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Phospholipases C from *Pseudomonas aeruginosa* and *Bacillus cereus* isolates, chromosome-mediated enzymes with roles in virulence

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Abstract: Phospholipases C (PLCs) from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101, 2 clinical isolates from 2 pus specimens, were partially purified by ammonium sulfate precipitation followed by dialysis and used to study the possible role of PLC in the virulence of the isolates. Partially purified PLC from both isolates induced lysis of Vero cells in the presence and absence of the producing bacterial cells. Noncytolytic dilutions of the partially purified PLC from *Pseudomonas aeruginosa* increased adherence of the producing cells to Vero cells but did not affect internalization. However, *Bacillus cereus* cells neither adhered to nor were internalized within the Vero cells in the presence or absence of noncytolytic dilutions of partially purified PLC from the isolate. Both PLC preparations were hemolytic to human red blood cells but did not induce human platelet aggregation. Thus, we can conclude that PLCs from *Pseudomonas aeruginosa* and *Bacillus cereus* are important virulence factors. PLC production by the 2 isolates was found to be chromosomal-mediated rather than plasmid-mediated.

Key words: Phospholipase C, *Pseudomonas aeruginosa*, *Bacillus cereus*, catalytic activity

1. Introduction

Phospholipases C (PLCs) have a special place in the history of bacterial toxinology because the α-toxin (PLC) of *Clostridium perfringens* was the first bacterial toxin shown to have an enzymatic mode of action (1). Bacterial PLCs comprise a diverse group of proteins that have a range of effects in vivo and in vitro, from minor alterations in cell membrane composition and function to lethality at low concentrations. Dissimilarity is more common than congruence; overlap in substrate specificity and the nature of substrate hydrolysis are seldom accompanied by obvious similarities in antigenic structure and effects in vivo or by homology in nucleic acid and protein sequences that delineate structural or functional parameters (2).

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a wide range of infections. It is the principal pathogen associated with chronic pulmonary infection in patients with cystic fibrosis (CF) (3). In addition to infections in CF patients, *P. aeruginosa* is recognized as a common source of many community-acquired and nosocomial infections (4). Until quite recently, there were only 2 known PLCs of this opportunist, PLC-H and PLC-N (5). However, the sequences of another 2 PLCs, in addition to a PLD and PLA_2 (ExoU), have since been determined (6).

Although food-borne gastroenteritis is the most common malady attributed to *B. cereus* (7), the most devastating is *B. cereus* endophthalmitis (8). *Bacillus cereus* is one of the most common causes of posttraumatic and metastatic bacterial endophthalmitis. It is considered one of the most destructive organisms to affect the eye. *B. cereus* endophthalmitis is quite refractory, and blindness often occurs, even when aggressive and appropriate antimicrobial therapy is instituted before the loss of visual acuity. Even if the infecting organisms are killed, sufficient intraocular toxin concentrations may continue to damage tissue and promote inflammation (9). A capacity of *B. cereus* to release nongastrointestinal infections was described more recently than its gastroenteric effects (10,11). In fact, there has been a noticeable tendency to dismiss aerobic spore-formers in general as harmless saprophytes and secondary wound contaminants (10). In contrast to food poisoning, infections may be severe in these cases. Potentially severe infections have been described in immunologically compromised patients. These infections usually become manifest as bacteremia or septicemia, necrotizing pneumonia, or a combination of these (12). Fatalities have even been reported (11). Invasive infections may also occur in patients with intact immunity. Such infections may become manifest as bacteremia, meningitis

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plus septicemia, endocarditis, or panophthalmitis (12). The general feeling is that *B. cereus* produces exclusively phospholipases of the C type (13). Slein and Logan (14) demonstrated the presence of 4 different phospholipases C in the culture filtrate of *Bacillus cereus*: the so-called phospholipase C that hydrolyzes phosphatidylcholine (PC) and phosphatidylethanolamine, sphingomyelinase, and phosphatidylinositol-specific phospholipase C. PC-preferring and sphingomyelinase-encoding genes form a gene cluster, which is not positioned in close proximity to the gene encoding phosphatidylinositol-specific PLC (15). The contribution of phospholipases to the virulence of *B. cereus* has not been investigated in animals. Although PLC of this bacterium is considered to be nontoxic, data suggestive of its involvement in host cell lysis have been reported (16). Gilmore et al. (15) suggested that by creating a duplex hemolysin, named cereolysin AB, the PC-preferring phospholipase and the sphingomyelinase act in concert to cause hemolysis. Wazny et al. (17) showed that *B. cereus* strains producing PLC cause degranulation of human neutrophils with a dose-dependent release of lysosomal enzymes, which may mediate tissue damage. Finally, it has been suggested that *B. cereus* protects itself against phagocytosis by releasing phospholipases (16).

