGE of ^{35}S-amino-acid-labeled cell lysates, using the T7 RNA polymerase expression system to express *plcN*. Standards at the left are given in kilodaltons. Lanes: 1, pGEM1; 2, pGEM1/PLC-N; 3, pGEM2; 4, pGEM2/PLC-N. Arrows point out insert-specific proteins. The 29-kDa protein is β-lactamase.

double digested with *Bam*HI and *Eco*RI and subjected to electrophoresis through a 0.8% agarose gel, and three fractions of DNA in the area of 8 kb were sliced from the gel. The DNA was purified, and a small amount (1 μg) of each fraction was probed with the *plcS* fragment in a Southern hybridization. The hybridizing fraction was cloned into *Eco*RI-*Bam*HI double-digested pUC18 and pUC19 (40). Tightly regulated genes from *P. aeruginosa* are not usually transcribed from their own promoters in *E. coli*. Since the direction of transcription of *plcN* was unknown, both pUC18 and pUC19 were used to allow cloning of the *P. aeruginosa* fragments downstream of a vector promoter in both orientations.

Because a single band from the *plcSR* mutant hybridized to the *plcS* probe, it was likely that the entire *plcN* gene was contained on the cloned fragment. In addition, very little background PLC activity is present in *E. coli*. Therefore, the *E. coli* clones were screened for PLC activity first, rather than hybridization to the *plcS* probe. A total of 800 clones (400 for each vector) were tested for PLC activity by using the NPPC assay in microdilution dishes. Eleven clones were identified as PLC positive, and they all contained inserts cloned into pUC18. The inserts had identical restriction enzyme digestion patterns, and the same-sized fragment hybridized to the *plcS* probe (data not shown). The PLC activity of the *plcN* clone chosen for further studies was 1.46 U/ml of cells per A_{590}, which is 4.3-fold higher than the value for the vector control. *plcN* was subcloned within a 3-kb *Cla*I-*Bam*HI fragment into *Acc*I–*Bam*HI double-digested pGEM1 and pGEM2.

Molecular weight determination of PLC-N. The 3-kb *Cla*I-*Bam*HI *plcN* fragment was cloned into *Acc*I–*Bam*HI double-digested pGEM1 and pGEM2. This allows the expression of *plcN* directed by the T7 promoter of these vectors. pGEM2/PLC-N contains *plcN* cloned in the same orientation as the T7 promoter, whereas pGEM1/PLC-N contains *plcN* in the opposite orientation. The T7 RNA polymerase-directed expression system was used to induce high-level synthesis of PLC-N. [^{35}S]methionine- plus [^{35}S]cysteine-labeled plasmid-encoded proteins from cell lysates were examined by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). A 78-kDa protein is produced in an insert- and orientation-specific manner (lane 4). A smaller, 18-kDa, protein is insert specific but not orientation specific. The large, dark area of the gel at 29 kDa is the vector-encoded β-lactamase.

Two deletion subclones of the 3-kb *plcN* fragment which were missing either 1.1 or 1.4 kb at the 3′ end of the fragment were tested by using the T7 expression system. Cell lysates of the clones were subjected to SDS-PAGE, and truncated proteins of 44 and 35 kDa were produced, respectively (data not shown). The sizes of the truncated proteins are consistent with the identification of the 78-kDa protein as PLC-N. These and other *plcN* subclones were tested for PLC activity (Fig. 3B). None of the 3′-deleted clones retained PLC activity. Taken together, the size of the truncated proteins and the PLC activity data predict the start of *plcN* to be approximately 0.7 kb from the *Cla*I end of the clone and the 3′ end of *plcN* to be about 0.2 kb from the *Bam*HI end of the clone.

