PlcR1 and PlcR2 Are Putative Calcium-Binding Proteins Required for Secretion of the Hemolytic Phospholipase C of *Pseudomonas aeruginosa*

ADELA COTA-GOMEZ,¹ ADRIANA I. VASIL,¹ JAGATH KADURUGAMUWA,² TERRANCE J. BEVERIDGE,²
HERBERT P. SCHWEIZER,³ AND MICHAEL L. VASIL^{1*}

Department of Microbiology, University of Colorado Health Sciences Center, Denver,¹ and Department of Microbiology, Colorado State University, Fort Collins,³ Colorado, and Department of Microbiology, Center of Canadian Bacterial Diseases, College of Biological Sciences, University of Guelph, Ontario, Canada²

Received 21 January 1997/Returned for modification 12 March 1997/Accepted 18 April 1997

The *plcHR* operon of *Pseudomonas aeruginosa* includes the structural gene for the hemolytic phospholipase C, *plcH* (previously known as *plcS*), and two overlapping, in-phase, genes designated *plcR1* and *plcR2*. Hemolytic and phospholipase C (PLC) activities produced by *Escherichia coli* and *P. aeruginosa* T7 expression systems were measured in strains carrying both *plcH* and *plcR* genes and in strains carrying each gene separately. When *plcH* was expressed by itself in the *E. coli* T7 system, the area of the hemolytic zone on blood agar was less than twice the area of growth. By contrast, when *plcR* was coexpressed with *plcH* in this system, the area of the hemolytic zone was approximately 10 times that of the area of the growth on blood agar. Native polyacrylamide gel electrophoretic analyses of PlcH activity expressed in either the *E. coli* or the *P. aeruginosa* T7 system carrying *plcH* alone, or along with the *plcR* genes, suggest that PlcR either posttranslationally alters the physical or biochemical nature of PlcH or releases PlcH from a complex in the cell so that it can be secreted. The hypothesis that PlcR is involved in the secretion of PlcH is supported by the observation that the ratio of extracellular to cell-associated PlcH activity produced by *P. aeruginosa* strains containing an in-frame deletion in the chromosomal *plcR* genes is significantly reduced in comparison with this ratio seen with the wild-type parental strain. This defect in the secretion of PlcH can be complemented by the *plcR* genes in *trans*. Additional data suggest that PlcR does not directly affect the secretion of the nonhemolytic phospholipase C (PlcN). PlcR is highly similar to a calcium-binding protein (CAB) from *Streptomyces erythraeus*. PlcR and CAB contain typical motifs (EF hands) characteristic of eucaryotic calcium-binding proteins, including calmodulin. *P. aeruginosa* naturally produces membrane vesicles (MVs) containing extracellular proteins including PLC. MVs from the PAO1WT strain contained at least 10-fold more PLC specific activity than those isolated from a strain carrying a deletion of *plcR* (PAO1ΔR). Immunogold electron microscopy of PAO1WT and PAO1ΔR whole cells revealed a distribution of PlcH in these strains consistent with the hypothesis that PlcR is required for the secretion of PlcH.

Pseudomonas aeruginosa is a gram-negative pathogen capable of causing great morbidity and mortality in patients such as burn victims, those with cystic fibrosis, and immunocompromised patients. It produces and secretes a number of extracellular virulence factors including exotoxins (13, 14, 35), several distinct proteolytic activities (9, 16, 17), hemolysins (18), and phospholipases (22, 24). Of the two hemolysins produced and secreted one is a heat-stable glycolipid (32) and the other is a heat-labile phospholipase C (PLC) known as the hemolytic phospholipase C (PlcH). A second PLC was designated the nonhemolytic PLC (PlcN) because it does not have any hemolytic activity on any erythrocyte thus far tested (22). Nevertheless, PlcH and PlcN are highly homologous (>40% identity), and both are capable of cleaving phosphatidylcholine, which is present in the outer leaflet of mammalian erythrocytes (6). Presently, it is not clear why PlcH is hemolytic and PlcN is not, but it is known that only PlcH cleaves sphingomyelin, the other major phospholipid in the outer leaflet of human erythrocytes. PlcN, but not PlcH, acts on phosphatidylserine (22), which is

located only in the inner leaflet of the phospholipid bilayer in human erythrocytes. PlcH and PlcN will hydrolyze the synthetic substrate *p*-nitrophenylphosphorylcholine (NPPC), which was used in several of the experiments described in this report.

Both PlcH and PlcN have relatively long, somewhat unusually highly charged (6⁺ and 4⁺, respectively) signal sequences that are cleaved from the mature PlcH or PlcN when they are secreted to the culture supernatant.

The structural gene for the hemolytic phospholipase C, *plcH*, is part of an operon that includes two other genes, *plcR1* and *plcR2* (26, 30). The *plcR* genes are located 3' to *plcH*, and although they overlap, they are in the same reading frame. The protein encoded by *plcR1* (207 amino acids) is slightly larger than that encoded by *plcR2* (151 amino acids). PlcR1 is synthesized with a signal sequence, while PlcR2, which starts at an initiation codon internal to *plcR1*, lacks a signal sequence (30). Shen et al. demonstrated that when the *plcR* genes are expressed in *Escherichia coli*, PlcR1 is located in the periplasm while PlcR2 remains in the cytoplasm (30). The predicted mature PlcR1 (without its signal sequence) is larger than PlcR2 by 36 amino acids, 10 of which are prolines and 15 of which are other hydrophobic amino acids (i.e., Ala, Val, Phe, and Ile). There are no charged amino acids in this 36-amino-acid region. PlcR2 is completely identical to PlcR1 except for the 20-amino-acid signal sequence of PlcR1 and the 36 amino

* Corresponding author. Mailing address: Department of Microbiology, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Box B175, Denver, CO 80262. Phone: (303) 315-8627. Fax: (303) 315-3112. E-mail: Mike.Vasil@uchsc.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
<i>E. coli</i> BL21(DE3)	<i>hsdS gal ompT</i> r^{-} m^{-} b^{-} (λ CI857 <i>ind1</i> Sam7 <i>nin5 lacUV5</i> T7 gene 1)	34
<i>P. aeruginosa</i>		
PAO1	Prototroph, <i>chl-2</i>	12
PAO1-ADD1976	Prototroph, <i>chl-2</i> , Tc ^r	4
PAO1ΔR	Prototroph, <i>chl-2</i> , Δ <i>plcR::tet</i>	This study
PAO1ΔR2-1C	Prototroph, <i>chl-2</i> , Δ <i>plcR-186</i>	This study
Plasmids		
<i>E. coli</i>		
pGEM2	Ap ^r	Promega Bio- tech Co.
pGEM2:: <i>plcHR</i>	Ap ^r , 6.1-kb <i>Bam</i> HI- <i>Bam</i> HI <i>plcHR</i>	This study
pGEM2:: <i>plcH</i>	Ap ^r , 3.6-kb <i>Bam</i> HI- <i>Bgl</i> II <i>plcH</i>	This study
pGEM2:: <i>plcR</i>	Ap ^r , 2.5-kb <i>Pst</i> I- <i>Bam</i> HI <i>plcR</i>	This study
<i>P. aeruginosa</i>		
pADD3268	Broad host range, Cb ^r	4
pUCP19	Broad host range, Cb ^r	37
pACG1	Cb ^r , pADD3268:: <i>plcHR</i>	This study
pACG2	Cb ^r , pADD3268:: <i>plcH</i>	This study
pUCR	Cb ^r , pUCP19:: <i>plcR</i>	This study

acids mentioned above. For purposes of clarity, and because they are not expressed separately in these previous studies, in this report *plcR1* and *plcR2* are frequently designated the *plcR* gene and their products as PlcR.