The purpose of this study is investigating the role PLCs play in the virulence of *Pseudomonas aeruginosa* and *Bacillus cereus*, besides finding out whether their production is plasmid- or chromosome-mediated.

2. Materials and methods

2.1. Chemicals

All chemicals were of high quality from available grades supplied (unless otherwise indicated) by El-Nasr Chemicals (ADWIC), Egypt. Adenosine diphosphate, bovine serum albumin fraction V, ethidium bromide, fetal bovine serum, p-nitrophenylphosphorylcholine, and RNase A were products of Sigma-Aldrich Co., St. Louis, MO, USA. Tissue culture media were purchased in powdered form from Sigma, filter sterilized, and dispensed. The DNA ladder (1.0 kb) and DNA loading buffer were products of Invitrogen, Germany. Lysozyme was a product of Winlab, Laboratory Chemicals, Leicestershire, UK.

2.2. Bacterial strains and maintenance

Isolates D101 and D183 were obtained through screening of 230 clinical isolates for phospholipase production. Both isolates were recovered from pus specimens obtained from patients of El-Demenrdash Hospital, Cairo, Egypt (18). The purified clinical isolates were maintained on nutrient agar slants at 4 °C and subcultured every month. Glycerol stocks were used for the long-term preservation of the isolates (19). A recombinant *Escherichia coli* DH5α (20) harboring the pUC18 plasmid (21) was kindly provided by Dr Khaled Abushanab, Lecturer of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University. This strain was used as a positive control in the plasmid extraction and detection experiments.

2.3. Vero cell line (ATCC No. CCL-81)

Kidney epithelial cells were derived from the African green monkey (Vero cells). The cell line used in this study was an ATCC cell line kindly provided by the Microbiology Department of the Faculty of Medicine for Girls, Al-Azhar University.

2.4. Maintenance of Vero cell line

Mammalian cells were grown in 50-mL sterile tissue culture flasks (Nunclon, Denmark) containing 10–15 mL of BTC medium (Eagle's minimum essential medium with Earle's balanced salts) supplemented with 2% fetal bovine serum and subcultured every 96 h.

2.5. Preparation of mammalian cell monolayer in microtiter plates

Mammalian cells maintained in tissue culture flasks were detached and suspended in propagation tissue culture medium (BTC supplemented with 5% fetal bovine serum). Aliquots of 100 µL of mammalian cell suspension were transferred to wells of a tissue culture plate. The plate was sealed with Parafilm and then incubated at 37 °C for 24 h to form a confluent monolayer.

2.6. Preparation of bacterial cells for cell lysis and adherence/internalization assays

Eighteen-hour trypticase soy broth (Oxoid) cultures of tested microorganisms were centrifuged, washed twice with sterile saline, and then standardized to 2×10^8 CFU/mL using BTC medium. The count was adjusted turbidimetrically at 640 nm with reference to calibration curves constructed for each isolate between the turbidity of the bacterial suspension and the count determined using the viable count technique.

2.7. Enzyme production

Cells from 1-day-old cultures on nutrient agar slants were adjusted to about 2×10^8 CFU/mL turbidimetrically at 640 nm with reference to calibration curves constructed for each isolate. The growth medium used for the production of PLC from *Pseudomonas aeruginosa* D183 was phosphate-starved Tris minimal medium [100 mM Tris-HCl (pH 7.2), 11 mM glucose, 5 mM NH_4Cl, 0.01 mM KH_2PO_4, 0.5 mM K_2SO_4, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$], and from *Bacillus cereus* D101 the same medium was used but was supplemented with 0.05% sodium cholate. Incubation was done at 37 °C in a platform orbital shaker (Orbital Shaker, SO1, Stuart Scientific, UK) at 225 rpm for 48 h.

2.8. Chromogenic enzyme assay

Phospholipase C activity was measured by the method of Kurioka and Matsuda (22). The assay was adapted to a

microtiter system by the method of Berka et al. (23). Four hundred units [1 unit of PLC activity was defined as the amount of enzyme that released 1 nM of p-nitrophenol by hydrolysis of NPPC per minute at 37 °C (23)] of the partially purified PLC was added to 90 µL of NPPC reagent in a microtiter test plate. The NPPC reagent contained 250 mM Tris (hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.2; Oxoid), 60% glycerol (wt/wt), 1.0 µM $ZnCl_2$, and 10 mM NPPC. The plates were then incubated at 37 °C for 17 h before the absorbance at 405 nm was measured with a MicroReader 4 Plus microplate reader (Hyperion. Inc., USA). A yellow color developed in positive cases. A blank containing 10 µL of the clear growth supernatant fluid and 90 µL of NPPC reagent that lacked the chromogenic substrate, and a control containing 10 µL of the uninoculated culture medium and 90 µL of NPPC reagent, were treated similarly and included in runs of the enzymatic assay.