Substrate specificity assays of PLC-N and PLC-H. Some PLC enzymes use sphingomyelin as a substrate. These enzymes, including *Clostridium perfringens* α-toxin (20), are often hemolytic. Other PLCs which cannot cleave sphingomyelin, such as the *Bacillus cereus* PLC, are usually nonhemolytic (8). The PLC and sphingomyelinase activities of the pGEM2/PLC-N and pGEM2/PLC-H clones were measured by using the T7 RNA polymerase-directed expression system. The PLC activities of pGEM2/PLC-N and pGEM2/PLC-H were 108.3 and 166.7 U/ml of cells per A_{590}, respectively. PLC-H has sphingomyelinase activity (87.5 U/ml of cells per A_{590}) but PLC-N does not (<1.0 U/ml of cells per A_{590}). This observation also holds true in *P. aeruginosa*. Wild-type PAO1 culture supernatants contain sphingomyeli-

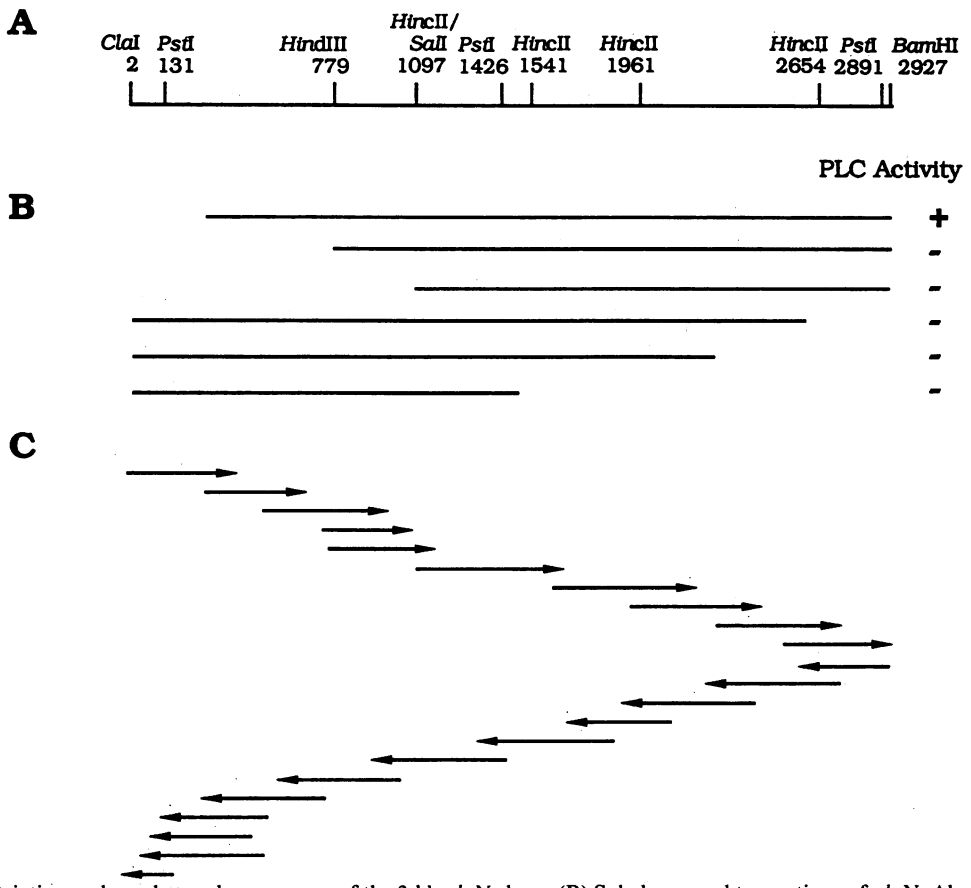

FIG. 3. (A) Restriction endonuclease cleavage map of the 3-kb *plcN* clone. (B) Subclones and truncations of *plcN*. Also shown is the PLC activity of each clone. (C) Sequencing strategy for *plcN*.

nase activity, but the *plcSR* deletion mutant supernatants do not (data not shown).