Preliminary experiments relating to *plcR* implicated these genes in the regulation of *plcH* expression; however, other more definitive data suggested a posttranslational role for PlcR. In this report we describe data strongly supporting this latter hypothesis and indicate that the *plcR* genes encode proteins which are specifically involved in the secretion of PlcH, but not PlcN, from *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals. Sphingomyelin, NPPC, and choline were obtained from Sigma Chemical Co., St. Louis, Mo. Radiolabeled sphingomyelin was obtained from Dupont, NEN Research Products, Boston, Mass. Isopropyl β-D-thiogalactopyranoside (IPTG), all restriction enzymes, and T4 DNA ligase were obtained from Bethesda Research Laboratories, Bethesda, Md. Acrylamide for all native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses was obtained from National Diagnostics, Atlanta, Ga.

Bacterial strains and plasmids. The *E. coli* strain used for overexpression of the *plcHR* genes was BL21(DE3) (34). This strain was transformed by the classical method by using calcium chloride (19) with pGEM2 plasmids containing the different *plcHR* genes 5' to the T7 promoter. All *P. aeruginosa* strains were derived from the prototrophic strain PAO1 (12). For overexpression of the *plcHR* genes, the PAO1 derivative ADD1976 was used, which is identical to PAO1 except that the T7 polymerase gene under the control of the *lacUV5* promoter was integrated into the chromosome of ADD1976 (4). T7 expression studies in this strain were performed by using pADD3268, a plasmid that contains a multiple cloning site (MCS) 3' to the T7 promoter and several T7 transcriptional terminators that are located 3' to the MCS reported by Brunschwig and Darzins (4). The PAO1ΔR mutant was constructed from a wild-type PAO1 parent by interruption of the gene via insertion of a 2.8-kb DNA fragment containing a *tet* gene (*tet* cassette) at the *Bgl*II sites between bases 3624 and 3810. A second PAO1ΔR mutant, PAO1ΔR2-1C, was constructed by deletion of the 186-bp *Bgl*II fragment between bases 3624 and 3810, but no *tet* cassette was inserted at this site as was done in PAO1ΔR. Selection of this mutant was accomplished by using the sucrose sensitivity phenotype of strains carrying the *sacB* gene on the pMOB plasmid designed by Schweizer (29). This mutant therefore carries an in-frame deletion of both *plcR* genes. All bacterial strains and plasmids used in this study are outlined in Table 1.

Culture conditions. Bacto-agarose and brain heart infusion (BHI) media (Difco Laboratories, Detroit, Mich.) were used for the growth of all cultures with

the exception of the cultures for the induction of chromosomal expression of PlcH in *P. aeruginosa*. These cultures were grown in HEPES minimal medium supplemented with 20 mM PO₄ and 0.2% choline at 32°C for 8 h (31). These conditions induce expression of PlcH and repress PlcN expression (8, 30). Antibiotics for *E. coli* were used in the following concentrations: 100 μg of ampicillin/ml, 40 μg of kanamycin/ml, 15 μg of gentamicin/ml, 200 μg of rifampin/ml, and 25 μg of chloramphenicol/ml. Antibiotics for *P. aeruginosa* were used in the following concentrations: 750 μg of carbenicillin/ml, 100 μg of tetracycline/ml, and 75 μg of gentamicin/ml.

Cloning and overexpression of *plcHR* genes. The genes of the *plcHR* operon of *P. aeruginosa* were cloned separately into the MCS of the pGEM2 plasmid directly 3' of the T7 promoter. A *P. aeruginosa* 6.1-kb *Bam*HI fragment containing the entire *plcHR* operon (35) was cloned into the *Bam*HI site of the pGEM2 plasmid. The *plcH* gene alone was isolated on a *Bam*HI-*Bgl*II DNA fragment from a plasmid carrying the 6.1-kb *Bam*HI fragment. This 3.6-kb fragment which contains only *plcH* was ligated into *Bam*HI-*Bgl*II-digested pGEM2. Finally, the *plcR* genes alone were cloned into the MCS of pGEM2 after the 6.1-kb *Bam*HI fragment of *plcHR* and the pGEM2 vector were digested with *Pst*I and *Bam*HI. The resulting plasmids were transformed into the BL21(DE3) *E. coli* strain that carries the T7 polymerase gene in the chromosome under the control of the IPTG-inducible *lacUV5* promoter. All digestions and ligations were done according to the instructions of the manufacturers. For expression, logarithmically growing cells at an *A*₅₉₀ of 0.5 to 0.7 were induced with 1 mM IPTG for 2 to 4 h, and chromosomal transcription was inhibited with 200 μg of rifampin/ml. Cells were collected by centrifugation, resuspended in a 1/10 volume of Tris-EDTA buffer at pH 8.0, and lysed by sonication. The cell lysates were diluted 1:10, and these dilutions were assayed for PLC activity. For overexpression in *P. aeruginosa* the genes were cloned in the same manner into pADD3268 (developed by Brunschwig and Darzins [4]) and transformed into PAO1 ADD1976 (4), which contains the T7 promoter in the chromosome under the control of the IPTG-inducible *lacUV5* promoter. With this *P. aeruginosa* system, culture supernatants were also collected after centrifugation of the cells in order to compare secreted versus cell-associated PLC activity.

Extracellular and cell-associated PlcH in PAO1ΔR mutants. In the PAO1ΔR mutants PlcH activity was assayed as described below after specific induction for expression of the *plcH* gene product. These experiments were done with HEPES minimal medium. Cells were grown overnight in HEPES supplemented with 20 mM phosphate by incubation at 37°C with the appropriate antibiotic when needed. These cultures were diluted to an *A*₅₉₀ of 0.1 in HEPES medium supplemented with 20 mM phosphate and 0.2% choline (no antibiotics were used at this point) and were grown at 32°C with vigorous shaking. These conditions induce expression of PlcH but not PlcN. Samples were harvested at 8 h, and the cell supernatants were collected after centrifugation. The *A*₅₉₀ of the cultures was recorded at the 8-h time point. The cell pellets were washed with 1× phosphate-buffered saline and centrifuged. The cells were resuspended in 1 ml of TE buffer containing 50 mM Tris and 2 mM EDTA at pH 8.0. The resuspended cells were lysed by sonication on ice.

Hemolysis assay. Analysis of the hemolytic activity of *E. coli* BL21(DE3) with the various constructs was performed by spotting the cells on tryptic soy agar plates containing 5% sheep blood. The plates were incubated overnight at 37°C, and the sizes of the zones of hemolysis were compared.

PLC assays. PLC activity was measured by two separate assays. The chromogenic synthetic substrate NPPC was used as previously described (3, 8). The cleavage of NPPC by PLC releases phosphorylcholine and the chromogenic p-nitrophenol, which later was measured spectrophotometrically at a wavelength of 410 nm (3). Hydrolysis of SM was used to specifically detect PlcH activity because PlcN does not hydrolyze this substrate. This assay is a modification of a procedure described by Goldfine et al. (7) which uses sphingomyelin containing [¹⁴C]choline. Briefly, one mixed micelle substrate solution was made per assay with both labeled and unlabeled lipids by mixing 288 μg of unlabeled sphingomyelin with 0.25 μCi of radiolabeled sphingomyelin. These lipids were dried with nitrogen and resuspended in 100 μl of a buffer containing 100 mM morpholinepropanesulfonic acid-morpholineethanesulfonic acid and 1.84% Triton X-100 at pH 7.4. The resuspended lipids were sonicated for 1 min in a bath and vortexed briefly three times to ensure complete dissolution. The 100-μl substrate solution was added to a prewarmed (37°C) assay tube (12 by 75 mm) and then mixed with 100 μl of sample. The substrate and enzyme were incubated at 37°C for 2 h. The reaction was stopped by the addition of 800 μl of chloroform-methanol (1:1) two times and the addition of 800 μl of 0.1 N HCl once. The mixture was vortexed briefly to create an emulsion, and the released labeled phosphorylcholine was extracted by centrifugation at 1,200 rpm in a Beckman GSA15 centrifuge for 7 min at 4°C. A 600-μl aliquot of the upper aqueous phase was transferred to a scintillation vial, and 5 ml of Ecocint scintillation fluid was added. The counts per minute were measured on a Beckman LS-7000 scintillation counter. The activity was expressed as counts per minute/*A*₅₉₀. For detection of the PLC activity in the overexpression experiments, samples of the cell lysates and culture supernatants were loaded onto native PAGE (N-PAGE) gels and electrophoresed overnight at 100 V as described previously. The gels were then reacted with NPPC for 30 to 60 min at 37°C and photographed. This method only detects PlcH activity in extracts or supernatants because PlcN does not migrate into the gel with this buffer system.

