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A *Pseudomonas aeruginosa* Mutant Non-Derepressible for Orthophosphate-Regulated Proteins

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Using a rapid screening assay based on the hydrolysis of *p*-nitrophenylphosphorylcholine, we isolated several mutants of *Pseudomonas aeruginosa* deficient in the production of phospholipase C. One, designated strain A50N, was also markedly deficient in the synthesis of alkaline phosphatase and several unidentified extracellular proteins. Because strain A50N produces these proteins under conditions of derepression at levels equal to those produced by the parental strain PAO1 grown in medium containing excess phosphate, it appears to have a mutation in a genetic element involved in the derepression of phosphate-repressible proteins.

Among the extracellular substances which are postulated to have a role in infections with *Pseudomonas aeruginosa* are two hemolysins (1, 17). One is a detergent-like, heat-stable glycolipid whose composition has been determined (10). The other is a heat-labile phospholipase C (PLC), an enzyme which liberates phosphorylcholine from lecithin (5). The two substances are produced maximally in the stationary phase under growth-limiting phosphate conditions (11, 17, 21). Phosphate starvation also induces an alkaline phosphatase (APase) (4, 9). It has been proposed by Kurioka and Liu (14) that these products function cooperatively as a phosphate-scavenging mechanism. Upon phosphate starvation, the two hemolysins and APase are induced concomitantly. Heat-stable hemolysin solubilizes phospholipids, thus facilitating the action of PLC. The liberated phosphorylcholine is then hydrolyzed by the phosphatase, thus making P_i available to the organism. We note here that it has been shown that APase may be either primarily a periplasmic or extracellular protein, depending on environmental conditions (4), and that under our culture conditions most APase is extracellular (unpublished observations).

Previous assays for *P. aeruginosa* PLC were indirect and time-consuming and therefore not well suited to the screening of large populations for PLC-deficient mutants. Although it should be possible to isolate PLC-deficient mutants by their inability to grow on a medium containing L-α-phosphatidylcholine as the sole source of phosphate, we decided against this for two reasons. First, phospholipids are highly insoluble in aqueous media, and second, such an assay would not be useful in the isolation of several classes of PLC mutants, such as constitutives or hyperproducers. Therefore, a method based on an enzymatic assay was preferred. Kurioka (13) and Kurioka and Matsuda (15) first described the use of *p*-nitrophenylphosphorylcholine (NPPC) for the assay of *Clostridium perfringens* PLC. NPPC is cleaved by the enzyme, yielding phosphorylcholine and the yellow chromogen *p*-nitrophenol. These workers warned that because various PLC differ in their substrate specificities, some of them might be inactive towards NPPC. However, the similarities between the substrate spectra of the PLC produced by *P. aeruginosa* and that produced by *C. perfringens* (1) suggested that NPPC might be hydrolyzed by the former enzyme. We found this to be the case and further observed that the assay conditions described by the Japanese workers were well suited to the assay of *P. aeruginosa* PLC.

Of the survivors of nitrosoguanidine mutagenesis (95% killing) (2) of PAO1 (8) selected for growth on tryptose minimal medium (TMM) (21), about 0.8% were PLC negative, and one was also deficient in APase. In light of Liu's observation that PLC and APase might share common controls (17), this mutant, designated A50N, was chosen for further study.

The availability of a mutant negative in the NPPC assay offered us an opportunity to test whether NPPC hydrolysis in *P. aeruginosa* is due to a true PLC activity or to other reactions which release *p*-nitrophenol, a possibility against which Kurioka and Matsuda have cautioned (15). Fig. 1 shows that the NPPC-positive strain

FIG. 1. *Thin-layer chromatography of lecithin and phosphorylcholine digested by TMM culture supernatants of strain PAO1 and mutant A50N. Thin-layer chromatography was performed by a previously described method (20). Lane a, Lecithin standard; lane b, phosphorylcholine standard; lane c, choline standard; lane d, lecithin plus C. perfringens PLC; lane e, lecithin plus PAO1 supernatant; lane f, lecithin plus A50N supernatant; lane g, phosphorylcholine plus PAO1 supernatant; lane h, phosphorylcholine plus A50N supernatant.*

PAO1 hydrolyzes lecithin, whereas the NPPC-negative mutant A50N is almost totally inactive towards lecithin. These data strongly suggest that NPPC hydrolysis measures PLC activity in *P. aeruginosa*. The failure of A50N to hydrolyze phosphorylcholine (Fig. 1) is in striking contrast to the behavior of the wild-type strain and clearly shows that the capacity to produce APase has been lost concomitantly with PLC production in the mutant.