Phosphatidylserine is a constituent of eucaryotic membranes, and its hydrolysis by PLC-H and PLC-N was tested with samples produced in the T7 RNA polymerase system

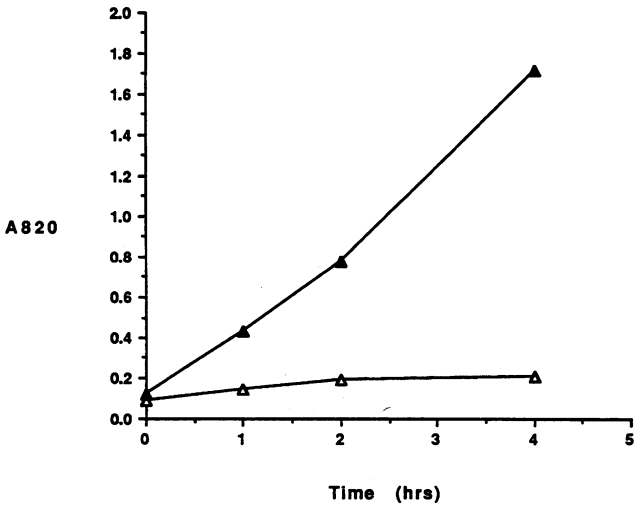

FIG. 4. Hydrolysis of phosphatidylserine by PLC-H (△) and PLC-N (▲).

(Fig. 4). Only PLC-N, but not PLC-H, was capable of cleaving phosphatidylserine.

Construction and analysis of PLC-N cointegrates. To conclusively demonstrate that a gene had been cloned which affected PLC production in *P. aeruginosa*, we constructed a gene interruption mutation in strain PAO1. Two *Hin*cII fragments of 444 and 420 base pairs from *plcN* were chosen for construction of the mutation. These fragments are arranged tandemly approximately 300 bp from the beginning of *plcN*, with the 444-bp fragment upstream of the 420-bp fragment (Fig. 3A). *Eco*RI linkers were ligated onto the blunt ends of these fragments, and they were each cloned individually into the *Eco*RI site of pSUP203, which results in inactivation of the Cm^r gene, and transformed into *E. coli* S17-1. The plasmids were transferred to *P. aeruginosa* PAO1 in mating experiments, and Tc^r recombinants were selected. Tc^r is vector encoded, and since these plasmids do not replicate in *P. aeruginosa*, the conversion to Tc^r is a result of homologous recombination between the chromosomal *plcN* locus and the small *plcN* fragment on the plasmid (Fig. 5). A single recombinational event results in cointegration of the entire plasmid into the chromosome, producing two incomplete copies of the *plcN* gene interrupted by vector sequences. The successful integration of these plasmids at the *plcN* locus was confirmed by Southern hybridization (data not shown). We are currently attempting to construct a strain with a mutation in both genes, but because of the high level of natural resistance of *P. aeruginosa* to many antibiotics, it has been difficult to identify an