FIG. 1. Blood agar plate demonstrating the hemolytic activity of *plcH* and *plcR* expressed together and separately from the T7 promoter in *E. coli* BL21(DE3). *E. coli* BL21(DE3) pGEM2 (A), *E. coli* BL21(DE3) pGEM2::*plcR* (B), *E. coli* BL21(DE3) pGEM2::*plcH* (C), and *E. coli* BL21(DE3) pGEM2::*plcHR* (D) are shown. IPTG (final concentration, 1 mM) was added to the blood agar plates to induce expression of the *plcHR* genes under the control of the T7 promoter.

Radiolabeling and SDS-PAGE analysis. The protein products of the overexpression from *E. coli* cells were radiolabeled in vivo and resolved by SDS-PAGE on 5% acrylamide gels. Briefly, expression was induced by addition of 1 mM IPTG to shaking cultures growing in mid-log phase at 37°C. Chromosomal expression was interrupted by using 200 µg of rifampin/ml. Cells were then allowed to grow at 37°C with shaking for 20 min before addition of 5 µCi of ¹⁴C-labeled L-amino acid mixture (Dupont, NEN Research Products). Cells were labeled for 1 h at 37°C with continuous shaking. After 1 h of labeling, the cells were harvested by centrifugation, and the pellets were resuspended in 50 µl of sample running buffer which included β-mercaptoethanol. Samples were resolved on an SDS-5% PAGE gel. The gel was dried and subjected to autoradiography as described previously (23).

Isolation and quantification of MVs. The *P. aeruginosa* strains tested for PLC activity in the membrane vesicles (MV) were grown in Trypticase soy broth to the early stationary growth phase (ca. 10⁸ CFU/ml) on an orbital shaker at 37°C with an agitation rate of 125 rpm. To support the production of PLC, 0.2% (wt/vol) choline ([2-hydroxyl methyl] trimethyl ammonium chloride salt; Sigma Chemical Co.) was added to the culture medium (31). MVs were recovered from broth cultures as described previously by Kadurugamuwa and Beveridge (15).

Immunogold electron microscopy. Immunogold electron microscopy was done essentially as described by Kadurugamuwa and Beveridge (15). Briefly, cells were embedded in 2% molten Noble agar, mildly fixed with LR white embedding regimen (London Resins), and indirectly labeled with protein A-gold and rabbit antibodies against purified native PlcH from *P. aeruginosa*.

RNAse protection assays. RNAse protection assays to detect specific *plcH* transcripts were performed exactly as previously described (28). Total RNA was isolated from log-phase cultures of bacteria grown in HEPES medium with the addition of 0.2% choline by the acid lysis-hot phenol method and treated with DNase I. Ten micrograms of total RNA was hybridized with ³²P-labeled runoff transcript prepared from *Xba*I-digested pGEM-7Z::*plcH* by using the Gemini II core runoff transcript kit of Promega and T7 polymerase as recommended by the manufacturer. Hybridization mixtures were then digested with RNases A and T1, and the protected RNA fragment was purified and resolved by PAGE.

RESULTS

Expression of *plcH* and *plcR* in an *E. coli* T7 expression system. During the initial characterization of the *plcHR* operon it was noted that when *plcH* was expressed from the Tac promoter in *E. coli*, without *plcR*, the area of the hemolytic zone expressed was considerably smaller than the area of the hemolytic zone expressed by *E. coli* carrying both *plcH* and *plcR* expressed from the same promoter. In order to obviate any

issues regarding differential expression of *plcH* with or without *plcR*, these genes were cloned together and separately in pGEM plasmids so that their expression would be directed by the strong T7 promoter. The plasmids were transformed into *E. coli* BL21(DE3), which carries the T7 polymerase gene under the control of the *lacUV5* promoter in the chromosome. As seen in Fig. 1, when *plcH* and *plcR* on the same plasmid are expressed in *E. coli* BL21(DE3) under the control of the T7 promoter, there is a zone of hemolysis on sheep or human blood agar which extends considerably beyond the area of the growth. In contrast, when the *plcH* gene alone is expressed from the same promoter in *E. coli*, the zone of hemolysis extends just beyond the area of growth. *E. coli* BL21(DE3) carrying the pGEM vector alone or *plcR* alone does not show any evidence of hemolytic activity, even under the area of growth (Fig. 1). These data could indicate that either *E. coli* BL21(DE3) pGEM::*plcH* expressed less PlcH or it was not able to secrete or release PlcH like the strain [BL21(DE3) pGEM2::*plcH*] expressing both *plcH* and *plcR*. It seemed less likely that the *E. coli* BL21(DE3) strain, which only carries the *plcH* gene, produces less PlcH because, in all cases, expression of *plcH* and *plcR* was directed by the T7 promoter. Curiously, when broth cultures of the strains shown in Fig. 1 were examined for PLC expression, only cell-associated PLC or hemolytic activity was detected in strains carrying the *plcH* gene alone or *plcH* and *plcR* together. No detectable PLC or hemolytic activity was present even in concentrated culture supernatants of strains carrying both the *plcH* and *plcR* genes (data not shown). Quantitative analysis of cell-associated PLC activity in *E. coli* BL21(DE3) expressing either *plcH* alone or *plcH* and *plcR* revealed that both strains expressed essentially the same level of PLC-specific activity (23.1 U/mg of protein for *E. coli* BL21(DE3) pGEM2::*plcH* versus 24.1 U/mg of protein for *E. coli* BL21(DE3) pGEM2::*plcHR*). Moreover, as seen in Fig. 2B, when *plcH* was expressed in *E. coli* BL21(DE3) with and with-

FIG. 2. (A) Detection of PLC activity in N-PAGE gel by NPCC. Extracts of *E. coli* BL21(DE3) expressing *plcH* and *plcR*, together and separately, were separated on an N-PAGE gel, and the gel was reacted with NPCC, the synthetic substrate for PLC activity. Lane 1, 100 µl of *E. coli* BL21(DE3) pGEM2::*plcHR* extract; lane 2, 10 µl of *E. coli* BL21(DE3) pGEM2::*plcHR* extract; lane 3, 50 µl of *E. coli* BL21(DE3) pGEM2::*plcH* extract mixed for 15 min at 37°C with 50 µl of *E. coli* BL21(DE3) pGEM2::*plcR* extract after cells were lysed separately; lane 4, 100 µl of *E. coli* BL21(DE3) pGEM2::*plcH* extract; lane 5, 100 µl of *E. coli* BL21(DE3) pGEM2::*plcR* extract. (B) Autoradiography of SDS-5% PAGE of ¹⁴C-labeled PlcH expressed in *E. coli* BL21(DE3) pGEM2::*plcHR* (lane 1) or in *E. coli* BL21(DE3) pGEM2::*plcH* (lane 2). The PlcR1 and PlcR2 proteins are not seen in lane 1 because they were run off of this gel. They have been shown in a previous study (30).

