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Studies of Phospholipase C (Heat-Labile Hemolysin) in *Pseudomonas aeruginosa*

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The chromogenic substrate *p*-nitrophenylphosphorylcholine was used to measure phospholipase C (heat-labile hemolysin) production by clinical strains of *Pseudomonas aeruginosa*. Analysis of strains from various types of infection showed that phospholipase C production by urinary tract isolates was significantly greater than that by lung, blood, or other isolates (e.g., wounds, etc.). The above method was also found to be effective for the isolation of several classes of phospholipase C mutants.

Pseudomonas aeruginosa elaborates several extracellular products which may contribute to its virulence, including two adenosine diphosphate ribosyltransferases (8, 9, 16, 21), several proteases (6, 10, 17), and two hemolysins (16). The role of hemolysins in the pathogenesis of *P. aeruginosa* infections is not well understood. One of the hemolysins is a heat-labile phospholipase C (3), which catalyzes the hydrolysis of phosphatidylcholine, yielding phosphorylcholine and diacylglycerol. The other is a heat-stable glycolipid (22). Both hemolysins are produced during stationary phase in low-phosphate cultures (11, 19). The proposed physiological role of these hemolysins in the organism is to act cooperatively with alkaline phosphatase in liberating inorganic phosphate from phospholipid (11).

Previous studies have implicated the heat-labile hemolysin as a virulence determinant in the pathogenesis of lung infections (11). Significant amounts of phospholipase C are produced when saline bronchial washings from laboratory animals are used as sole nutritional source for *P. aeruginosa* (19). This is particularly interesting because the lungs of humans and animals are covered with surfactant, which is mostly phospholipid. Liu (19) hypothesized that the combination of hemolytic glycolipid and phospholipase C may produce considerable cytopathology to lung tissues during pulmonary infections. Furthermore, Southern et al. postulated that phospholipase C may enhance colonization of lung tissue by *P. aeruginosa* (23). Phospholipase C may also play a significant role in the formation of skin lesions of *P. aeruginosa*. The lesions produced by some live cultures on the skin of rabbits resemble the pathology produced by partially purified preparations of phospholipase C, and not protease- or exotoxin-A-induced pathology (18). Phospholipase C (α-toxin) is also a major dermonecrotic toxin for *Clostridium perfringens* (20).

Genetics and pathogenesis studies of phospholipase C have been hampered by lack of a direct, rapid, and quantitative assay for enzymatic activity. Conventional assays employing phosphatidylcholine hydrolysis do not measure phospholipase C directly and are cumbersome because of substrate insolubility (12, 20). Egg yolk assays are also unsuitable for the study of *P. aeruginosa* phospholipase C because the detergent-like glycolipid hemolysin solubilizes the reaction products (14). We report here the application of a colorimetric assay using *p*-nitrophenylphosphorylcholine (NPPC). An assay of this type was developed by Kurioka and Matsuda (13) for studies of phospholipase C (α-toxin) of *Clostridium perfringens* and is based on the release of the yellow chromogen, *p*-nitrophenol, after hydrolysis of NPPC by phospholipase C. We found the assay to be sensitive and specific for phospholipase C in *P. aeruginosa* (5) and adapted the assay to a microtiter system which permits rapid screening for phospholipase mutants and characterization of clinical strains.

Method 1: phospholipase C production by clinical strains. *P. aeruginosa* strain PAO1 (B. W. Holloway, Monash University, Clayton Victoria, Australia) was used as the standard for comparative purposes in this study. We found that PAO1 produces about the same amount of phospholipase C as PA103 and has a well-characterized genetic map. Clinical strains of *P.*

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aeruginosa were provided by J. Michael Janda (The Mount Sinai Hospital, New York, N.Y.), and cystic fibrosis isolates were obtained from Mary Jane Thomassen (Case Western Reserve, Cleveland, Ohio). Isolated colonies from 5% sheep blood agar plates (Pasco Laboratories, Wheatridge, Colo.) were used to inoculate 2 ml of tryptose minimal medium (24) in capped test tubes (16 by 150 mm). The tubes were shaken vigorously at 32°C for 24 h, and the optical density of each culture was measured at 540 nm. Cells were removed from 1 ml of culture by centrifugation (Beckman Microfuge), and approximately 10 mg of decolorizing carbon was added to each supernatant to remove pigment. This treatment did not remove phospholipase C activity from the supernatant. After centrifuging, 10 μl of the clear supernatant fluid was added to 90 μl of NPPC reagent in a microtiter test plate (96 wells per plate). The NPPC reagent contained 250 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.2), 60% glycerol (wt/wt), 1.0 μM $ZnCl_2$, and 10 mM NPPC (Sigma Chemical Co., St. Louis, Mo.) (13). The plates were then incubated at 37°C for 1 h before the absorbance at 405 nm was measured with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., Rockville, Md.).

