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Mutations in the Hemolytic-Phospholipase C Operon Result in Decreased Virulence of *Pseudomonas aeruginosa* PAO1 Grown under Phosphate-Limiting Conditions

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The phospholipase C (PLC) operon of *Pseudomonas aeruginosa* consists of *plcS*, which encodes a heat-labile secreted hemolysin, and two in-phase, overlapping genes, *plcR1* and *plcR2*, which may encode P_i-regulatory genes. A 2.8-kilobase-pair deletion mutation in this operon was constructed, and a tetracycline resistance (Tc^r) cartridge replaced the deleted sequences. A deletion mutant of strain PAO1 was obtained through recombination between the flanking regions of the mutated cloned PLC operon and the homologous chromosomal regions. The deletion of the chromosomal PLC operon and its replacement by the Tc^r cartridge was confirmed by Southern hybridization. The deletion strain, PLC SR, is nonhemolytic. However, it retains PLC activity when measured on a synthetic substrate. A second mutant strain, PLC R, contains a deletion in the *plcR* genes. This mutant is more hemolytic and produces more enzymatic activity than PAO1. The virulence of both of these mutants was compared with that of PAO1 in the mouse burn model of infection. When mice were infected with cultures grown in a high-P_i medium, there was a 10-fold increase in the 50% lethal dose of the mutants compared with PAO1. In contrast, when the inoculum originated from low-P_i cultures, there was a 200- to 10,000-fold increase in the 50% lethal dose of the mutants over PAO1.

Pseudomonas aeruginosa is an important opportunistic pathogen which elaborates several extracellular products that may contribute to pathogenesis. Many of these products are produced when specific environmental factors are limiting. For example, exotoxin A, elastase, and alkaline protease are maximally produced under low-iron conditions (4). Several exoproducts are synthesized when the P_i concentration is low, including alkaline phosphatase, two hemolysins (one a glycolipid and one a phospholipase C [PLC-H]), and a nonhemolytic PLC (PLC-N) (15, 18). Environmental conditions have been shown to coordinately regulate the production of several virulence factors by other pathogenic organisms, including yersiniae (5), *Bordetella pertussis* (11), and *Vibrio cholerae* (24).

Liu (15) proposed that the P_i-regulated products of *P. aeruginosa* function cooperatively as a P_i scavenging system. This system may be critical to pathogenesis, because it has been observed that in humans infected with gram-negative pathogens, the quantity of P_i in plasma is reduced to a level suboptimal for bacterial growth (27).

In addition, the PLC-H may be an important toxic factor. Berk et al. (2) have shown that purified PLC-H causes paralysis, dermonecrosis, vascular permeability, and death when injected into mice. PLC-H also causes human platelet aggregation (7). The primary substrate for both PLC-H and PLC-N is phosphatidylcholine, which is the major component of lung surfactant (3, 8, 18). Degradation of surfactant may provide essential nutrients including P_i, enhance lung colonization, and contribute to atelectasis (14).

The structural gene encoding PLC-H (*plcS*) has been cloned and sequenced (6, 16, 19, 25) and is part of a three-gene operon regulated by P_i at the level of transcription (19, 20). *plcS* is the first gene of the operon and encodes an 82.6-kilodalton protein, which may be processed to yield the secreted 78.2-kilodalton hemolysin (19). Downstream of *plcS* are two in-phase, overlapping genes, *plcR1* and *plcR2* (19, 20). The function of the *plcR* gene products is not clear, but preliminary evidence suggests a role in the regulation of PLC-H and other secreted P_i-regulated proteins (R. Ostroff and M. Vasil, unpublished results).

To investigate the role of PLC-H in pathogenesis and to study its regulation, an insertional mutation was created in the *plcS* gene of the *P. aeruginosa* chromosome (18). Analysis of this mutant led to the identification of a second PLC enzyme, which is P_i regulated and secreted, but nonhemolytic (18). In the present study, two additional mutations in the *plcSR* operon are described: (i) a deletion mutation in all three genes of the operon, and (ii) a deletion mutation in the *plcR* region of the operon. Gene replacement methods were used to incorporate the mutated sequences into the *P. aeruginosa* chromosome in place of the wild-type *plcSR* operon in strain PAO1. The virulence of these mutants when grown under high- or low-P_i conditions was compared with that of the wild-type strain by using the mouse burn model of infection.