FIG. 3. Detection of PLC activity in an N-PAGE gel by NPCC. Whole-cell extracts or culture supernatants of *P. aeruginosa* ADD1976 expressing *plcH* and *plcR* from the T7 promoter in plasmid pADD3268, together and separately, were separated on an N-PAGE gel, and the gel was reacted with NPCC, the synthetic substrate for PLC activity. The same restriction fragments as in the *E. coli* T7 system were cloned into the pADD3268 plasmid (in the case of *plcHR* and *plcH*) or the pUCP19 plasmid (in the case of *plcR*). (A) Lane 1, 100 μ l of whole-cell extract of ADD1976 pADD3268::*plcHR*; lane 2, 100 μ l of culture supernatant of pADD3268::*plcHR*; lane 3, 100 μ l of whole-cell extract of ADD1976 pADD3268::*plcH*; lane 4, 100 μ l of culture supernatant of ADD1976 pADD3268::*plcH*. (B) Lane 1, whole-cell extract of ADD1976 pADD3268::*plcH* mixed with a whole-cell extract of ADD1976 pADD3268::*plcR* after the cells were lysed separately; lane 2, whole-cell extract of ADD1976 pADD3268::*plcR*; lane 3, whole-cell extract of ADD1976 pADD3268::*plcH* mixed with a whole-cell extract of *E. coli* BL21(DE3) pGEM2::*plcR*, after the cells were lysed separately. This lane was from a separate N-PAGE gel so the PlcH appears to migrate faster than the activity seen in lane 1 in this panel.

out *plcR*, and PlcH was radiolabeled with 14 C-amino acids, the amount of radiolabeled PlcH in both cases was essentially the same. These data suggested that *plcR* has no obvious effect on transcription or translation of *plcH*.

We previously reported that the synthetic substrate NPCC can be used to detect PlcH activity in N-PAGE gels (31). PlcH activity cannot be detected with this substrate in SDS-PAGE gels. Figure 2A, lane 1, shows PLC activity in an N-PAGE gel of cell lysates from *E. coli* BL21(DE3) expressing both the *plcH* and *plcR* genes together. The activity migrates well into the separating gel. Two bands of activity are seen, but it is not clear why this is so. Even highly purified preparations of PlcH from *P. aeruginosa* exhibit this behavior (35b) in N-PAGE gels. Even on blood agar with cultures of *E. coli* BL21(DE3) pGEM::*plcHR* (Fig. 1) or when purified *plcH* from either *E. coli* or *P. aeruginosa* is spotted on this medium, there are two apparent zones of hemolysis, one less intense, but faster migrating, and the other showing a more complete hemolysis. There actually appears to be a sharp demarcation between the two zones. Nevertheless, as seen in Fig. 2B, with SDS-PAGE only one band is detected when PlcH is expressed in *E. coli* BL21 (DE3). When *plcH* is expressed without the *plcR* genes in this expression system and the PLC activity is examined in cell lysates run on an N-PAGE gel, in contrast to what is found for cells expressing both *plcH* and *plcR* genes, the activity in these extracts barely migrates past the interface of the stacking and separating gels (Fig. 2A, lane 4). Moreover, much of the activity still remains in the well and does not even migrate into the stacking gel, such that it appears this strain is producing less total PlcH. At this point, it was considered that *plcR* could be expressing a protein which posttranslationally modifies PlcH. For example, PlcR could be involved in glycosylation, phosphorylation, or acylation of PlcH. If this was true, it is possible that PlcR-modified PlcH might migrate differently on an SDS-PAGE gel than unmodified PlcH. Previously reported data had suggested that this is not the case; however, since PlcH is a relatively large protein (78 kDa), small differences between the migration of modified and unmodified PlcH might not be detected in the SDS-PAGE systems used in those studies (i.e., 10 to 12.5% acrylamide). Nonetheless, as shown in Fig. 2B, even in an SDS-PAGE gel containing only 5% acrylamide, PlcR-modified PlcH does not migrate any differently than the unmodified form. The PlcR proteins are not seen in lane 1 be-

cause they are relatively small in comparison to PlcH and in this acrylamide concentration they would run off the bottom of the gel.

While there does not appear to be any alteration of the mass of PlcH by PlcR that can be detected in SDS-PAGE analysis, the additional results presented in Fig. 2A suggest that PlcR posttranslationally affects the physical nature of PlcH. As shown in lanes 2 and 3 of this figure, when *plcH* is expressed by itself in *E. coli* BL21(DE3) and the lysate of these cells is mixed with a lysate from *E. coli* BL21(DE3) expressing only the *plcR* genes, the PLC activity in this preparation now migrates like the PLC activity in lysates of cells expressing both *plcH* and *plcR* at the same time. This does not occur if the lysate containing only PlcH is mixed with a lysate from *E. coli* BL21 (DE3) carrying only the pGEM vector, nor does it occur if a heated lysate (100°C, 5 min) of cells expressing *plcR* is used (data not shown). Collectively, these data clearly indicate that PlcR, directly or indirectly, posttranslationally alters the physical properties of PlcH. PlcR has no apparent effect on the migration of PlcN expressed in *E. coli* (data not shown).

Expression of *plcH* and *plcR* in a *P. aeruginosa* T7 expression system. A T7 expression system for *P. aeruginosa* similar to the *E. coli* system employed in this study was used to express the *plcHR* genes in the PAO1-derivative ADD1976 with the pADD3268 expression plasmid (4). While ADD1976 still carries chromosomal copies of the *plcHR* operon and the *plcN* gene, it is possible to use this system to study expression of these genes directed by the T7 promoter on the pADD3268 vector because the conditions used in these experiments do not result in the induction of either chromosomal gene. In fact, no PLC activity can be detected in culture supernatants of ADD1976 carrying only the vector plasmid when it is grown under the conditions used in these experiments. In order to be certain that the phenomena of posttranslational modification of PlcH we observed by using the *E. coli* T7 expression system could occur in the genus that naturally carries these genes, we performed similar experiments using the *P. aeruginosa* T7 expression system. Induced culture supernatants and cell lysates were assayed for PLC activity on N-PAGE gels. Figure 3 shows the results of these experiments. Expression of the *plcH* gene alone had two results. First, the retarded migration of PlcH as seen from the overexpression in *E. coli* was also observed in *P. aeruginosa* (Fig. 3A, compare lanes 1 and 3). Second, there was

TABLE 2. Ratios of cell-associated and extracellular PLC activity in PAO1WT and PAO1 Δ R and complementation of secretion defect in PAO1 Δ R with *plcR*^a

Strain	SMase activity (S/L ratio $\pm$ SEM)	NPPC activity (S/L ratio $\pm$ SEM)
PAO1WT	3.60 $\pm$ 0.693	5.84 $\pm$ 0.994
PAO1 Δ R	1.19 $\pm$ 0.033	0.542 $\pm$ 0.098
PAO1 Δ R2-1c	0.556 $\pm$ 0.029	0.437 $\pm$ 0.203
PAO1 Δ R pUCP19	1.19 $\pm$ 0.107	0.574 $\pm$ 0.133
PAO1 Δ R pUCR	2.27 $\pm$ 0.250	2.77 $\pm$ 0.046
PAO1 Δ R2-1c pUCP19	0.547 $\pm$ 0.184	0.181 $\pm$ 0.052
PAO1 Δ R2-1c pUCR	5.44 $\pm$ 0.356	2.97 $\pm$ 0.433

^a Each PLC assay was performed three to four times. Mean values of specific PLC activity in the culture supernatants (extracellular) and the whole-cell lysates (cell associated) were used to calculate the ratio of supernatant to lysate (S/L) activity. NPPC assay units, $\Delta A_{410}/\text{min}/A_{590}$; SM assay units, cpm/A_{590} .

no detectable PlcH in the culture supernatant (compare lanes 2 and 4). The vector control had no detectable PLC activity (data not shown). Furthermore, a mixture of cell lysates from cells expressing PlcR and PlcH separately also restored the migration of the activity whether the PlcR was expressed in *E. coli* or *P. aeruginosa* (Figure 3B, lanes 1 and 3). These data are consistent with the data from the *E. coli* expression experiments.