2.9. Partial purification of PLC

Partial purification was carried out as described by Ostroff and Vasil (24). Cells were removed from 500 mL of culture by centrifugation at 6000 rpm for 20 min. Solid ammonium sulfate was added very slowly, while stirring at 4 °C, to the cell-free supernatant until 70% saturation (472 g/L), and the mixture was stirred slowly at 4 °C overnight. The resulting precipitate was pelleted at 18,000 rpm for 20 min at 4 °C using a Beckman J2-HS cooling centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The formed pellet was suspended in 25 mL of 10 mM Tris-hydrochloride, pH 7.2. The suspension was loaded in dialysis tubing (molecular weight cutoff, 6–8000 Da) and dialyzed for 48 h in the cold against the same buffer, which was replaced by fresh buffer after 24 h. Protein concentrations for concentrated culture supernatants were measured by the method of Lowry et al. (25) using bovine serum albumin (Sigma) as a standard.

Partially purified PLC preparations were used for investigating the possible role of PLC from the 2 isolates, D183 and D101, in virulence. The partially purified preparations were tested for protease and lipase activities before use.

2.10. Detection of protease activity of the partially purified enzyme preparations

Protease activity was detected by the gelatin hydrolysis assay on plates as described by Marokhazi et al. (26). Gelatin nutrient agar plates were spot-inoculated with 20 µL of partially purified enzyme preparations. After overnight incubation at 37 °C, the plates were flooded with 5.0 mL of mercuric chloride reagent ($HgCl_2$, 15.0 g; HCl, 20 mL; distilled H_2O to 100 mL), which reveals gelatin hydrolysis as clear (no precipitation) zones around the spots.

2.11. Detection of lipase activity of the partially purified enzyme preparations

Lipase activity was detected by the Tween agar plate technique, as described by Thaler et al. (27). In this method, 20 µL of partially purified enzyme preparation was spot inoculated on the surface of Tween agar plates and incubated at 37 °C overnight. Positive lipase activity was indicated by the presence of an opalescent zone around the enzyme spot.

2.12. Mammalian cell lysis assay

This was done by the crystal violet staining method of Kueng et al. (28). First, the supermerging medium in the wells of the microtiter plates was decanted off the mammalian cell monolayer. In order to study the cytolytic effect of bacterial cells, aliquots (100 µL) of bacterial cell suspension were added to wells of the microtiter plate. To study the cytolytic effect of PLC in the presence of cells of the producing isolate, 100 µL of various dilutions of the partially purified PLC from the isolates D183 and D101 in the BTC medium was added to the corresponding cell suspensions. To study the cytolytic effect of PLC in the absence of cells of the producing isolate, 100 µL of various dilutions of the partially purified PLC from each isolate in the BTC medium was added to the monolayer. Control wells contained 100 µL of the BTC medium and were done in parallel. Incubation was done for 2 h at 37 °C, after which the bacterial cells and/or enzyme together with the detached lysed cells were decanted. The nonlysed cells were fixed with 110 µL of 1% glutaraldehyde in phosphate-buffered saline (PBS). After incubation for 15 min, plates were washed 3 times by submersion in water. Plates were then air-dried and stained by the addition of 100 µL of a 0.1% crystal violet solution (crystal violet 0.1% in distilled water dissolved by shaking at 225 rpm for 30 min, then filtered twice using Whatman No. 1 filter paper) and incubated at room temperature for 20 min. At the end of the incubation period, excess dye was removed by extensive washing with water and plates were air-dried prior to bound dye solubilization in 100 µL of 10% acetic acid. The optical density of dye extracts was measured in plates using a microplate reader at 540 nm.

The percentage of cytotoxicity was calculated according to the following equation:

$$\text{Percentage cytotoxicity} = \frac{(\text{Absorbance of control} - \text{absorbance of test})}{\text{Absorbance of control}} \times 100$$

2.13. Bacterial adherence to and internalization within mammalian cells

An adherence assay was carried out as described by Plotkowski et al. (29). The medium supermerging the mammalian cell monolayer was first discarded. Aliquots of 100 µL of bacterial suspension plus 100 µL of the BTC medium or 100 µL of bacterial suspension plus 100

μL of various dilutions of the corresponding partially purified PLC in the BTC medium were added to the wells of the tissue culture plate containing the mammalian cell monolayer. After incubation for 2 h, the monolayer with adherent bacteria was washed 3 times with PBS. Quantitative determination of the adherent viable bacterial cells was carried out depending on the difference between the total number of bacterial cells (adherent to and uptaken by the mammalian cells) and the number of uptaken bacterial cells. The total number of the cells was determined as follows: after washing the monolayer with PBS, lysis of mammalian cells was carried out by treatment with a lysis solution (0.025% trypsin and 1% Tween 20 in PBS) for 30 min at 37 °C. Aliquots of the cell lysates were properly diluted and placed onto nutrient agar plates for determination of viable count. The number of uptaken cells was determined as follows: after washing the monolayer with PBS, aliquots of 250 μL of gentamycin solution (300 μg/mL) in the BTC medium were added to wells and the plate was left at room temperature for 1 h to allow killing of the adherent bacteria. After removal of the gentamycin solution, the mammalian cells were washed 5 times with PBS and then treated with the lysis solution, and then the number of uptaken cells was determined by viable count technique of the cells on nutrient agar plates.