To test whether the mutation in strain A50N affected the production of extracellular proteins nonspecifically, we compared the proteins produced by A50N and PAO1 in a medium known to give optimal levels of extracellular protein (DTSB) (18). Table 1 shows that both strains produced equivalent amounts of exotoxin A, protease, and total extracellular protein. Furthermore, no differences in the relative quantities of many other extracellular proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were found (data not shown).

Because PLC and APase are known to be produced in growth-limiting phosphate conditions, the possibility that these enzymes were non-derepressible in mutant A50N was considered. Table 2 shows that phosphate starvation of PAO1 caused approximately six- and eightfold increases in APase and PLC (as measured by NPPC hydrolysis) levels, respectively, but failed to stimulate production of these enzymes in A50N. These results strongly suggest that the failure of the mutant to produce wild-type levels of PLC and APase is due to a mutation in a genetic element responsible for phosphate derepression of these enzymes. Furthermore, it appeared that there are other proteins which have also become non-derepressible in A50N because the influence of the mutation, with respect to phosphate concentration, on total extracellular protein parallelled its effect on PLC and APase. A comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of

TABLE 1. *Extracellular proteins produced in DTSB supernatants by strain PAO1 and mutant A50N*[a]

Strain	Exotoxin A (μg/ml per OD unit)	Total protease (U/ml per OD unit)	Total protein (μg/ml per OD unit)
PAO1	0.185	0.50	31.3
A50N	0.209	0.59	34.5

[a] Cultures were grown for 24 h at 32°C (absorbance at 540 nm, ~12) in dialyzed Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented to 1% with glycerol and to 0.05 M with monosodium glutamate (16). Exotoxin A was measured as previously described (3, 19), protease was assayed by the method of Wretlind et al. (22), and total protein was measured as the absorbance at 280 nm, with bovine gamma globulin as a standard. OD, Optical density.

TABLE 2. *Extracellular products of strain PAO1 and mutant A50N in TMM or TMM with 10 mM P_i*[a]

Strain	Activity of:									
	PLC		APase		Heat-stable hemolysin		Heat-labile hemolysin		Total protein	
	$-P_i$	$+P_i$	$-P_i$	$+P_i$	$-P_i$	$+P_i$	$-P_i$	$+P_i$[b]	$-P_i$	$+P_i$
PAO1	56.0	6.6	8.9	1.4	0.5	0.0	84.4	0.0	16.0	1.7
A50N	6.0	5.3	1.1	1.3	0.6	0.0	13.0	0.0	1.4	1.2

[a] PLC activity is expressed as 10^{-5} U/ml per optical density unit of culture. APase activity, measured by the method of Garen and Levinthal (6), is expressed as 10^{-4} U/ml per optical density unit of culture. Hemolytic activities are expressed as units per 25 μl of culture supernatant. A unit of heat-stable hemolysin is defined as 1 mm^2 of hemolysis produced by boiled supernatants on 4°C sheep blood agar plates (Pasco Laboratories, Wheatridge, Colo.). Total hemolysis is defined as the hemolytic area produced by unboiled supernatants on 37°C plates. A unit of heat-labile hemolysin is defined as 1 mm^2 of total hemolysis minus hemolysis due to heat-stable glycolipid. Total protein is expressed as micrograms per milliliter per optical density unit of culture. $-P_i$, Phosphate-deficient medium; $+P_i$, phosphate-supplemented medium.

[b] Because heat-stable hemolysin is known to strongly potentiate the activity of PLC toward (water-insoluble) phospholipids (14), the absence of heat-labile hemolytic activity in excess-phosphate medium may be largely due to the coordinate repression of heat-stable hemolysin.

culture supernatants of A50N and PAO1 strongly supported this idea (Fig. 2). At least six proteins appeared to be derepressed in PAO1 by phosphate starvation (positions indicated by arrows). Interestingly, mutant A50N, under growth-limiting phosphate conditions, failed to produce these same proteins at derepressed levels but appeared to be unaltered with respect to production of proteins not regulated by phosphate. In contrast, the phosphate regulation of the heat-stable (glycolipid) hemolysin appeared to be unaffected by the mutation (Table 2).