MATERIALS AND METHODS

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

Media and antibiotics. *Escherichia coli* cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C. Tris minimal medium (TMM) (23) or peptone medium (1% peptone, 1% NaCl, 1% glycerol) (12) was used for production of PLC from *P. aeruginosa* with or without the addition of 10 mM P_i. Blood agar (5% sheep erythrocytes) (Pasco Laboratories, Wheatridge, Colo.) was used to screen hemolytic activity. Pseudomonas isolation agar (Difco) supplemented with the appropriate antibiotics was used to select for *P. aeruginosa* in the mating experiments. Antibiotics were used in the following concentrations

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
Strain		
E. coli S17-1	*thi pro hsdR hsdM*$^+$ *recA* integrated RP4-2-Tc^r::Mu Kn^r::Tn*7*	21
P. aeruginosa PAO1	Prototroph *chl-3*	10
P. aeruginosa PLC S	*plcS*::Tc^r	18
P. aeruginosa PLC R	Δ*plcR*::Tc^r	This study
P. aeruginosa PLC SR	Δ*plcSR*::Tc^r	This study
Plasmid		
pGV26	Ap^r-Cb^r *plcSR*	25
pSUP203	Ap^r-Cb^r Cm^r Tc^r *mob*	21
ColE1::Tn*5-132*	Tc^r	1
pRR2	pSUP203 with Δ*plcR*::Tc^r	This study
pRR3	pBR322 with Δ*plcSR*::Tc^r	This study
pRR4	pSUP203 with Δ*plcSR*::Tc^r	This study

[a] Tc^r, tetracycline resistance; Kn^r, kanamycin resistance; Ap^r-Cb^r, ampicillin-carbenicillin resistance; Cm^r, chloramphenicol resistance; *mob*, P-type specific recognition site for mobilization.

(milligrams per liter): for *E. coli*, carbenicillin, 100, and tetracycline, 20; for *P. aeruginosa*, carbenicillin, 1,000, and tetracycline, 200.

Isolation and manipulation of DNA and Southern blot hybridization. Conditions for DNA purification and manipulation for cloning were as described previously (17). Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as indicated by the supplier. Genomic DNAs of *P. aeruginosa* strains were isolated and digested as previously described (26). Southern blot hybridization and high-stringency washes were also performed as previously described (26).

Transfer of plasmids from *E. coli* to *P. aeruginosa*. Plasmid transfer matings were performed as previously described (18).

PLC assay. PLC was measured by using the method of Kurioka et al. (13), in which the hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC; Sigma Chemical Co., St. Louis, Mo.) by supernatants from cultures grown in TMM medium with or without 10 mM added P_i is monitored.

Hemolytic titers. Serial twofold dilutions of supernatants from cultures grown in peptone medium with or without the addition of 10 mM P_i were added to 0.4% sheep erythrocytes. The hemolytic activity was estimated visually by determination of the release of hemoglobin after 24 h of incubation at 37°C.

Mouse burn model of infection. The bacteria were grown in peptone medium overnight with or without 10 mM P_i added. The cultures were prepared, and CBA mice were inoculated as described by Wretlind et al. (29). The CFUs of the log-phase cultures were determined, and 0.1 ml of serial 10-fold dilutions was injected into each animal (five animals per dilution). The 50% lethal doses (LD_{50}s) were calculated by the Spearman-Kärber method (9).

RESULTS

Construction of deletion mutants. The *plcSR* operon is composed of three genes, *plcS*, which encodes a hemolytic PLC, and two in-frame, overlapping genes, *plcR1* and *plcR2*. (Fig. 1). Insertion and deletion mutations have been constructed in this operon and introduced into the chromosome in place of the wild-type locus through homologous recombination. A tetracycline resistance (Tc^r) cartridge has been inserted into *plcS*, resulting in a nonhemolytic mutant of strain PAO1, designated PLC S (18). However, PLC S retains PLC enzymatic activity when measured on synthetic substrates.