2.14. Hemolytic activity on mammalian RBCs

The hemolytic activity of the test isolates was assessed as described by Cortajarena et al. (30) and was carried out as follows: a standard human erythrocyte suspension was prepared in PBS by centrifuging 100 μL of blood bank packed red blood cells at 6000 rpm for 5 min. The supernatant was discarded and the pelleted cells were washed twice with PBS before resuspension in 1 mL of PBS. The count was adjusted to 1.5×10^6 red blood cells/mL using a hemocytometer.

For construction of a standard curve for hemolytic activity, 1.5-fold serial dilutions of the standard human erythrocyte suspension in PBS were prepared, to which 1 mL of water was added, vortexed, and incubated for 45 min. The dilutions were then centrifuged at 6000 rpm for 5 min to pellet nonlysed cells. The absorbance of the supernatants was measured at 412 nm. The highest absorbance was assigned the value of 100% hemolysis and the constructed standard curve (Figure 1) was used to deduce the % hemolysis of the other absorptions. Equal volumes (500 μL) of standard erythrocyte suspension and the partially purified enzyme, after its dilution in PBS, were mixed. The mixtures were vortexed and incubated at 37 °C for 45 min, then centrifuged at 6000 rpm for 5 min. The absorbance of the supernatants was measured at 412 nm. Control experiments were carried out as above except that the partially purified enzymes were replaced by 10 mM Tris-hydrochloride (pH 7.2). Measurements were done against blanks containing PBS instead of the partially purified enzyme.

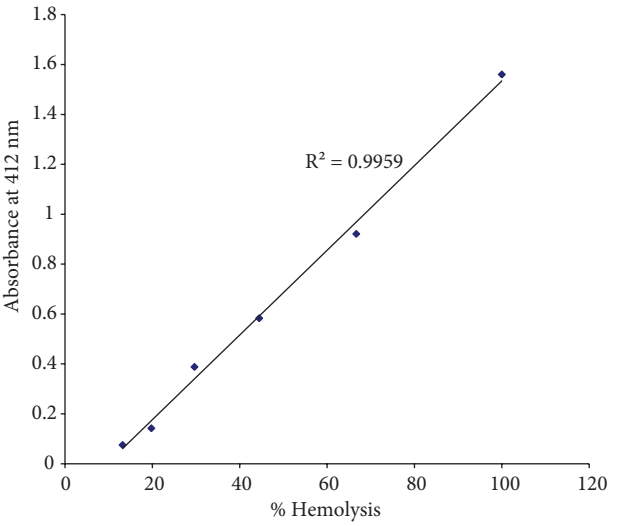

Figure 1. Standard curve for hemolytic activity.

2.15. In vitro platelet aggregation.

The ability of PLC from the tested isolates to induce platelet aggregation was assessed as described by Born and Cross (31) and carried out as follows:

2.15.1. Preparation of platelet-rich plasma

The blood was collected into polypropylene tubes containing a 1/10 volume of 3.8% sodium citrate as the anticoagulant. Platelet aggregation tests were done within 2 h of sample collection to avoid loss of platelet activity. The blood sample was centrifuged at 800 rpm for 10 min. The upper-most layer of the plasma, i.e. platelet-rich plasma (PRP), was aspirated using a micropipette. Platelet counts were performed on a Beckman Coulter Counter Gen-S System 2 (Beckman Coulter, Pasadena, CA, USA). PRP was then diluted with platelet-poor plasma (PPP) to obtain a final count of 300,000 platelets/mL.

2.15.2. Preparation of platelet-poor plasma

After separating PRP, the remaining lower portions were centrifuged at 2000 rpm for 30 min to separate PPP.

2.15.3. In vitro platelet aggregation testing

Platelet aggregation was measured by using a Whole Blood Aggregometer (Chronolog Corporation, Havertown, PA, USA). The reaction mixture consisted of 450 μL of PRP and 50 μL of the partially purified enzyme of the test isolates. Stirring speed was fixed to 800 rpm. The difference in light transmittance between the reaction mixtures and the blank (PPP) was recorded over 1 h on a Chronolog Chart Recorder. Adenosine diphosphate (ADP), 5 μM, was used as a positive control for induction of platelet aggregation.