PLC expression in PAO1 Δ R mutants. Specific chromosomal induction of the *plcH* gene can be achieved by growing the cultures in minimal medium supplemented with 20 mM phosphate and 0.2% choline. Choline induces expression of PlcH, while this concentration of phosphate represses expression of the nonhemolytic PLC (31). The PAO1 Δ R and PAO1 Δ R2-1C mutants lacking wild-type *plcR* genes were grown along with the parental PAO1 strain in HEPES medium supplemented with phosphate and choline as described in Materials and Methods. Whole-cell extracts and supernatants taken 8 h after induction were assayed for PLC activity by using either the synthetic substrate NPPC or the natural substrate for PlcH, SM, as described previously (3, 8). Table 2 shows the results from this study. The ratio of secreted versus cell-associated PLC activity was calculated. A great majority of the PLC activity from the wild-type strain was detected in the culture supernatant as expected. Secretion of PLC activity into the culture supernatant was affected in both the PAO1 Δ R and PAO1 Δ R2-1C mutants. These mutants secreted at least 10-fold less PLC activity than wild-type PAO1 into the culture medium. These data suggest that in the absence of PlcR some secretion of PlcH may occur, but it is much less efficient. They are also entirely consistent with the previous data from the overexpression experiments. Moreover, it is possible to complement the secretion defect by expressing wild-type PlcR in *trans*. The results of these experiments are also shown in Table 2. The pUCR plasmid carrying the *plcR* genes complements the defect in these mutants, as demonstrated by restoration of extracellular PLC activity measured by both hydrolysis of NPPC and cleavage of SM, the specific substrate for PlcH.

While the ratio of extracellular activity to cell-associated activity in the first mutant that was constructed (PAO1 Δ R) is significantly reduced in comparison to wild-type PAO1, this mutant expresses significantly greater levels of total (cell-associated plus extracellular) PLC activity than the wild type (3.9 and 6.0 U of total PLC activity for PAO1WT and PAO1 Δ R, respectively). This was true whether PLC activity was measured with NPPC or with radiolabeled SM as a substrate. This observation was of some concern because it could be that

overexpression of PlcH simply results in a nonspecific defect in secretion of PlcH by blocking the *sec*-dependent secretion pathway, for example. Therefore another mutant, Δ R2-1C, was constructed with the same 186-bp in-frame deletion of the *plcR* genes as the original PAO1 Δ R mutant, but it does not carry the tetracycline resistance (*tet*) cassette. This new mutant was created by using the *sac* selection system described by Schweizer (29). As shown in Table 2, the ratio of extracellular to cell-associated PLC activity is significantly reduced in Δ R2-1C in comparison to PAO1 wild type, and Δ R2-1C does not express higher total levels of PLC activity than the wild-type strain PAO1 (total NPPC activity in these experiments: Δ R2-1C, 1.1 U; PAO1WT, 3.9 U). Furthermore the defect in secretion of PlcH is like that of the original mutant PAO1 Δ R. These observations cogently support the hypothesis that the *plcR* gene products affect the secretion of PlcH but not its expression.

The reason for the differences in these mutants was not clear. One possibility was that insertion of the 2.8-kb *tet* cassette in the place of the *plcR* deletion in PAO1 Δ R in some way affected the stability of the *plcH* mRNA, thereby increasing the overall level of *plcH* expression. It seemed that the in-frame deletion without an insertion of a large fragment (i.e., *tet*) in the *plcHR* operon in Δ R2-1C would be less likely to have such an effect. This possibility was examined by using RNase protection analysis of *plcH* specific mRNA in the two mutants and the PAO1 wild type. As shown in Fig. 4 there were considerably higher levels of mRNA detected in the PAO1 Δ R mutant with the tetracycline cassette than in the PAO1 Δ R2-1C mutant or the wild-type strain at the later time point (e.g., 5 h). These experimental results indicate that the higher levels of total PlcH activity (~twofold) in PAO1 Δ R in comparison to wild type or the PAO1 Δ R2-1C mutant is most probably a consequence of the significantly increased levels of *plcH*-specific message in this strain. The higher levels of total PlcH activity are also consistent with an increased stability of *plcH*-specific message resulting from the insertion of the *tet* cassette in the *plcHR* operon. In contrast, PAO1 Δ R2-1C only contains an in-frame 186-bp deletion in the *plcHR* operon. Insertion of the

FIG. 4. RNase protection analysis of *plcH* mRNA isolated from PAO1WT (lanes A), PAO1 Δ R (lanes B), and PAO1 Δ R2-1C (lanes C) at the indicated times after addition of 0.2% choline. The numbers on the left are values for the RNA base standards. The undigested probe indicated was loaded at a 100-fold dilution to avoid overexposure of the autoradiograph.

TABLE 3. Distribution of PLC activity in LPS MVs and whole-cell extracts^a

Strain	Sample	PLC activity (U/mg [$\pm$ SD])
PAO1WT	MV	7.62 $\pm$ 1.0
PAO1WT	CE	0.21 $\pm$ 0.05
PAO1 Δ HR	MV	0.30 $\pm$ 0.18
PAO1 Δ HR	CE	0.17 $\pm$ 0.22
PAO1 Δ RN	MV	0.0015 $\pm$ 0.004
PAO1 Δ RN	CE	0.15 $\pm$ 0.04
PAO1 Δ R	MV	0.8 $\pm$ 0.16
PAO1 Δ R	CE	0.22 $\pm$ 0.04

^a Assays were done using NPPC as the substrate (units, ΔA_{405} of 0.01 per h at 37°C) in triplicate to obtain mean values ($\pm$ standard deviation). CE, whole-cell extracts.

tet cassette in other loci (e.g., *tox4*) in the PAO1 genome has no effect on the secretion or expression of PlcH (35a). These data suggest that considerable caution should be exercised when such large DNA fragments (2.8 kb) are introduced into a locus to select for a mutant strain. In the case described above, insertion of this cassette would not be predicted to have a polar effect because the *plcR* genes are located at the very 3' end of the *plcHR* operon. Nevertheless, it did have a significant anomalous effect on the transcription of this operon (Fig. 4) and led to the early erroneous hypothesis that *plcR* had a regulatory effect on the expression of PlcH.

Analysis of PLC Activity in MVs. *P. aeruginosa* has been shown to shed bleb-like structures which contain portions of lipopolysaccharide (LPS) (15). These structures, known as MVs, are detected during logarithmic growth, and their production is enhanced by sublethal concentrations of antibiotics such as gentamicin. However, the purpose of these phenomena, which have also been observed in other gram-negative microbes (25), is not understood. More recently, it was shown that MVs from *P. aeruginosa* contain active enzymes including PLC, and it was proposed that the release of MVs by *P. aeruginosa* could result in the delivery of endotoxin as well as other virulence factors to sites distal to a primary site of infection (15). Because PLC activity had been detected in the MVs the effect of the *plcHR* mutations on the PLC activity inside *P. aeruginosa* MVs was examined. Examination of the *plcHR* mutants showed that the amount of PLC activity in the MVs from the mutant strains was decreased by 96 to 99.9% (Table 3). These results clearly indicate that there is a requirement for PlcR to localize PlcH in the MVs. Deletion of *plcR* has no apparent effect on LPS synthesis because the LPS patterns (i.e., smooth) of PAO1WT and PAO1 Δ R were identical by silver-stained PAGE of whole cells (data not shown).