To eliminate the possibility that the PLC activity was due to a truncated *plcS* product, we constructed a deletion mutant (Fig. 2). The *plcSR* operon was cloned as a 6.1-kilobase-pair (kb) *Bam*HI fragment (25) into pBR322. This plasmid, pGV26, was digested with *Sma*I, *Bgl*II linkers were ligated to the blunt *Sma*I ends, and then pGV26 was digested with *Bgl*II. The resulting plasmid contains a deletion in the *plcSR* sequences extending from 14 base pairs upstream of

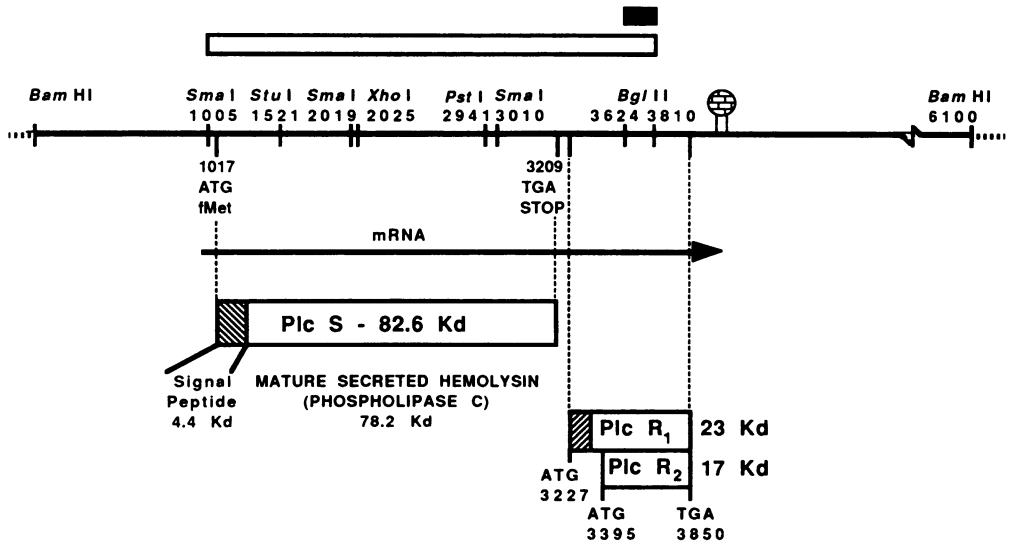

FIG. 1. Diagram of the 6.1-kb *Bam*HI-*Bam*HI DNA fragment containing the *plcSR* operon. The transcriptional terminator is indicated by the stem-loop structure. Symbols: ■, boundaries of the deletion in PLC R; □, extent of the deletion in PLC SR. These deleted sequences were replaced by a Tc^r cartridge as described in the text. Diagrammed below the restriction map are the mRNA and proteins synthesized from this fragment (19, 20). Kd, Kilodaltons.