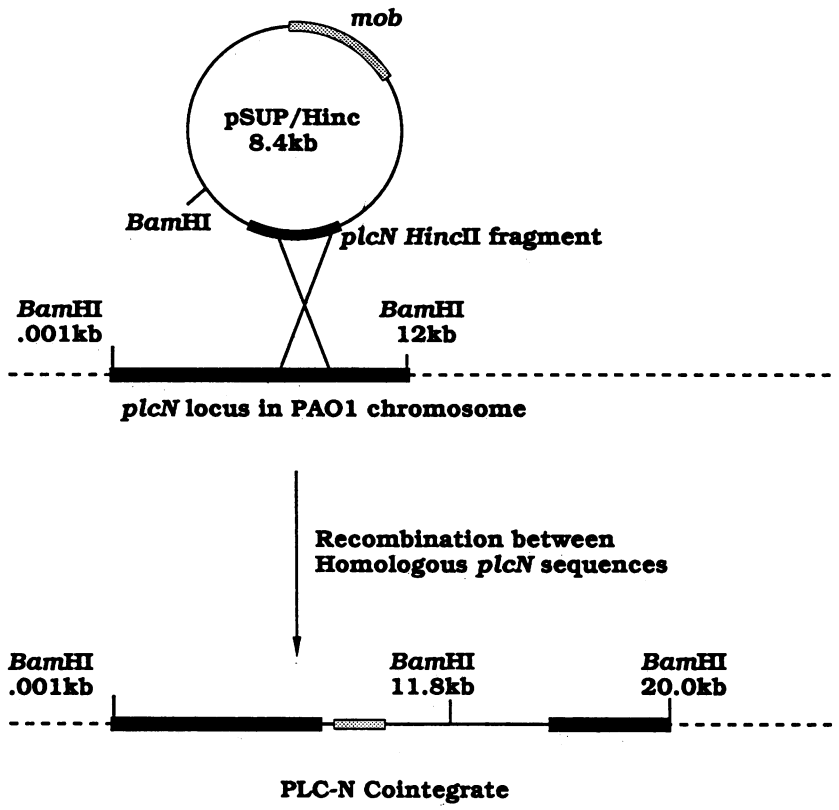

FIG. 5. Construction of the *plcN* cointegrate mutant strains.

antibiotic resistance marker other than Tc^r which we can use for selection of the mutant phenotype in this strain since production of PLC is not a selectable phenotype.

The PLC activity of the PLC-N cointegrates was measured (Table 2). As predicted above, the total PLC activity decreased by approximately 55% from that in PAO1 under low-P_i growth conditions. This observation is consistent with the conclusion that the gene encoding the second PLC, *plcN*, has been successfully mutated by using the cloned sequences. Therefore, the structural gene for PLC-N has been cloned.

The pattern of P_i regulation is also consistent with the inactivation of *plcN*. As illustrated by the *plcSR* mutant, the synthesis of PLC-N is tightly regulated by P_i (>30-fold repressed by 10 mM P_i). The PLC activity of the PLCN-C1 and PLCN-C2 cointegrates originates from PLC-H expression, which is not as tightly repressed by P_i (eightfold repressed by 10 mM P_i). The PLC-N cointegrates remain hemolytic, further confirming that the expression of PLC-H has not been altered.

TABLE 2. PLC activity produced by mutant and wild-type strains

Strain	PLC activity[a]		Relative % activity[b]	
	$+P_i$	$-P_i$	$+P_i$	$-P_i$
PLCN-C1	1.0	8.0	5.3	42.1
PLCN-C2	1.1	9.3	5.8	48.9
PLC SR	0.3	10.0	1.6	52.6
PAO1	2.0	19.0	10.5	100.0

[a] PLC activity is reported in units per milliliter of culture supernatant per A_{590}.

[b] Relative percent activity is the value of each mutant compared with that of PAO1 in low-P_i medium.

Sequence of *plcN*. Overlapping sequence data were obtained primarily by using synthetic oligonucleotide primers. The DNA sequence of the entire *Cla*I-*Bam*HI *plcN* cloned fragment was determined (Fig. 6). Both DNA strands were sequenced by using either single-stranded or double-stranded templates (Fig. 3C).

A 2,075-bp open reading frame was identified between positions 759 and 2834. This is large enough to encode a 77-kDa protein, which is in close agreement with the size predicted from the protein expression studies. The positions of the initiation and termination codons are consistent with those predicted by the subclone expression data. A potential Shine-Dalgarno ribosome-binding sequence (GAG) was identified 7 bp upstream of the ATG initiation codon.

The overall G+C content of the coding region is 67.3%, which is consistent with the predicted genomic G+C content of *P. aeruginosa* (67%). There is a 91-bp stretch of A+T-rich sequence (62.6% A+T) beginning 191 bp upstream of the initiation codon. The codon usage bias of *plcN* adheres very well to that predicted for *P. aeruginosa* (39) and results in a preference for C in the third position.