Method 2: isolation of phospholipase C mutants. All mutants were derived from strain PAO1 by nitrosoguanidine mutagenesis as described previously (1). Survivors of nitrosoguanidine mutagenesis (95 to 98% killing) were plated on tryptose minimal agar and incubated 48 h at 32°C. Individual colonies were picked from these plates and inoculated into microtiter wells (96 wells per plate) containing 0.1 ml of sterile tryptose minimal medium. After incubation at 32°C for 24 h, the isolates were screened for phospholipase C production by adding one drop (~50 μl) of NPPC reagent to each well. The microtiter plates were then incubated at 37°C for 1 h. Microtiter wells containing phospholipase C producers turned bright yellow under these conditions, whereas phospholipase C-deficient mutants were indicated by colorless or pale yellow reactions. Phospholipase C mutants were found at a frequency of about 0.2%. It should be noted that we attempted to incorporate the artificial substrate NPPC into agar plates and agar overlays; however, the yellow color was rapidly diffusable, and the growth heterogeneity of mutagenized cell populations made screening for mutants on agar plates difficult.

Table 1 shows phospholipase C production by various clinical isolates of *P. aeruginosa*. All of the strains tested produced phospholipase C, but the amount varied with the site of isolation. Surprisingly, urinary tract isolates produced higher levels of phospholipase C than either lung isolates ($P < 0.025$; Student t test) or a category combining blood and other isolates ($P < 0.01$). This finding is interesting in that hemolysin production is also a common property among *Escherichia coli* urinary tract isolates (7). Similarly, Evans et al. found that hemolytic *E. coli* was more frequently isolated from urine than from blood (4). On the average, lung isolates in this study produced levels of phospholipase C approximately equal to those of the standard strain PAO1 (originally isolated from a wound), and blood isolates produced less. However, several of these lung isolates produced very high phospholipase levels. Unfortunately, we lacked the information necessary to determine whether

TABLE 1. *Comparison of phospholipase C production by clinical isolates of P. aeruginosa*

Source	No. of strains tested	No. of positive strains	Range of relative activity[a]	Mean ± SEM[b]	% Greater than PA01 standard[c]
Lung					
Noncystic	15	15	0.1–2.7	0.99 ± 0.21	40
Cystic[d]					
Mucoid	5	5	0.2–1.4	0.79 ± 0.19	20
Nonmucoid	5	5	0.4–2.8	1.67 ± 0.43	80
Urine	10	10	0.5–9.5	3.06 ± 0.96	80
Blood	8	8	0.1–1.4	0.60 ± 0.19	25
Others[e]	6	6	0.2–1.0	0.58 ± 0.14	17

[a] Activity was calculated as the absorbance at 405 nm with NPPC, divided by the optical density of the culture at 540 nm. Relative activity was defined as the ratio of activity of the clinical strains to the activity of PA01.

[b] SEM, Standard error of the mean.

[c] Refers to the percentage of strains from each source producing more phospholipase C activity than PA01.

[d] Cystic fibrosis strains were paired, i.e., one mucoid and one nonmucoid isolate were obtained from each of five patients.

[e] Other isolates included the following: Meatus (1), bile (1), throat (1), tracheal secretion (1), bone (1), and leg ulcer (1).

these high-producing strains were representative of true pneumonias or were only transient respiratory residents. Such information would be useful in deciding whether there is a correlation between phospholipase C production and overt infection rather than transient colonization. Among cystic fibrosis strains of *P. aeruginosa*, we found that 80% of nonmucoid strains and only 20% of mucoid isolates from the same patients produced levels of phospholipase C greater than PAO1. This difference was statistically significant ($P < 0.05$, chi-square analysis) and may be related to primary colonization of cystic patients by nonmucoid strains (2). Southern et al. (23) found that only the high-phospholipase-C-producing strains of *P. aeruginosa* multiplied and that they were cleared more slowly from the lungs of mice than were weak phospholipase producers. It is possible that phospholipase C is important in establishing pulmonary infections by providing nutrients for early growth from phospholipids in surfactant.

A genetic approach offers an opportunity to study phospholipase C pathogenicity and phosphate-scavenging mechanisms. The prospect that phospholipase C may be an important colonization factor of *P. aeruginosa* suggests the potential usefulness of mutants in the development of an effective vaccine. The methods described in this paper may be applied to the isolation of structural (e.g., temperature-sensitive) and other regulatory (e.g., constitutive) mutants, which will be useful in addressing these questions. Table 2 shows the phenotypes of several classes of phospholipase C-deficient mutants. It is important to emphasize that the NPPC assay is the only rapid method available for the isolation of these three mutant classes. A striking aspect of these data is that type I and II mutants are deficient in alkaline phosphatase, as well as phospholipase C. This finding is consistent with the hypothesis that these two enzymes are coordinately regulated (15). It might be argued that these mutants have multiple mutations due to the effects of nitrosoguanidine; however, careful genetic analysis suggests that this is not likely (G. L. Gray and M. L. Vasil, Mol. Gen. Genet., in press). Type I and II mutants will be useful in evaluating the importance of the phosphate-scavenging system (i.e., phospholipase C and alkaline phosphatase) in the establishment of experimental infections. Type III mutants appear to be phospholipase specific and will be used to determine the precise role of phospholipase C in the pathogenesis of *P. aeruginosa* infections.

TABLE 2. *Phospholipase C-deficient mutants of P. aeruginosa*

Mutant class	Phospholipase C	Alkaline phosphatase[a]
Wild-type	++++	++++
I	−	−
II	+	+
III	±	+++

[a] Alkaline phosphatase activity was measured as described previously (5). Kurioka and Liu (11, 19) noted that phospholipase C and alkaline phosphatase appeared to be coordinately expressed.

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