Since Esselmann and Liu (5) first identified the heat-labile hemolysin as a PLC, its role in the physiology and pathogenesis of *P. aeruginosa* has been a subject of continued speculation. However, the study of this PLC has been severely restricted by the unavailability of a convenient assay. The application of Kurioka's NPPC assay to *P. aeruginosa* as described here should greatly facilitate physiological and genetic studies of PLC in this organism. For example, it may be used for the isolation of PLC mutants of various classes.

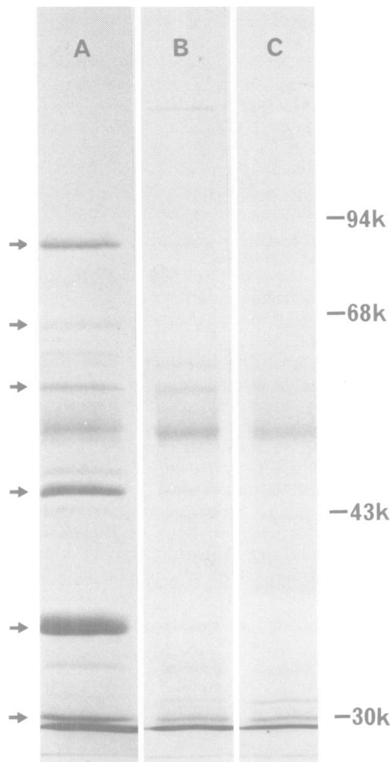

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins produced in TMM or TMM with 10 mM P_i by P. aeruginosa parental strain PAO1 and mutant A50N. The electrophoresis was performed as elsewhere described (7). Arrows indicate positions of phosphate-repressible proteins. Lane A, PAO1 grown in TMM; lane B, PAO1 grown in TMM with 10 mM P_i; lane C, A50N grown in TMM. Positions of molecular weight markers are shown.*

Because P_i starvation enhances the production of PLC, APase, and heat-stable hemolysin, it has been suggested that these extracellular substances might be functionally related as a phosphate-scavenging mechanism. It therefore seemed likely to us that their syntheses might be regulated coordinately at the genetic level. This hypothesis predicts that single mutations might result in a deficiency in two or more of these products. Mutant A50N is deficient in both PLC and APase but appears to be unaltered in its synthesis of hemolytic glycolipid. This suggests that although all three products are phosphate repressible, regulation of the synthesis of heat-stable hemolysin is at least partially independent of that of PLC and APase. Because A50N secretes the same types and quantities of proteins into DTSB as the parental strain, it appears not to be deficient in protein secretion. The possibility that strain A50N has a phosphate-dependent secretory defect was ruled out by showing that the relative proportions of cell-associated and extracellular PLC and APase in A50N were similar to those in PAO1 (data not shown). The possibility that the parental strain PAO1 produces larger amounts of PLC, APase, and total extracellular protein in phosphate-deficient medium than in phosphate-supplemented medium due to increased cell lysis and that therefore strain A50N might have a mutation leading to increased cell wall stability in low-phosphate medium was considered. However, this appeared not to be the case for two reasons. First, microscopic examination of PAO1 cells grown in TMM revealed no cell lysis. Second, sonicates of PAO1 cells grown in TMM supplemented with P_i contained little PLC or APase. It seems more likely that A50N is non-derepressible for PLC, APase, and several unidentified extracellular proteins because it synthesizes them in both limited phosphate and excess phosphate growth conditions at the repressed level of PAO1 (Table 2 and unpublished observations).

Because strain A50N was isolated by nitrosoguanidine mutagenesis, it was necessary to consider whether its pleiotropic changes in extracellular products were due to multiple mutations. However, PLC-deficient mutants which were also deficient in APase were too frequent (about 50%) to be accounted for by comutation. Furthermore, mapping of the mutation in A50N showed that transfer of APase deficiency always accompanies PLC deficiency in recipient strains (G. L. Gray, and M. L. Vasil, Mol. Gen. Genet.,

in press). These data strongly suggest that PLC and APase deficiencies in strain A50N result from a single mutation.