The predicted protein is very hydrophilic. A putative signal peptide was identified which contains the properties predicted for signal sequences (5, 37, 38). There is an 11-amino-acid charged NH_2-terminal region of *plcN* with a net charge of 4+, followed by a 17-amino-acid hydrophobic core region. A polar C-terminal region ends with the sequence Ala-Leu-Ala 8 amino acids after the hydrophobic core. Signal peptidase cleavage is inferred to occur at this position. Alanine is very abundant in procaryotic signal

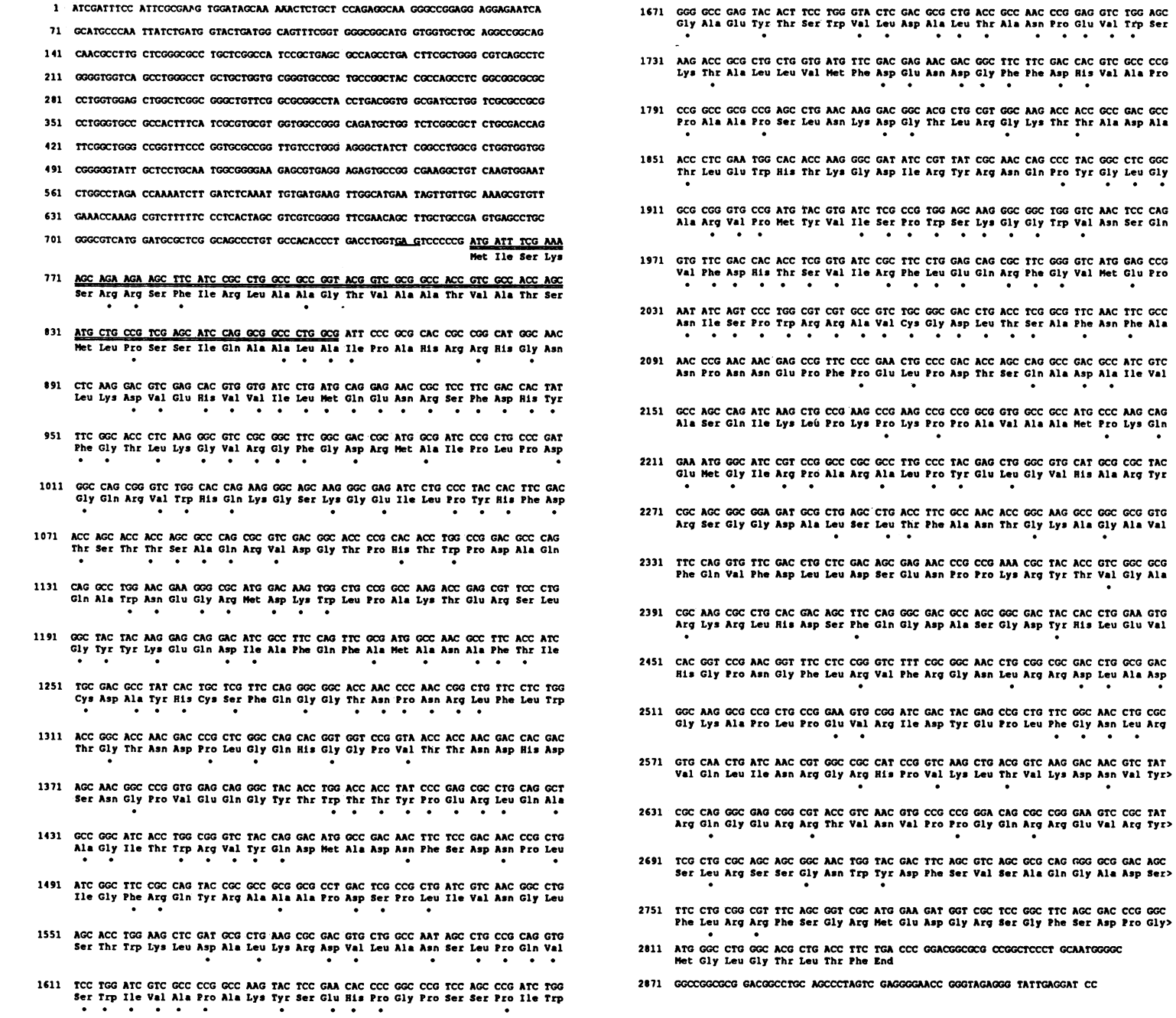

FIG. 6. Nucleotide and amino acid sequence of the *Cla*I-*Bam*HI *plcN* cloned fragment. The predicted Shine-Dalgarno ribosome-binding site is underlined. The predicted signal peptide is double underlined. Dots indicate identical amino acids in PLC-H.

TABLE 3. Comparison of mature PLC proteins[a]

Amino acid	No. of residues in:	
	PLC-N	PLC-H
Ala	53	54
Val	41	47
Leu	50	57
Ile	20	18
Pro	50	44
Met	12	10
Phe	32	24
Trp	17	23
Gly	59	59
Ser	40	45
Thr	36	32
Cys	3	6
Tyr	22	39
Asn	32	34
Gln	27	34
Asp	44	44
Glu	26	38
Lys	25	19
Arg	49	47
His	19	18
Total	657	692

[a] All values are predicted from the DNA sequence data. Molecular weights are 73,455 and 78,352 for PLC-N and PLC-H, respectively. Predicted pI values are 8.8 and 5.5 for PLC-N and PLC-H, respectively.

sequences (38), and the PLC-N signal sequence contains eight alanine residues. The entire proposal signal peptide is 35 amino acids, which is long in comparison with other procaryotic signal sequences but close to PLC-H in length (38 amino acids) (26). It is also interesting that both putative signal sequences contain the amino acid phenylalanine. This amino acid is not usually found in procaryotic signal sequences, although pilin, which is the subunit of some bacterial pili, contains an *N*-methylphenylalanine at the +1 position.