Immunogold electron microscopy. PAO1WT and PAO1 Δ R grown under inducing conditions (i.e., in 0.2% choline) were reacted with immunogold beads coupled to anti-PlcH antibodies. As shown in Fig. 5, PlcH is mainly associated with the MVs and the outer-facing surface of the cell periphery. In contrast,

FIG. 5. Immunogold electron microscopic detection of PlcH in wild-type *P. aeruginosa* PAO1WT (A) and PAO1 Δ R (B) with rabbit antiserum against native PlcH purified from *P. aeruginosa*.

in the mutant the immunogold beads are considerably more cell associated and are found deeper in the cells and less in the outer-facing surface than in the wild-type strain. MVs from PAO1 Δ R also appear to contain less PlcH. These data clearly support the hypothesis that PlcR serves a posttranslational function and is required for the efficient secretion or release of PlcH from *P. aeruginosa*.

Effect of PlcR on secretion of PlcN. Previous studies demonstrated that deletion of both *plcH* and *plcR* has no apparent effect on secretion of PlcN because PAO1 Δ HR expresses approximately one-half the level of PLC activity as PAO1WT. This is in fact how PlcN was discovered because PAO1 Δ HR still expressed extracellular PLC activity under phosphate-limiting conditions as measured by the synthetic substrate NPPC. Moreover, culture supernatants from PAO1 Δ HR contained as much PLC activity as PAO1WT when a natural substrate of PlcN, phosphatidylserine, was used for assay of PLC activity (35b). However, deletion of *plcR* does appear to have an indirect effect on the secretion of PlcN because culture supernatants of PAO1 Δ R do not contain detectable levels of PLC in assays using NPPC as the substrate (data not shown). These data suggest that the blockage of the secretion pathway by PlcH in the absence of PlcR can affect the secretion of PlcN, but that PlcR specifically facilitates only the secretion of PlcH because in the absence of PlcH, PlcN secretion is unaffected.

DISCUSSION

Bacterial pathogens produce and secrete a number of extracellular virulence determinants which are often necessary for successful colonization and/or pathogenesis. The complex envelope of the gram-negative pathogens represents a formidable barrier since the proteins which traverse the envelope of these microorganisms must cross the inner membrane, the peptidoglycan layer, the periplasm, and then the outer membrane to be fully secreted into the medium. Microbial pathogens have therefore devised accessory mechanisms to specifically secrete certain proteins to the extracellular spaces. Many, but not all, extracellular proteins of gram-negative microbes are secreted via a complex signal-sequence-dependent process that may occur in two distinct steps (27) (type II secretion). The first step involves the transport of the protein through the inner membrane by the *sec*-dependent pathway and into the periplasmic space. The signal sequence is cleaved during this process. The second step involves recognition of the outer membrane and subsequent transfer of the protein past this barrier. This latter process is often accomplished with the aid of specific accessory functions (2). These functions have also been called extragenic factors and are remarkable in that they often facilitate secretion of a single or perhaps only a few proteins destined to the extracellular spaces (38). Many bacteria, including *P. aeruginosa*, which secretes a variety of extracellular virulence determinants, express such accessory factors. These accessory proteins may have enzymatic activities such as HlyB, an ATPase of *E. coli* that is required for secretion of hemolysin (11), or the lipase required by many species of enteric bacteria for the secretion of colicin (33). Extracellular secretion of the mature elastase in *P. aeruginosa* is dependent on the proteolytic activity of a portion of the cell-associated proelastase (propeptide) which must autocatalytically cleave the mature elastase from this precursor to ultimately achieve release of the mature active elastase into the culture supernatant (20). The cleaved propeptide appears to act as a chaperone for the secretion of the mature elastase. Not all accessory factors act enzymatically. Some act by affecting the folding of the secreted protein to prevent it from adopting

a conformation that could lead to a secretion-incompetent state (e.g., chaperonin). Secretion of *Vibrio cholerae* enterotoxin appears to involve this type of activity (10).

PlcH is synthesized with a highly positively charged (6⁺) N-terminal signal sequence, which suggests that secretion of this virulence determinant occurs via the type II pathway and requires the genes of the *xcp* locus (16). Additionally, it appears that secretion of PlcH by *P. aeruginosa* requires an accessory factor because experimental results reported in the present study demonstrate that *E. coli* expressing PlcH does not release PLC or hemolytic activity into culture supernatants of broth-grown cultures. These results could indicate that there are elements missing in *E. coli* that are required for extracellular secretion of PlcH in this organism. However, it appears that under certain circumstances (i.e., in solid medium) PlcH can be released or secreted from *E. coli* but only when it is co-expressed with PlcR. The reason for this different behavior is not clear but it is not at all unprecedented. In *Yersinia* spp. outer proteins are not normally released from the microbe into the surrounding environment until the organism makes contact with a eucaryotic cell. Further experimental results from the present study clearly indicate that PlcR is required for the extracellular secretion or release of PlcH, but not PlcN, in the natural host *P. aeruginosa*. Cumulative data clearly suggest that PlcR functions as a specific accessory factor for the secretion of PlcH, but at the present time its mechanism is not clear. PlcR could be a specific chaperonin for PlcH and directly interact with PlcH to maintain it in a secretion-competent conformation as it traverses both the inner and outer membranes. Previous studies established that PlcR1 is secreted to the periplasm in *E. coli* (30). It is predicted to have a classical signal sequence, and pulse-chase experiments and cell fractionation studies demonstrated that PlcR1 is predominately found in the periplasm after cleavage of the signal sequence of pre-PlcR1 (30). PlcR2, on the other hand, does not have a classical signal sequence, and pulse-chase experiments and cell fractionation studies indicate that this form remains in the cytoplasm. Hydrophobicity analyses of the 151 common amino acids of PlcR1 and PlcR2 predict that they are hydrophilic proteins and are not likely to be membrane associated. The most remarkable difference between PlcR1 and PlcR2, besides the signal sequence, is that of the 36 amino acids present in the mature PlcR1, but absent in PlcR2, 10 are proline residues and 15 are other hydrophobic residues. This domain unique to PlcR1 could be a membrane anchor, but by and large, these proteins are most probably soluble, hydrophilic proteins of the periplasm and cytoplasm. One hypothesis that could explain the compartmentalization of the PlcR proteins is that PlcR2 may be involved in escorting PlcH through the inner membrane, while PlcR1 is required for secretion through the outer membrane, possibly by affecting the activity of chaperonins or a specific secretion apparatus in each membrane. Such an elaborate chaperone system for PlcH may be required for several reasons. Among them would be that PlcH is a relatively large protein with significant potential, by virtue of its natural enzymatic activity toward phospholipids, to interact in a deleterious way with the membrane phospholipids of the inner and outer membranes. PlcR proteins could specifically and directly bind to PlcH and chaperone it through both membranes or through a two-membrane secretion complex. Such a scenario would suggest that the PlcR proteins might interact concomitantly with the presecreted form of PlcH during the secretion process and perhaps alter its conformation. This form of PlcH might occur so that it will not interact nonspecifically with membrane phospholipids or perhaps other components it might encounter on this journey, including the peptidoglycan layer or mem-

brane proteins. However, the predicted hydrophilic nature of the PlcR proteins would preclude them from blocking interactions during passage through the inner and outer membranes or from forming a pore-like structure in these membranes. At the present time it is not known if the PlcR proteins are monomeric or multimeric in their natural state.

One of the most revealing experiments regarding the mechanism of PlcR in the present study demonstrated that extracts of *E. coli* BL21(DE3) pGEM2::plcR were capable of altering the migration of PlcH on N-PAGE gel when PlcH was expressed in *E. coli* in the absence of PlcR. Moreover, supporting the view that PlcH, expressed without PlcR, is avidly complexed with other cellular components is the biochemical and biophysical behavior of PlcH expressed in the presence or absence of PlcR. PlcH in extracts of *E. coli* BL21(DE3) pGEM2::plcHR can bind tightly to a MonoQ column and then it can be eluted as a soluble protein with increasing NaCl concentrations (~250 mM). This purified PlcH from *E. coli* BL21(DE3) pGEM2::plcHR behaves in every way like soluble PlcH purified from culture supernatants of *P. aeruginosa*. In contrast, PlcH activity in extracts of *E. coli* BL21(DE3) pGEM2::plcH does not even enter and bind to a MonoQ column. If an extract from *E. coli* BL21(DE3) pGEM2::plcR is added to MonoQ column the PlcH activity in this bipartite extract then behaves as PlcH expressed in *E. coli* BL21(DE3) pGEM2::plcHR (35b). This behavior suggests that in the absence of PlcR, PlcH becomes tightly complexed with cellular components, but that in vitro, PlcR can release PlcH from this complex. While it is possible that PlcR permits proper folding of PlcH in some way like classical chaperonins, it is likewise possible that PlcR in some manner facilitates the postfolding release of PlcH from a membrane complex which could include inner and outer membranes as well as peptidoglycan.