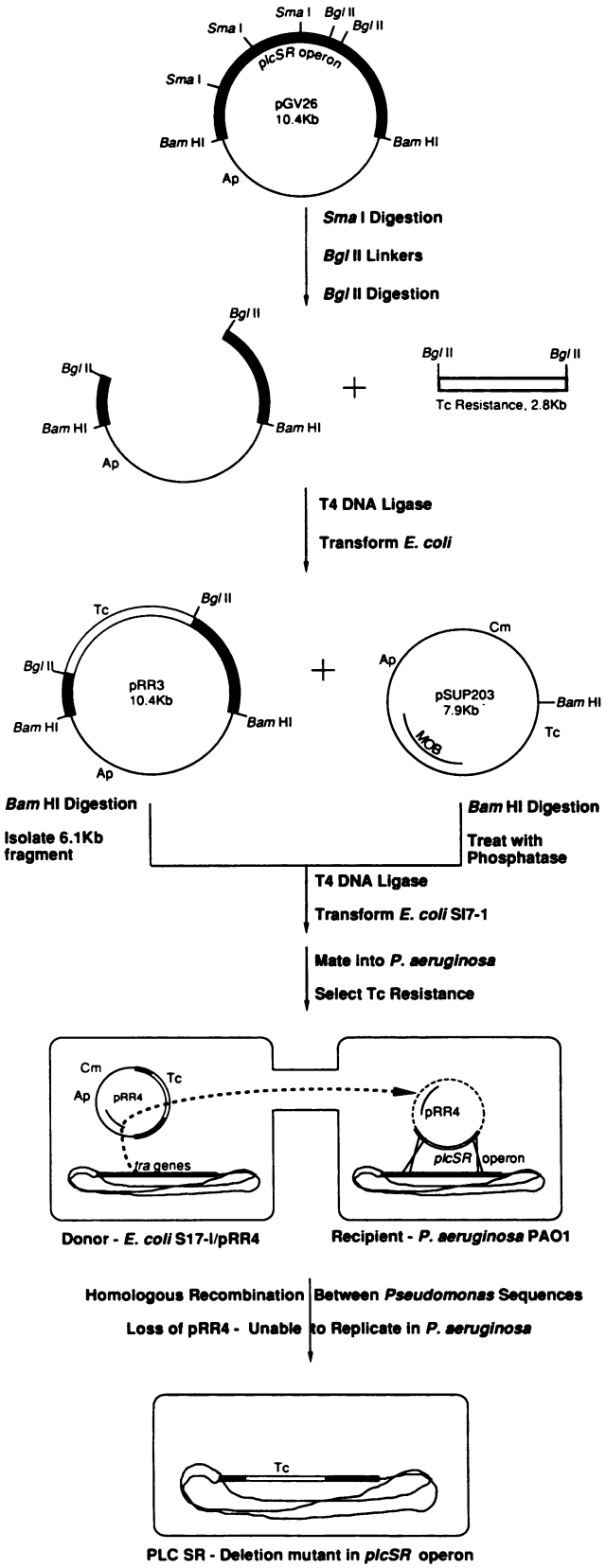

FIG. 2. Construction of the deletion mutant strain PLC SR. See text for details.

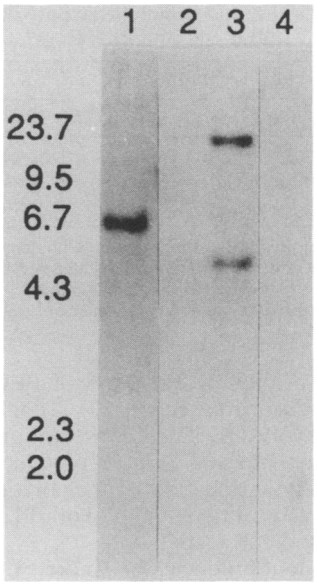

FIG. 3. Southern blot of PAO1 and PLC SR hybridized with the nick-translated 1.4-kb *StuI-PstI plcS* probe. Lanes: 1, PAO1 digested with *Bam*HI; 2, PLC SR digested with *Bam*HI; 3, PAO1 digested with *Xho*I; 4, PLC SR digested with *Xho*I. Molecular size markers are indicated in kilobases.

the *plcS* initiation codon to 38 base pairs 5′ of the *plcR1* and *plcR2* termination codon. These deleted sequences were replaced by a 2.8-kb Tc^r fragment from Tn*5-132*, resulting in pRR3. The 6.1-kb *Bam*HI fragment containing the deletion construction was cloned into the *Bam*HI site of pSUP203, resulting in pRR4. pSUP203 is a derivative of pBR325 which contains the *mob* site from RP4 (21). When this vector is present in the *E. coli* host S17-1, transfer of the plasmid is facilitated by the action of the transfer proteins, encoded by a chromosomally integrated RP4, on the *mob* site. This system allows efficient transfer of plasmids between gram-negative organisms such as *E. coli* and *P. aeruginosa*. Plasmid pRR4 was transferred to PAO1, and Tc^r Ap^s *P. aeruginosa* strains were selected. Since pRR4 is unable to replicate in *P. aeruginosa*, Tc^r Ap^s clones result from recombination on either side of the sequences flanking the *plcSR* deletion in the plasmid and the homologous chromosomal sequences.