Comparison of PLC-N and PLC-H. The nucleotide sequence of *plcN* is 58.7% homologous to that of *plcS*. The amino acid sequences of PLC-N and PLC-H are also quite homologous (Fig. 6). The overall amino acid homology is 40% identical. The homology is greatest at the NH_2 ends of the two proteins, in which the first two-thirds of the proteins are 47% identical. There are several short stretches of perfect identity in this area. The last one-third of the proteins share only 23% identity.

Several properties of PLC-N are similar to those of PLC-H (Table 3). The molecular weights of the two proteins are very close, and the predicted amino acid compositions are extraordinarily similar. However, the predicted pIs of the two proteins are quite different (Table 3, footnote *a*). PLC-H is an acidic protein (pI 5.5), whereas PLC-N is basic (pI 8.8). The pI difference can be accounted for by the smaller number of glutamic acid residues and the larger number of lysine residues in PLC-N in comparison with PLC-H. The mobility of PLC-H and PLC-N on nondenaturing polyacrylamide gels (pH 7.5) is consistent with their predicted pIs (data not shown). PLC-N fails to enter these gels, either because it is hydrophobic or aggregated or because the positive charge of the molecule prohibits its migration toward the cathode. The latter is consistent with a basic pI. PLC-H has a predicted pI of 5.5, which is reflected in its efficient migration in nondenaturing gels.

DISCUSSION

These studies describe the cloning and characterization of the gene encoding a 77-kDa nonhemolytic secreted PLC from *P. aeruginosa*, PLC-N. The cross-hybridization of *plcN* to a *plcS* probe was a convenient tool for cloning *plcN*. Like the hemolytic PLC, PLC-H, expression of PLC-N is P_i regulated and secreted.