2.16. Isolation and detection of plasmids

Plasmid extraction was performed using the alkaline lysis methods of Birnboim and Doly (32). *Escherichia coli*

DH5α/pUC 18 (20,21), being a standard strain bearing the pUC plasmid, was used as a positive control. The extraction process was done as follows: 5 mL of LB broth in a 50-mL Erlenmeyer flask was inoculated with a loopful of the test organism and incubated at 37 °C for 12 h with shaking at 250 rpm. An aliquot (1.5 mL) of the obtained culture broth was centrifuged at 10,000 × *g* for 5 min. The cell pellet was resuspended in 100 µL of a GTE buffer [900 mg glucose, 2.5 mL Tris-HCl (121 mg/mL, pH 8), 2 mL Na_2EDTA (186 mg/mL, pH 8), distilled H_2O to 100 mL, sterilized by filtration] and then vortexed gently. In the case of the *Bacillus cereus* isolate only, lysozyme was added to the GTE buffer at 2 mg/mL just before use. A volume of 200 µL of NaOH/SDS lysis solution [1 mL NaOH (40 mg/mL), 0.5 mL SDS (100 mg/mL), distilled H_2O to 100 mL] was added and the tubes were mixed by inversion 6–8 times followed by the immediate addition of 150 µL of a high-salt solution (potassium acetate 29.43 g, glacial acetic acid 11.5 mL, distilled H_2O to 100 mL, sterilized by autoclaving at 121 °C for 15 min). The tubes were then centrifuged at 10,000 × *g* for 15 min. The plasmid (if any) was precipitated with 0.5 mL of isopropanol while keeping the tubes in ice for 10 min. The tubes were then centrifuged at 10,000 × *g* for 30 min. The pellets were dissolved in 40 µL of a TE buffer [1 mL Tris-HCl (121 mg/mL, pH 8), 2 mL Na_2EDTA (186 mg/mL, pH 8), distilled H_2O to 100 mL, pH adjusted to 7.5 with HCl] before adding 10 µL of RNase A solution [200 mg RNase A in 3.3 µL NaOAc (408 mg/mL, pH 4.8), sterile distilled H_2O to 10 mL, and the solution was boiled for 10 min to get rid of DNase]. The tube was vortexed and incubated at 37 °C for 20 to 30 min.

Agarose gel electrophoresis was carried out as described by Sambrook and Russell (33). Electrophoresis was conducted at 200 mA. The electrophoresed gel was stained with ethidium bromide solution (5 mg/mL in distilled water) and visualized using long-wave UV light (302 nm).

3. Results and discussion

3.1. Partial purification of PLC from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101 culture supernatants

The different parameters measured for the partially purified enzyme preparations, as well as the growth supernatants of the selected 2 isolates, are summarized in the Table.

3.2. Investigating the possible role of PLC from the 2 isolates *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101 in virulence

In order to check that the partially purified enzyme preparations were free of proteases and lipases, the other 2 membrane-damaging enzymes (16), tests were performed to detect protease and lipase activities. This was important for studying the possible role of PLC in virulence of the 2 tested isolates.

3.2.1. Detection of protease activity of the partially purified enzyme preparations

The failure of partially purified enzyme preparations to develop clear (no precipitation) zones around their spots on gelatin agar plates after incubation and flooding of mercuric chloride reagent indicated that the preparations were free of protease activity. These results were obtained for the enzyme preparations from both test isolates.

3.2.2. Detection of lipase activity of the partially purified enzyme preparations

The partially purified enzyme preparations from both test isolates did not possess lipase activities, as indicated by the absence of opalescent zones around their spots on Tween agar plates after overnight incubation at 37 °C.

3.3. Effect on mammalian cell lysis

Washed bacterial cells alone caused low cell lysis (4.9% for *Pseudomonas aeruginosa* D183 cells and 16.5% for *Bacillus cereus* D101 cells); however, when partially purified PLC was added, a concentration-dependent increase in cell lysis

Table. Different parameters measured for the partial purified PLC preparations from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101 in comparison to growth supernatants.

Isolate	Fraction	Volume	Total protein	Total activity	Specific activity	Recovery
		(mL)	(mg)	(units)	(units/mg protein)	(%)
P. aeruginosa D183	Growth supernatant	480	141.12	40,680,000	288,265.3	100
	Partially purified preparation	25	16.3	29,687,500	1,821,319.01	72.98
B. cereus D101	Growth supernatant	480	58.79	24,312,000	413,539.71	100
	Partially purified preparation	25	18.55	4,460,000	240,431.27	18.35

was detected until a plateau was reached at 15,000 units for *Pseudomonas aeruginosa* D183 PLC and 5000 units for *Bacillus cereus* D101 PLC (Figures 2a and 2b). Partially purified PLC alone, without bacterial cells, was also tested and similar results were obtained. This may indicate that direct contact between mammalian and bacterial cells is not required for cytotoxicity in this case (34–36). Phospholipids and proteins represent the major chemical constituents of the host cell envelope. Therefore, enzymes capable of hydrolyzing these chemical classes, such as phospholipases and proteinases, are likely to be involved in the membrane disruption processes. By cleaving phospholipids, phospholipases destabilize the membrane and cell lysis results. Consequently, phospholipases have been included among the virulence factors that damage host cells (16).