The deletion of the chromosomal *plcSR* operon and its replacement by the Tc^r cartridge was confirmed by Southern hybridizations. Figure 3 is a Southern blot in which an internal *StuI-PstI plcSR* probe was used; it illustrates that the deletion mutant does not hybridize to this probe. The absence of vector sequences was also confirmed by Southern hybridizations (data not shown). This mutant is designated PLC SR.

Similar methods to those described above were used to construct a mutant with another mutation in the *plcSR* operon, PLC R. PLC R contains a deletion of the 186-base-pair *Bgl*II fragment of the *plcR* genes. A Tc^r cartridge was also cloned into the *plcSR* operon in place of these deleted sequences, and the same gene replacement system was used to produce a chromosomal mutation in the *plcR* genes.

PLC and hemolytic activities of PAO1, PLC SR, and PLC R. The *plcSR* deletion mutant is nonhemolytic, yet it retains PLC enzymatic activity when measured on the synthetic

TABLE 2. PLC and hemolytic activity of PAO1, PLC SR, and PLC R

Strain	PLC activity[a]		Hemolysis[b]	
	+10 mM P_i	$-P_i$	+10 mM P_i	$-P_i$
PAO1	2.35	26.34	64	939
PLC SR	0.30	18.28	<2	<2
PLC R	1.90	38.33	352	1,024

[a] PLC activity is expressed as the hydrolysis of the synthetic substrate NPPC by culture supernatants in units per milliliter per A_{540} of the culture.
[b] Hemolytic titers are expressed as the mean of the reciprocal of the highest dilution of culture supernatant yielding the release of hemoglobin from sheep erythrocytes.

substrate NPPC (Table 2). The level of PLC activity produced by PLC SR is decreased by 31% as compared with the level produced by PAO1. These observations confirm our earlier hypothesis that *P. aeruginosa* produces two different PLCs, one hemolytic and one nonhemolytic (PLC-H and PLC-N, respectively). Interestingly, both PLCs are P_i regulated and are found extracellularly.

The PLC R mutant produces 31% more PLC activity than PAO1 does. The hemolytic activity appears to be greater and less well regulated by P_i in PLC R than in PAO1.

Virulence of PLC mutants and PAO1 in the mouse burn model of infection. The LD_{50} for each PLC operon mutant and PAO1 was determined by using the mouse burn model of infection. Cultures were grown overnight with or without the addition of 10 mM P_i, and then various dilutions were used for infection. There was no difference in the growth rate of the mutants versus the wild type in either high- or low-P_i media (data not shown). There was approximately a 10-fold difference between the LD_{50}s of the mutant strains and PAO1 when the strains were grown under P_i-sufficient conditions (Table 3). However, under P_i-limiting conditions, there was about a 200-fold decrease in the LD_{50} of PAO1 compared with that of PLC S and PLC R. Additionally, the decrease in the LD_{50} of PAO1 compared with that of PLC SR grown under low-P_i conditions was 10,000-fold. The difference in the number of animal deaths induced by injection of low-P_i cultures, all mutants versus wild type, was significant ($P < 0.01$) by the Fischer exact test.

It should also be noted that virulence in these studies is P_i dependent. Therefore, the decreased virulence of the mutants is not simply due to the presence of the Tc^r gene cartridge. In addition, similar gene replacement mutations in the exotoxin A gene of *P. aeruginosa* M2 containing the Tc^r cartridge are not altered in virulence in this animal model (data not shown).

DISCUSSION

We have presented data illustrating the role of the *plcSR* operon in the virulence of *P. aeruginosa* in mouse burn infection. Well-defined mutants which are isogenic with the wild-type PAO1 except at the specific locus of the *plcSR* operon targeted for mutagenesis were tested. When the strains were grown under P_i-limiting conditions, the virulence of *plcSR* operon mutants was decreased 200- to 10,000-fold in comparison with PAO1. There was not as large a difference between the LD_{50}s of the mutants and the wild type when the strains were grown in high-P_i media.