Since *plcS* is deleted in the strain used for cloning *plcN*, the cloned PLC activity originates from *plcN*. Internal fragments from the cloned DNA were used to generate gene interruption mutants which produced 55% less total PLC activity than the wild-type strain. The hybridization data, the PLC activity expressed in *E. coli*, and the decrease in PLC activity of the gene interruption mutant all support the conclusion that *plcN* was successfully cloned.

In contrast to the many similarities of PLC-N to PLC-H, PLC-N is nonhemolytic. Although both enzymes hydrolyze phosphatidylcholine, PLC-N does so only 30 to 50% as efficiently as PLC-H. Perhaps PLC-N is nonhemolytic because it 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 *B. cereus* is nonhemolytic to intact erythrocytes (22). However, it can hydrolyze a broad spectrum of phospholipids in in vitro assays in which the phospholipids are detergent solubilized (22).

The cereolysin of *B. cereus* is hemolytic and is a combination of PLC and a sphingomyelinase (8). PLC-H possesses both PLC and sphingomyelinase activity. Similarly, the hemolytic *C. perfringens* α-toxin is both a PLC and a sphingomyelinase (33). Neither PLC-N nor the *B. cereus* PLC are sphingomyelinases, nor are they hemolytic. These examples suggest that the hemolytic activity of a PLC is dependent upon the combined ability to hydrolyze phospholipids such as phosphatidylcholine and sphingomyelin.

Phosphatidylserine is the only major constituent of eucaryotic membranes which carries a net negative charge. The basic (pI 8.8) nature of PLC-N may contribute to its ability to hydrolyze phosphatidylserine. PLC-H is acidic (pI 5.5) and does not hydrolyze phosphatidylserine as it does other phospholipid substrates, such as phosphatidylcholine and sphingomyelin. Phosphatidylserine is found primarily in the inner leaflet of the erythrocyte membrane and therefore is unavailable for hydrolysis as long as the outer leaflet remains intact. PLC-N may be nonhemolytic because the phospholipid components of the outer leaflet of the erythrocyte membrane (phosphatidylcholine and sphingomyelin) are not hydrolyzed efficiently by PLC-N. In contrast, the phospholipid substrates hydrolyzed by PLC-H are major constituents of the outer leaflet. This difference could explain the hemolytic nature of PLC-H and the nonhemolytic property of PLC-N. In this regard it should be mentioned that just because PLC-H has sphingomyelinase activity and activity against phosphatidylcholine, these activities do not fully account for the hemolytic activity of PLC-H. We have recently found that one or both of the products of the *plcR* genes (29) posttranslationally modifies PLC-H. Modified PLC-H is more hemolytic and migrates faster in nondenaturing PAGE than the unmodified version. In denaturing PAGE (SDS-PAGE) there is no detectable difference in their migration. Although the precise nature of the modification is not known, it is clear that it is necessary for the full hemolytic activity of PLC-H (36; M. L. Vasil, R. M. Ostroff, and A. I. Vasil, unpublished observations).

Both of the PLC enzymes are synthesized maximally by

P. aeruginosa under low-P_i growth conditions. Other proteins, such as alkaline phosphatase and P_i transport proteins, are similarly regulated (10). Perhaps the varied substrate specificities of the two PLCs allow for more efficient degradation of eucaryotic membrane phospholipids in order to acquire P_i. The two enzymes could work sequentially and synergistically. PLC-H would begin degradation of the erythrocyte membrane, exposing the inner leaflet. PLC-N could then hydrolyze phosphatidylserine. The products of phospholipid hydrolysis (e.g., phosphorylcholine) would be digested by alkaline phosphatase to release P_i.