Mutations in *Escherichia coli* leading to nonderepressibility of APase and at least three other unidentified periplasmic proteins (18) have been shown by complementation analysis to be in two cistrons encoding two diffusible proteins which positively regulate the synthesis of these periplasmic proteins (12). The exact nature of the mutation in mutant A50N is unknown at present. A genetic analysis of additional, newly isolated mutants with the PLC-negative phenotype of A50N is presently being conducted and should help to further define the genetics of regulation of phosphate-repressible proteins in *P. aeruginosa*.

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LITERATURE CITED

1. **Avigad, G.** 1976. Microbial phospholipases, p. 99-168. *In* A. W. Bernheimer (ed.), Mechanisms of bacterial toxinology. John Wiley & Sons, Inc., New York.
2. **Baine, W. B., M. L. Vasil, and R. K. Holmes.** 1978. Genetic mapping of mutations in independently isolated nontoxinogenic mutants of *Vibrio cholerae*. Infect. Immun. **21:**194-200.
3. **Bjorn, M. J., B. H. Iglewski, S. K. Ives, J. C. Sadoff, and M. L. Vasil.** 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA-103. Infect. Immun. **19:**785-791.
4. **Cheng, K.-J., J. M. Ingram, and J. W. Costerton.** 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of *p*H. J. Bacteriol. **104:**748-753.
5. **Esselmann, M. T., and P. V. Liu.** 1961. Lecithinase production by gram-negative bacteria. J. Bacteriol. **81:** 939-945.
6. **Garen, A., and C. Levinthal.** 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochem. Biophys. Acta **38:**470-483.
7. **Gray, G. L., and M. L. Vasil.** 1981. Isolation and genetic characterization of toxin-deficient mutants of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **147:**273-279.
8. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbial. Rev. **43:**73-102.
9. **Hou, C. I., A. F. Gronlund, and J. J. R. Campbell.** 1966. Influence of phosphate starvation on cultures of *Pseudomonas aeruginosa*. J. Bacteriol. **92:**851-855.
10. **Jarvis, F. G., and M. J. Johnson.** 1949. A glycolipid produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. **71:**4124-4126.
11. **Johnson, M. K., and D. Boese-Marrazzo.** 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect. Immun. **29:**1028-1033.
12. **Kreuzer, K., C. Pratt, and A. Torriani.** 1975. Genetic analysis of regulatory mutants of alkaline phosphatase of *E. coli*. Genetics **81:**459-468.
13. **Kurioka, S.** 1968. Synthesis of *p*-nitrophenylphosphorylcholine and the hydrolysis with phospholipase C. J. Biochem. (Tokyo) **63:**678-680.
14. **Kurioka, S., and P. V. Liu.** 1967. Effect of the hemolysin of *Pseudomonas aeruginosa* on phosphatides and on phospholipase c activity. J. Bacteriol. **93:**670-674.
15. **Kurioka, S., and M. Matsuda.** 1976. Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75:**281-289.
16. **Liu, P. V.** 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. **128:**506-513.
17. **Liu, P. V.** 1979. Toxins of *Pseudomonas aeruginosa*, p. 63-88. *In* R. G. Doggett (ed.), *Pseudomonas aeruginosa*. Academic Press, Inc., New York.
18. **Morris, H., J. Schlesinger, M. Bracha, and E. Yagil.** 1974. Pleiotropic effects of mutations involved in the regulation of *Escherichia coli* K-12 alkaline phosphatase. J. Bacteriol. **119:**583-592.
19. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28:**899-908.
20. **Okawa, J., and T. Yamaguchi.** 1975. Studies on phospholipases from *Streptomyces*. III. Purification and properties of *Streptomyces hachijoensis* phospholipase C. J. Biochem. **78:**537-545.
21. **Stinson, M. W., and C. Hayden.** 1979. Secretion of phospholipase C by *Pseudomonas aeruginosa*. Infect. Immun. **25:**558-564.
22. **Wretlind, B., L. Sjöberg, and T. Wadström.** 1977. Protease deficient mutants of *Pseudomonas aeruginosa:* pleiotropic changes in activity of other extracellular enzymes. J. Gen. Microbiol. **103:**329-336.