Still, it is possible that the PlcR proteins constitute a specialized chaperonin system for PlcH. While it is still somewhat controversial where chaperonins affect the folding of their protein substrate, they are thought to participate in the folding process by either increasing the kinetics or fidelity of the folding process (5). Chaperonins are capable of discriminating between native and unfolded states of a target protein but they only bind to the latter. Even PlcH expressed by itself in *E. coli* is still enzymatically active and hemolytic. Quantitative measurement of specific PlcH activity in *E. coli* expressing only plcH with the soluble NPPC as substrate (which can penetrate even the cellular complex) indicates that this complexed PlcH has the same amount of specific PLC activity as PlcH expressed in *E. coli* in the presence of PlcR (see Results). While PlcH expressed alone in *E. coli* is hemolytic on blood agar, it was not possible to measure specific hemolytic activity with erythrocytes because of the insoluble nature of PlcH from cells expressing PlcH alone and the particulate nature of the substrate (erythrocytes) used in this assay. While PlcH expressed in *E. coli* BL21(DE3) pGEM2::plcHR behaves as a soluble protein after the cells are simply lysed by sonication, PlcH expressed in *E. coli* BL21(DE3) pGEM2::plcH always remained in an insoluble complex with the cell membranes. All efforts to disassociate it from the complex in the absence of PlcR resulted in inactivation of PLC activity. While these extracts exhibit hemolytic activity on erythrocytes and on blood agar, which can be neutralized with antibodies against native PlcH purified from *P. aeruginosa*, it was not possible to obtain an accurate specific hemolytic titer of these extracts. Nevertheless, collectively these data strongly suggest that in vitro, PlcR can mimic its in vivo function, a characteristic less likely to be associated with a classical procaryotic chaperonin that would probably not interact with the native form of its target protein. These re-

```

PlcR  MNTATAPDNLEQQLGE-FGRNAGQMSIERKQAAEGLIEQLKREVAVGADPRQTFFEEIQR
      .. : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
CAB   ALERADFEKEAQHIAEAFGKIDAGAAEVQTLKNAFGGLFDYLAKEAGVSGDGLSTEEQFIR

PlcR  LT---PYVEADARRREALDFEIMALKDNASVQQQAPTPEEEQLREYAQESDK-VIAEV
      .. : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
CAB   VTENLIFEQGEASFNRVLG-PVVKGTWCMCDKNADGQINADFAAWLTALGMSKAEAAEA

PlcR  LASVDDEEQRHAAIDERLKLARKQIFGEENPRLLQ
      .. : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
CAB   FNQVDITNGELSLDELLTAVRDFHFGRILDVELLG

```

FIG. 6. Alignment of the deduced amino acid sequences of PlcR1 and PlcR2 (first amino acid of PlcR shown is the first amino acid of PlcR2) with a calcium-binding protein of *S. erythraeus* (CAB). Putative EF hand motifs are underlined. Colons and periods indicate amino acid identity and similarity, respectively. Dashes indicate gaps in the sequence introduced for alignment.

sults, however, still do not rule out the possibility that the PlcR proteins could be chaperonin-like proteins that are capable of affecting the posttranslational folding of PlcH. While the data mentioned above would suggest that PlcH is already in its native state, even in extracts from *E. coli* BL21(DE3) pGEM2::plcH, there are not any credible data at this time to entirely rule out the possibility that there are regions or domains of PlcH that are still in the unfolded state that could be a target for a chaperonin-like molecule.

Alternatively, it is possible that PlcR acts in an enzymatic manner. For example, PlcR may enzymatically, posttranslationally modify PlcH so that it could be efficiently secreted or released. Such modifications could include acylation or glycosylation. There were, however, no modifications of PlcH detected that significantly altered its mobility on SDS-PAGE gels. More sensitive methods, such as mass spectrometry of PlcH purified from *P. aeruginosa*, could reveal if PlcR posttranslationally modifies PlcH and the nature of such a modification.

Another reasonable scenario is that PlcR acts enzymatically to alter the biochemical or biophysical nature of the cell envelope, including the inner and outer membranes and peptidoglycan. Such a modification does not necessarily have to directly involve PlcH, but it could be part of a secretion complex in the cell envelope that provides an avenue for PlcH to escape from the cell. Such a mechanism might include rearrangement of membrane phospholipids, alteration of peptidoglycan, or modification of other proteins involved in secretion. Such a secretion complex has been proposed for more complex toxins including pertussis toxin (36). It has been proposed that pertussis toxin secretion is accomplished through a membrane fusion mechanism that releases the toxin from the secretion complex into the medium. It is interesting that PlcH is found in the MVs which bud off of *P. aeruginosa* during growth. Considerably reduced levels of PlcH are found in these MVs in *P. aeruginosa* PAO1ΔR mutants. These data could suggest that PlcH secretion is associated with production of the MVs. Alternatively, it is possible that during secretion of PlcH some of this protein along with PlcN is simply trapped in these vesicles. The latter hypothesis seems to be more likely since most PlcH found in culture supernatants of *P. aeruginosa* is not associated with the vesicles but behaves as a soluble 78-kDa protein.

A BLAST search of GenBank revealed that the 151 amino acids common to PlcR1 and PlcR2 have significant homology (25% identity and 68% similarity over 147 amino acids) to a calcium-binding protein (GenBank accession no. M29700) from *Streptomyces erythraeus* (CAB) (Fig. 6). CAB and PlcR share some significant features with calcium-binding proteins of eucaryotic cells, including calmodulin. These small acidic

proteins each have one or more highly conserved calcium-binding domains referred to as EF hands. The typical EF hand is composed of a 12-residue loop which contributes six ligands to a Ca^{2+} ion, and the loop is flanked by two amphipathic α helices that pack against each other and stabilize the loop (1). PlcR1 and PlcR2 contain three such loops which have significant similarity (10 of 12 amino acids) to EF hands found in the *S. erythraeus* protein. The positions of these EF hands in PlcR align with those of EF hands in CAB, and the loops in PlcR are flanked by amphipathic α helices. Unfortunately, the function of this calcium-binding protein in *S. erythraeus* is not clear, but calmodulin-like proteins have been increasingly found in prokaryotic organisms, including *E. coli*, *Bacillus subtilis*, and *Myxococcus xanthus* (21). Based on this homology we attempted to determine if a calcium chelator might have an effect on the PlcR-induced migration of PlcH in N-PAGE gels. Preliminary results using the chelators EGTA and EDTA suggest that a divalent cation, perhaps calcium, is required for PlcR activity (4a). It is possible that if PlcR1 and PlcR2 bind calcium through their EF hands, like calmodulin, they might not need to directly interact with pre-secreted PlcH. They could participate in the secretion process by affecting the activities of enzymes (e.g., via phosphorylation) or proteins involved in secretion by modulating their activities through calcium. Calcium is associated with phospholipid metabolism in *E. coli*. The interaction of calcium with phospholipids is associated with the formation of non-bilayer structures and lipid scrambling. Calcium also has a structural role in stabilizing LPS (21). These associations between the possible calcium-binding nature of PlcR and the role of calcium in membrane processes suggest additional experimental approaches for dissecting the function of this accessory factor in the secretion of PlcH. With regard to the possible calcium-binding nature of PlcR, it has not escaped our notice that calcium-binding proteins can be involved in modulating the activity of calcium-dependent ATPases. Similar enzymes play a role in the secretion of hemolysins in *E. coli* (11).