Vasil (6) reported that it is extremely unlikely that the cytotoxicity of PLC is solely due to its ability to hydrolyze phospholipids. He presented data indicating that not only is the hemolytic PLC (PlcH) of *Pseudomonas aeruginosa* cytotoxic to live eukaryotic cells, it is selectively toxic (i.e. highly toxic to some kinds of cells and minimally toxic or nontoxic to others). He reported that highly purified preparations of PlcH induce programmed cell death (apoptosis) in an assortment of eukaryotic cell types. More exactly, even though it is highly cytotoxic to some cell types (e.g., endothelial cells), PlcH exhibits minimal cytotoxic effects to other cell types. These differences are vast. Because both susceptible and resistant cell lines contain PC in the outer leaflet of their cytoplasmic membranes, it is extremely unlikely that the cytotoxicity of PlcH is solely due to its ability to hydrolyze these phospholipids. Vasil (6) also indicated that PlcH interacts with a specific class of calcium-dependent eukaryotic cell receptors (i.e. integrins). This highly specific cytotoxic nature of PlcH adds a novel dimension to its role in *P. aeruginosa* virulence.

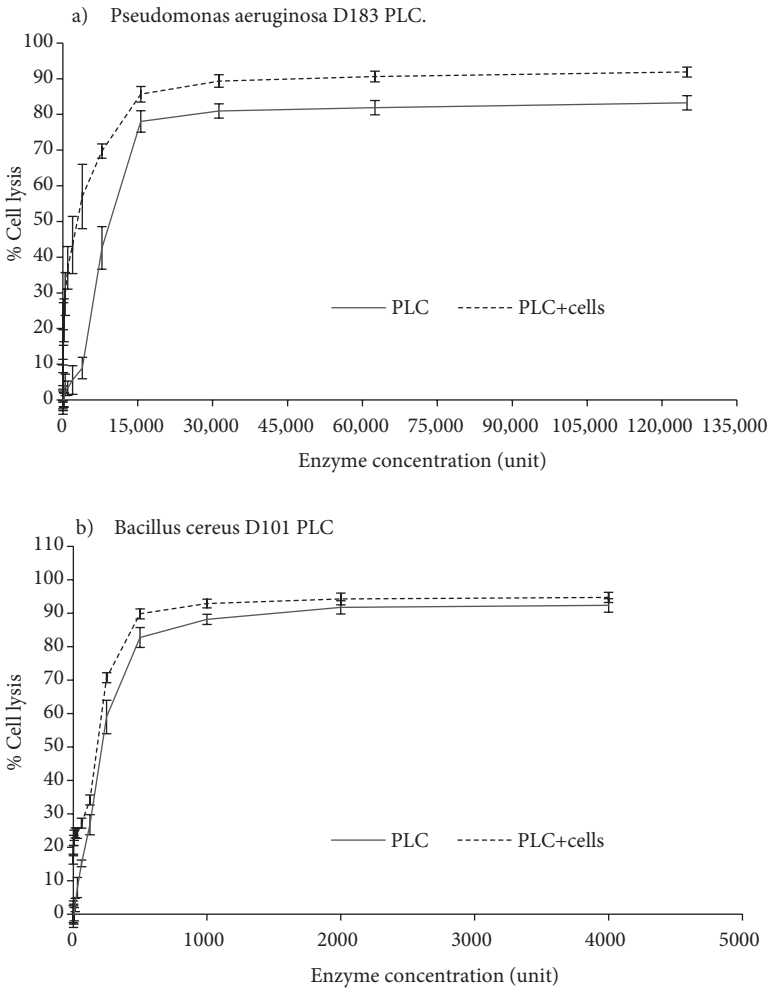

Figure 2. Effect of partially purified PLC from a) *Pseudomonas aeruginosa* D183 and b) *Bacillus cereus* D101 on Vero cell lysis.

Even though *Bacillus cereus* PLC is generally regarded as being of low lethality, especially when compared to the *Clostridium perfringens* α-toxin with which it has some amino acid sequence homology (37), more than one researcher has recorded cytolytic effects of *B. cereus* PLC on a variety of mammalian cell lines. Flores-Diaz et al. (38) provided evidence that PLC from *Bacillus cereus* causes lysis of Chinese Hamster ovary cells. Wazny et al. (17) demonstrated that *Bacillus cereus* PLC induced human neutrophil degranulation at low concentrations and toxicity at high concentrations. Moreover, Rowan et al. (36) reported a role for the enzyme in cytotoxicity against HEp-2 and Caco-2 cells.