The DNA sequence of *plcN* was determined. The 2,075-bp open reading frame is large enough to encode a protein of 77 kDa, the predicted size of PLC-N. No consensus procaryotic promoter was found upstream of *plcN*, which is not unusual for *P. aeruginosa* genes. The fact that *plcN* is not expressed in *E. coli* from its own promoter is also consistent with there being no consensus promoter. There are sequences upstream of *plcN* which may be analogous to the consensus *pho* box found upstream of *E. coli* genes which are regulated by P_i, but assignment of any function to these sequences must await further analysis. The promoter region of *plcN* may be contained within the unusually A+T-rich area 191 bp upstream of *plcN*. An A+T-rich stretch also occurs upstream of the exotoxin A gene and is proposed to facilitate the binding of RNA polymerase as a result of the lower energy required to denature the DNA in this area. A conserved dodecamer sequence is found in the promoter region of exotoxin A and *plcS* (9). No similarity to this dodecamer was found upstream of *plcN*.

There are several similarities between PLC-N and PLC-H. The proteins have similar sizes, are secreted, and are produced maximally in low-P_i medium during the same growth phase of the culture. Both enzymes hydrolyze phospholipids with quaternary ammonium groups, such as phosphatidylcholine, which is abundant in the eucaryotic cell membrane (1), and both have little activity toward phosphatidylethanolamine, a phospholipid found primarily in the procaryotic membrane (1).

The hybridization data predicted homology between the two proteins. The *plcS* probe used for the cloning of *plcN* is homologous to *plcN* in the region from 1263 to 2610. This area spans several regions of identity between PLC-N and PLC-H, including a stretch of 64 amino acids, encoded within nucleotides 1896 to 2097, which is 75% identical. The DNA from this region is 55% homologous.

The total nucleic acid homology between *plcN* and *plcS* is 58.7%. The entire amino acid sequence of PLC-N is 40% identical to PLC-H. There is also a striking parallel between the frequency of occurrence of amino acids. The homology is greatest in the first two-thirds to the proteins, which are 47% identical. The regions of strongest homology between the two proteins contain an equal mix of hydrophobic and hydrophilic amino acids.

The high degree of homology between *plcN* and *plcS* suggests a gene duplication event with subsequent mutagenic drift. Mutations could occur independently in duplicated genes, resulting in two very different enzymes that evolved from a common ancestor. For example, a mutated form of the aliphatic amidase of *P. aeruginosa* has a substitution of an isoleucine for a threonine residue, which broadens the substrate specificity of the enzyme (27), allowing utilization of a substrate not available to the parent enzyme. This process may result in an increase in metabolic diversity, allowing growth in novel environments. It should be noted however, that *plcN* and *plcS* are not the result of a relatively simple tandem duplication, because we have found by using transverse pulsed-field gel electrophoresis and genetic linkage studies that these genes are considerably distal to each other, on opposite sides (i.e., ca. 180° on a circular chromosome) of the *P. aeruginosa* chromosome (17; V. Shortridge, R. Fick, M. Pato, and M. Vasil, submitted for publication). *plcN* is located at approximately 34 min and *plcH* is located at approximately 67 min on the 75-min linkage map (23).

Some structure-function relationships can be inferred from the homology data. The majority of the homology lies within the first two-thirds of the proteins. Six strongly conserved areas occur within this region. These domains may be critical for PLC activity, either as active sites or by conferring a particular conformation to the proteins. Perhaps the hemolytic and substrate specificity domain of PLC-H is contained in the COOH terminus of the protein. Little homology exists between other genes and *plcN*, based on a GenBank search.

Further comparisons of PLC-N with PLC-H can be made in the future by using the sequence information. The homology data can be used to target specific sequences for site-directed mutagenesis in both PLC-N and PLC-H. These experiments may further define structure-function relationships between PLC activity, substrate specificity, and hemolytic activity.

ACKNOWLEDGMENTS

We thank Randy Berka for generously supplying oligonucleotide probes and Ron Gill and Arthur Pritchard for helpful discussions. We also thank Peter Neifert for excellent technical assistance.

This research was supported by Public Health Service grant AI15940 from the National Institute of Allergy and Infectious Diseases to M.L.V.

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