TABLE 3. LD_{50} of PAO1 and PLC mutants in the mouse burn model of infection

Strain	Log_{10} LD_{50}[a]	
	$+P_i$	$-P_i$
PAO1	5.53	2.99
PLC S	6.94	5.39
PLC R	6.11	5.53
PLC SR	6.80	7.02

[a] Cultures were grown overnight in peptone medium with or without the addition of 10 mM P_i and then diluted and injected into the burn wound of CBA mice.

P_i-limiting conditions induce the expression of PLC as well as several other P_i-regulated products such as the heat-stable glycolipid hemolysin, alkaline phosphatase, and P_i transport proteins. These products are proposed to act synergistically as a P_i-scavenging system (15). The absence of one of these components may impede the ability of the organism to acquire P_i during P_i limitation.

Similarly, the iron concentration of the culture medium used to prepare *P. aeruginosa* inocula influences pathogenesis. The corneal damage produced by PAO1 grown in a high-iron medium was considerably reduced in comparison with damage produced by PAO1 grown in a low-iron medium (28). Additionally, it has been shown that mutants defective in iron acquisition are unable to colonize injured corneas or mouse burns (22).

It is surprising that although PLC R is more hemolytic than PAO1, it is still less virulent when grown under P_i-limiting conditions. This result implies a role for the *plcR* gene products in pathogenesis. Each PLC mutant described has a mutation in the *plcR* genes. The PLC S insertional mutation may produce polar effects on the expression of the downstream *plcR* genes. PLC SR and PLC R both contain deletions in the *plcR* genes. At this time, the role of the *plcR* gene products remains unclear. Preliminary evidence suggests that the *plcR* products affect the expression of several P_i-regulated genes (Ostroff and Vasil, unpublished). Perhaps *plcR1* and *plcR2* encode regulatory proteins necessary for efficient expression or activation of P_i-regulated proteins. These observations stress that to understand pathogenesis, it is important to examine the mechanism for P_i regulation of the *plcSR* operon and other P_i-regulated products in addition to studying single virulence factors.

Another possibility is that the *plcR* products are required for colonization. The high LD_{50}s of each mutant under both high- and low-P_i growth conditions may indicate that these strains lack the ability to colonize in the mouse burn model. If this is true, it is difficult to assess the pathogenic potential of PLC-H or PLC-N in this infectious model by using the present mutants. Additional mutants in which the expression of the *plcR* genes is not affected must be used if these questions are to be answered. If an organism is unable to establish infection, it is difficult to assess the contribution of a specific toxin to pathogenesis. For example, Sokol (22) reported that a mutant defective in iron acquisition and lacking surface expression of ferripyochelin-binding protein was less virulent than the wild-type strain and was unable to disseminate in the mouse burn model, although it produced twice as much exotoxin A as the parental strain did.

Although PLC S and PLC SR are defective in PLC-H production, they still produce PLC-N. PLC-N is P_i regulated and is found extracellularly. It is known that supernatants of PLC-H mutants are capable of releasing P_i from detergent-solubilized phosphatidylcholine (18). Perhaps PLC-N plays a role in P_i scavenging after complex eucaryotic membrane structures are degraded by the hemolysins to more soluble phospholipid molecules. Interestingly, PLC-N shares some DNA homology with PLC-H (Ostroff and Vasil, unpublished). Further studies aimed at characterizing PLC-N in

comparison with PLC-H to examine structure-function relationships determining PLC enzymatic activity and hemolytic activity will be of interest.