3.4. Effect on bacterial adherence to and internalization within mammalian cells

The lowest dilutions of partially purified PLC from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101 that did not cause lysis of Vero cells, and 2-fold serial dilutions starting from these dilutions in the BTC medium were used. The addition of partially purified PLC from *Pseudomonas aeruginosa* D183 to the washed bacterial cells caused an increase in adherence to Vero cells but did not have an effect on internalization (Figure 3a). *Pseudomonas aeruginosa* D183 cells showed 1% adherence to the Vero cell monolayer, and this value increased to 10% upon the addition of dilutions of the partially purified PLC preparation. Likewise, Saiman et al. (39) reported that *Pseudomonas* supernatants containing protease, phospholipase C, and neuraminidase activity increased adherence to cystic fibrosis nasal polyp and normal healthy nasal polyp cells. Saiman et al. (40) also demonstrated that the adherence of *Pseudomonas* to bovine trachea epithelial monolayers was increased 60% in the presence of bacterial supernatants from phosphate-limited cultures and they correlated it with PLC activity in the supernatant. This increase in adherence may be due to the modification of the epithelial surface by *Pseudomonas* PLC (40).

On the other hand, the addition of the dilutions of partially purified PLC preparation did not affect internalization of *Pseudomonas aeruginosa* D183 within Vero cells (10% internalization for washed producing bacterial cells with or without the dilutions of partially purified PLC preparation). Similar results were obtained by Evans et al. (34) in their study of *Pseudomonas aeruginosa* invasion of immortalized rabbit corneal epithelial cells. They reported that the inhibition of *Pseudomonas aeruginosa* PLC did not affect its invasion of the mammalian cells. The participation of PLCs in invasion of host cells is most obvious in *Listeria monocytogenes*, which produces 2 PLCs, a phosphatidylinositol-specific PLC (PI-PLC) and a PC-preferring PLC (PC-PLC). The enzymes function together throughout the course of cellular infection to promote *L. monocytogenes* access to the host cytoplasm, both during the initial invasion of host cells and upon escape from the vacuole and subsequent cell-to-cell spread. A severe defect of the double PLC mutant was seen in the mouse LD_{50} assay. The mutant strain was 500-fold less virulent than the wild type (41).

However, *Bacillus cereus* D101 cells neither adhered to nor invaded Vero cells, and the addition of partially purified PLC preparation did not alter such behavior (Figure 3b). This agrees with the data presented by Ramarao and Lereclus (36), who provided evidence that adhesion of *B. cereus* group members to epithelial cells is strain-dependent and that they are poorly or not invasive.

3.5. Evaluation of hemolytic activity

As shown in Figure 4, partially purified PLC from both isolates caused concentration-dependent hemolysis until plateaus were reached. Action of PLC on the erythrocyte membrane might be divided into 3 steps, as postulated by Taguchi and Ikezawa (42): first, the enzyme attaches to the membrane surface, then it hydrolyzes phospholipids on the outer surface of the membrane, and, finally, as a result of product accumulation and substrate disappearance, the physical state of the membrane is altered. Hemolysis by PLC may be considered due to phase separation of reaction products and other components remaining in the membrane, because the polar parts of the phospholipid molecule (phosphorylamines) are split off by the action of PLC from the unpolar moieties of the substrates such as diglycerides or ceramides (42). The appearance of

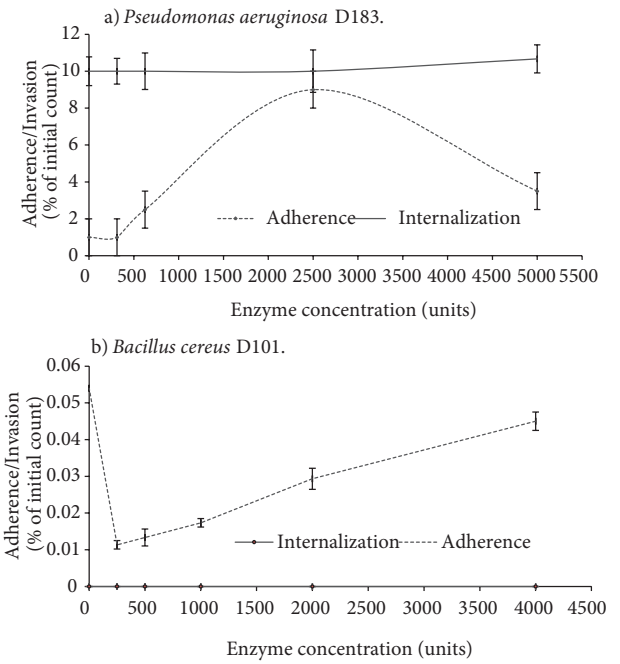

Figure 3. Effect of partially purified PLC from a) *Pseudomonas aeruginosa* D183 and b) *Bacillus cereus* D101 on adherence to and internalization into Vero cells.