Finally, with regard to the role of secretion of PlcH in the virulence of *P. aeruginosa*, it is noteworthy that PAO1 Δ PlcR was significantly reduced in virulence in a model *P. aeruginosa* infection. PAO1 Δ PlcR was nearly 200-fold less virulent in the mouse burn model than the parental strain PAO1, despite its ability to still express significant levels of cell-associated PlcH and normal levels of PlcN (24). These data strongly emphasize the importance of PlcR and PlcH in virulence of *P. aeruginosa* and the critical role of extracellular secretion in bacterial pathogenesis.

ACKNOWLEDGMENTS

We thank Zaiga Johnson for assistance with the RNase protection assays and Dorothy Pierson for assistance with the LPS silver-stained gels.

This work was supported by an NIH grant from the National Institute of Diabetes and Digestive and Kidney Diseases (DK46440) to Michael L. Vasil.

REFERENCES

- Banden, C., and J. Tooze. 1991. Introduction to protein structure. Garland Publishing Inc., New York, N.Y.
- Beckwith, J., and S. Ferro-Novick. 1986. Genetic studies on protein secretion in bacteria. *Top. Microb. Immunol.* **125**:5–27.
- Berka, R. M., G. L. Gray, and M. L. Vasil. 1981. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. *Infect. Immun.* **34**:1071–1074.
- Brunschwig, E., and A. Darzins. 1992. A two-component T7 expression system for expression of genes in *Pseudomonas aeruginosa*. *Gene* **111**:35–41.
- 4a. Cota-Gomez, A., and M. L. Vasil. Unpublished data.
- Ellis, R. J., and S. M. van der Vies. 1991. Molecular chaperones. *Annu. Rev. Biochem.* **60**:321–347.
- Esselman, M., and P. V. Liu. 1961. Lecithinase production by gram-negative bacteria. *J. Bacteriol.* **81**:939–945.
- Goldfine, H., N. C. Johnston, and C. Knob. 1993. Nonspecific phospholipase C of *Listeria monocytogenes*: activity on phospholipids in Triton X-100-mixed micelles and in biological membranes. *J. Bacteriol.* **175**:4298–4306.
- Gray, L. M., R. M. Berka, and M. L. Vasil. 1981. A *Pseudomonas aeruginosa* mutant non-derepressible for orthophosphate-regulated proteins. *J. Bacteriol.* **147**:675–678.
- Heckly, R. J. 1971. Toxins of pseudomonas, p. 473–491. In S. Aji (ed.), *Microbial toxins*, vol. 3. Academic Press, Inc., New York, N.Y.
- Hirst, T. R., and R. A. Welch. 1988. Mechanisms for secretion of extracellular proteins by gram-negative bacteria. *Trends Biochem. Sci.* **13**:265–269.
- Holland, I. B., B. Kenny, and M. Blight. 1990. Hemolysin secretion from *Escherichia coli*. *Biochimie* **72**:131–141.
- Holloway, B. W., V. Krishnapillai, and A. F. Morgan. 1979. Chromosomal genetics of *Pseudomonas*. *Microbiol. Rev.* **43**:73–102.
- Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. USA* **72**:2284–2288.
- Iglewski, B. H., J. Sadoff, M. J. Bjorn, and E. S. Maxwell. 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. *Proc. Natl. Acad. Sci. USA* **75**:3211–3215.
- Kadurugamuwa, J., and T. J. Beveridge. 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J. Bacteriol.* **177**:3998–4008.
- Kreger, A. S., and L. D. Gray. 1978. Purification of *Pseudomonas aeruginosa* protease and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. *Infect. Immun.* **19**:630–648.
- Liu, P. V. 1974. Extracellular toxins of *Pseudomonas aeruginosa*. *J. Infect. Dis.* **130**(Suppl.):594–599.
- Liu, P. V. 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of lethal toxins produced *in vitro* and *in vivo*. *J. Infect. Dis.* **116**:481–489.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y.
- McIver, K. S., E. Kessler, J. C. Olson, and D. E. Ohman. 1995. The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **18**:877–889.
- Norris, V., S. Grant, P. Freestone, J. Canvin, F. N. Sheikh, I. Toth, M. Trinei, K. Modha, and R. I. Norman. 1996. Calcium signalling in bacteria. *J. Bacteriol.* **178**:3677–3682.
- Ostroff, R. M., and M. L. Vasil. 1987. Identification of a new phospholipase C activity by analysis of insertional mutations in the hemolytic phospholipase C structural gene of *Pseudomonas aeruginosa*. *J. Bacteriol.* **169**:4597–4601.
- Ostroff, R. M., A. I. Vasil, and M. L. Vasil. 1990. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. *J. Bacteriol.* **172**:5915–5923.
- Ostroff, R. M., B. Wretling, and M. L. Vasil. 1989. Mutations in hemolytic phospholipase C operon result in decreased virulence of *Pseudomonas aeruginosa* PAO1 grown under phosphate-limiting conditions. *Infect. Immun.* **57**:1369–1373.
- Pettit, R. K., and R. C. Judd. 1992. Characterization of naturally elaborated blebs from serum-susceptible and serum-resistant strains of *Neisseria gonorrhoeae*. *Mol. Microbiol.* **6**:723–728.
- Pritchard, A. E., and M. L. Vasil. 1986. Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. *J. Bacteriol.* **167**:291–298.
- Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
- Sage, A. E., A. I. Vasil, and M. L. Vasil. 1997. Molecular characterization of mutants affected in the osmoprotectant-dependent induction of phospholipase C in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol.* **23**:43–56.
- Schweizer, H. P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis* *sacB* marker. *Mol. Microbiol.* **6**:1195–1204.
- Shen, B.-F., P. C. Tai, A. E. Pritchard, and M. L. Vasil. 1987. Nucleotide sequence and expression in *Escherichia coli* of the in-phase overlapping *Pseudomonas aeruginosa* *plcR* genes. *J. Bacteriol.* **169**:4602–4607.
- Shortridge, V. D., A. Lazdunski, and M. L. Vasil. 1992. Osmoprotectant and phosphate-regulated expression of phospholipase C of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **6**:863–871.
- Sierra, G. 1960. Hemolytic effect of a glycolipid produced by *Pseudomonas aeruginosa*. *Antonie Leeuwenhoek J. Microbiol. Serol.* **26**:189–192.
- Skrvinsky, R. C., R. L. Gilson, and R. Kolter. 1991. Signal sequence-independent protein secretion in gram-negative bacteria: colicin V and microcin B17, p. 205–221. In A. M. Tartakoff (ed.), *Methods in cellular biology*. Academic Press, Inc., San Diego, Calif.

34. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **198**:113–130.
35. **Vasil, M. L., R. M. Berka, G. L. Gray, and H. Nakai.** 1982. Cloning of a phosphate-regulated hemolysin gene (phospholipase C) from *Pseudomonas aeruginosa*. *J. Bacteriol.* **152**:431–440.
- 35a. **Vasil, M. L., and Z. Johnson.** Unpublished data.
- 35b. **Vasil, M. L., and A. I. Vasil.** Unpublished data.
36. **Weiss, A. A.** 1994. Unorthodox secretion by gram-negative bacteria, p. 341–349. *In* V. L. Miller, J. B. Kaper, D. A. Portnoy, and R. R. Isberg (ed.), *Molecular genetics of bacterial pathogenesis*. ASM Press, Washington, D.C.
37. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia coli*-*Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**:81–86.
38. **Wretling, B., and O. R. Pavlovskis.** 1984. Genetic mapping of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. *J. Bacteriol.* **158**:801–808.

Editor: P. E. Orndorff