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LITERATURE CITED

1. **Berg, D. E., C. Egner, B. J. Hirschel, J. Howard, L. Johnsrud, R. A. Jorgensen, and T. D. Tlsty.** 1980. Insertion, excision, and inversion of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. **45**:115–123.
2. **Berk, R. S., D. Brown, I. Coutinho, and D. Meyers.** 1987. In vivo studies with two phospholipase C fractions from *Pseudomonas aeruginosa*. Infect. Immun. **55**:1728–1730.
3. **Berka, R. M., and M. L. Vasil.** 1982. Phospholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J. Bacteriol. **152**:239–245.
4. **Bjorn, M. J., P. A. Sokol, and B. H. Iglewski.** 1979. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. J. Bacteriol. **138**:193–200.
5. **Brubaker, R. R.** 1972. The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. Immunol. **57**: 111–158.
6. **Coleman, K., G. Dougan, and J. P. Arbuthnott.** 1983. Cloning, and expression in *Escherichia coli* K-12, of the chromosomal hemolysin (phospholipase C) determinant of *Pseudomonas aeruginosa*. J. Bacteriol. **153**:909–915.
7. **Coutinho, I. R., R. S. Berk, and E. Mammen.** 1988. Platelet aggregation by a phospholipase C from *Pseudomonas aeruginosa*. Thromb. Res. **51**:495–505.
8. **Esselmann, M. T., and P. V. Liu.** 1961. Lecithinase production by gram-negative bacteria. J. Bacteriol. **81**:939–945.
9. **Finney, D. J.** 1964. Statistical methods in biological assay, 2nd ed. Charles Griffin & Co., Ltd., London.
10. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **45**: 73–102.
11. **Idigbe, E. O., R. Parton, and A. C. Wardlaw.** 1981. Rapidity of antigenic modulation of *Bordetella pertussis* in modified Hornibrook medium. J. Med. Microbiol. **14**:409–418.
12. **Johnson, M. K., and D. Boese-Marrazzo.** 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect. Immun. **29**:1028–1033.
13. **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.
14. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl):S94–S99.
15. **Liu, P. V.** 1979. Toxins of *Pseudomonas aeruginosa*, p. 63–88. *In* R. G. Doggett (ed.), *Pseudomonas aeruginosa*: clinical manifestations of infection and current therapy. Academic Press, Inc., New York.
16. **Lory, S., and P. C. Tai.** 1983. Characterization of the phospholipase C gene of *Pseudomonas aeruginosa* cloned in *Escherichia coli*. Gene **22**:95–101.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Ostroff, R. M., and M. L. Vasil.** 1987. Indentification of a new phospholipase C activity by analysis of an insertional mutation in the hemolytic phospholipase C structural gene of *Pseudomonas aeruginosa*. J. Bacteriol. **169**:4597–4601.
19. **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.
20. **Shen, B., P. C. Tai, A. E. Pritchard, and M. L. Vasil.** 1987. Nucleotide sequences and expression in *Escherichia coli* of the in-phase overlapping *Pseudomonas aeruginosa plcR* genes. J. Bacteriol. **169**:4602–4607.
21. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
22. **Sokol, P. A.** 1987. Surface expression of ferripyochelin-binding protein is required for virulence of *Pseudomonas aeruginosa*. Infect. Immun. **55**:2021–2025.
23. **Stinson, M. W., and C. Hayden.** 1979. Secretion of phospholipase C by *Pseudomonas aeruginosa*. **25**:558–564.
24. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. **84**:2833–2837.
25. **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.
26. **Vasil, M. L., C. Chamberlain, and C. C. R. Grant.** 1986. Molecular studies of *Pseudomonas* exotoxin A gene. Infect. Immun. **52**:538–548.
27. **Weinberg, E. D.** 1974. Iron and susceptibility to infectious disease. Science **134**:952–955.
28. **Woods, D. E., P. A. Sokol, and B. H. Iglewski.** 1982. Modulatory effect of iron on the pathogenesis of *Pseudomonas aeruginosa* mouse corneal infections. Infect. Immun. **35**:461–464.
29. **Wretlind, B., A. Björklind, and O. R. Pavlovskis.** 1987. Role of exotoxin A and elastase in the pathogenicity of *Pseudomonas aeruginosa* strain PAO experimental mouse burn infection. Microb. Pathog. **2**:397–404.