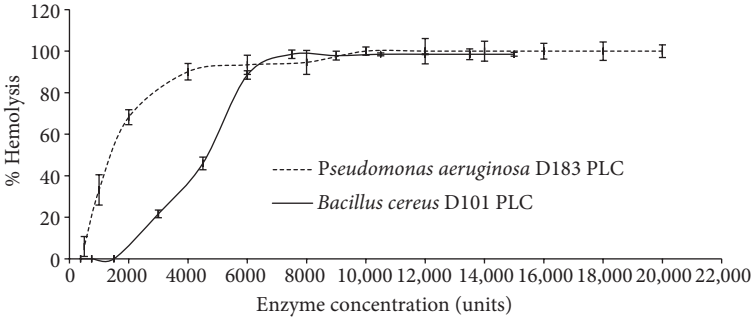

Figure 4. Hemolytic activity of partially purified PLC from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101. Distilled water was used as the positive control, inducing 100% hemolysis, and PBS was used as the negative control, inducing 0% hemolysis.

erythrocytes treated with PLC has often revealed electron-dense intralamellar droplets, which have been presumed to arise from aggregation of the ceramide or diglyceride products of phospholipid hydrolysis. These droplets disappear after digestion with pancreatic lipase or bound lipophilic dyes (38). Cold shock greatly accelerates this phase separation. Thus, hemolysis caused by PLC is classified as a hot-cold type of lysis (42).

Lindgren et al. (43) constructed an in vitro insertion mutation in the PLC gene of *Pseudomonas aeruginosa* PAO, which has been previously cloned and sequenced (44), and used gene replacement techniques to introduce the mutated gene in place of the wild-type gene. The resulting strain was designated PLC S and its colonies were unable to produce a zone of hemolysis on sheep blood agar plates. This led to the conclusion that PLC is responsible for hemolysis caused by *Pseudomonas aeruginosa* strains. For *Bacillus cereus* and *Bacillus thuringiensis*, a transcriptional activator that positively regulates the expression of PLC genes has been identified and named plcR (45). Salamitou et al. (46) constructed mutant strains in which the plcR gene was disrupted. These strains lacked phospholipase C and hemolytic activities (46).

3.6. In vitro platelet aggregation

Although the positive control, ADP (5 μM), induced 80% aggregation of platelets in PRP, none of the tested PLCs were able to induce platelet aggregation. The same results were obtained when the change in light transmittance caused by the tested PLCs was recorded over a whole hour. These results are in accordance with those recorded by Chap et al. (47) and Solberg et al. (48), who provided evidence that PLCs hydrolyze about 45% of the PC content of the outer leaflet of the platelet membrane without inducing aggregation. Conversely, Coutinho et al. (49) reported that PLC from *P. aeruginosa* induces aggregation of human platelets in a concentration-dependent manner.

3.7. Detection of plasmid(s) in *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101

Plasmids (neither of high nor low molecular weight) were not observed in either of the 2 test isolates by the technique used. Thus, it can be concluded that PLC coding genes are located in the chromosomal DNA of the tested isolates and are not plasmid-associated. These results are in agreement with results of studies conducted by Lereclus et al. (45) on PLC production by *Pseudomonas aeruginosa*, which proved that the structural gene for PLC is chromosomally located and not plasmid-located; it is particularly located in the so-called late region of the chromosome, where many of the genes for utilization of various carbon sources are located (43). Similarly, Durban et al. (50) reported that for *Bacillus cereus*, the PC-PLC gene has a length of 852 nucleotides and it is located on the bacterial chromosome. The possible roles of PLC from *Bacillus cereus* D101 and *Pseudomonas aeruginosa* D183 in virulence were studied using the partially purified enzyme preparations. Partially purified PLC from both isolates was found to be cytolytic to Vero cells, in the presence and absence of the washed producing bacterial cells. Dilutions of the partially purified PLC preparations that did not induce cytolysis were tested for an effect on adherence to and internalization within Vero cells. PLC from *Pseudomonas aeruginosa* D183 had a positive effect on adherence but did not affect internalization of the producing cells. However, *Bacillus cereus* D101 cells had negligible adherence to and no internalization within Vero cells, and this behavior was not affected by the addition of the partially purified PLC from the isolate. A hemolytic effect on human red blood cells was recorded for PLC preparations from both isolates, yet they failed to induce aggregation of human platelets. Studying the genetics of PLC production by the 2 isolates revealed that PLC production by each isolate is chromosomal-mediated rather than plasmid-mediated.

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