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Cloning, and Expression in *Escherichia coli* K-12, of the Chromosomal Hemolysin (Phospholipase C) Determinant of *Pseudomonas aeruginosa*

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A hemolysin determinant was cloned from *Pseudomonas aeruginosa* PA103 by inserting *Sau*3a-generated DNA fragments between the *Bam*HI sites of the λ replacement vector WL47.1. A 9.5-kilobase *Hin*dIII fragment encoding the hemolysin was subcloned from this phage and inserted into the plasmid vector pHC79 to generate the recombinant plasmid pKC95. *Escherichia coli* K-12 strains harboring pKC95 exhibited zones of hemolysis after several days of growth on blood agar plates. Hemolysis was shown to be due to phospholipase C activity by using the chromogenic substrate *p*-nitrophenylphosphorylcholine. Deletion mutants of pKC95 were isolated, and polypeptides expressed from these plasmids were examined by using the *E. coli* minicell system. A polypeptide of 78,000 daltons was associated with phospholipase C activity. The hemolytic activity was cell associated when expressed in *E. coli*.

Hemolysins are known to play an important role in infections caused by certain gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus pyogenes* (2). Circumstantial evidence suggests that hemolysins may also assist in the establishment or maintenance (or both) of gram-negative infections. The hemolytic phenotype is more often encountered in clinical as opposed to environmental strains of gram-negative bacteria, for example, *Escherichia coli* (9) and *Pseudomonas aeruginosa* (1), and acquisition of a cloned hemolysin determinant can increase the virulence of some *E. coli* strains in an animal model system (30).

Most strains of *P. aeruginosa* produce two hemolytic substances—a heat-stable glycolipid (17) and a protein hemolysin (20). The latter has previously been referred to as the heat-labile hemolysin (12), the lecithinase, or the phospholipase (8). The mechanism of pathogencity in *P. aeruginosa* is complex and multifactorial, and the role of the heat-labile hemolysin is unclear. A genetic approach to the study of this problem would be to construct isogenic derivatives of well-characterized *P. aeruginosa* strains with and without the hemolysin determinant. Such strains could be used to determine whether hemolysin contributes to virulence and, more importantly, how this factor may interact with other virulence factors to augment virulence.

As a first step toward this end, we describe here the cloning of a *P. aeruginosa* hemolysin determinant into *E. coli*, by using a λ replacement vector (22),and subsequently onto a plasmid. Unlike the situation in *P. aeruginasa* where the protein product is exported (28), the hemolysin remains cell associated in *E. coli*. Since the protein has not previously been purified, the cloning of this gene will facilitate characterization of the hemolysin.

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MATERIALS AND METHODS

Microorganism. The bacterial strains used are listed in Table 1. PA103 is a clinical isolate originally chosen by Liu (20) because it is an excellent exotoxin A producer, but is weakly proteolytic. This strain was the source of chromosomal DNA. The bacteriophage vector used in the experiments was λWL47.1 (22). Replacement of the central fragment of the phage with foreign DNA of approximately 10 kilobase pairs results in packageable structures which are infective and cause lytic infections in *E. coli* C600. Plate lysates were made by the methods of Davis et al. (5).

Media and culture conditions. *P. aeruginosa* PA103 cells were grown in trypic soya broth (Oxoid Ltd., United Kingdom). *E. coli* strains were grown in LB broth (Oxoid) with added NaCl (0.5% [wt/vol], plus any selective agents, if required). Blood agar was made by adding 2.5 ml of packed washed sheep erythrocytes to 100 ml of trypic soya agar tempered to 54°C. Solid and liquid media for the preparation of λ plate stocks and phage lysates were prepared as described by Murray et al. (24).

***P. aeruginosa* DNA preparation.** Genomic DNA from late-exponential cells of *P. aeruginosa* PA103 was isolated by a variation of the procedure of Marmur (23). Cells harvested from 100 ml of culture media

TABLE 1. List of bacterial strains

Organism	Relevant genotype	Source and reference
P. aeruginosa PA103	*tox*$^+$ *hly*$^+$	P. V. Liu (19)
E. coli K-12 C600	*thr leu thi lac*	This laboratory
JE5505	*lpo*$^-$	H. Yamada (16)
JE5506	*lpo*$^+$	
XPh302	*perA*$^-$ *rpsL*	J. Beckwith (29)
XPh303	*perA*$^+$ *rpsL*	
DS410	*rpsL minA minB*	This laboratory

were washed three times in cold 25 mM Tris-hydrochloride (pH 8.6) and suspended in 5 ml of the same buffer. A 15-ml volume of 10 mM Tris-hydrochloride–1 mM EDTA–20% (wt/vol) sucrose (pH 8.0) was then added, and the tubes were left on ice for 10 min. Lysozyme (0.5 mg/ml) and sodium dodecyl sulfate (SDS; 0.2%, wt/vol) were then added, and the cells were left on ice for an additional 10-min period. Tubes were left at room temperature for 5 min followed by 5 min of incubation at 70°C, after which time complete lysis occurred. Lysates were then centrifuged at 10,000 × *g* for 20 min, and the viscous supernatant fluid was carefully removed. An equal volume of phenol saturated with 10 mM Tris-hydrochloride–1 mM EDTA, pH 8.0 (TE buffer), was added and mixed gently by inversion at room temperature. Phase separation was achieved by centrifugation at 10,000 × *g* for 10 min at 18°C. After two phenol extractions, boiled RNase (type III; Sigma Ltd, Poole, United Kingdom) was added to a final concentration of 100 μg/ml, and the DNA solution was left at room temperature for 2 h. The phenol extractions were continued until no protein was visible at the interface. At this stage, 0.1 volume of 3 M sodium acetate (pH 5.5) was added, followed by 1 volume of cold (−20°C) isopropanol. The DNA was immediately visible as hairlike threads. The threads were spooled out of the tube with a glass rod and dissolved in 5 ml of TE buffer. Two additional rounds of phenol extraction and isopropanol precipitation were performed before the DNA was finally dissolved in about 1 ml of TE buffer, and the optical density at 260 nm was determined. Purified DNA was kept at −20°C until required.

Phage and plasmid DNA preparation. Phage DNA was prepared by phenol extraction of purified phage as described previously by Leonen and Brammar (22). Phage WL47.1 DNA was prepared by M. Kehoe. Plasmid DNA from *E. coli* was isolated by equilibrium density centrifugation in CsCl (5).

Restriction endonuclease cleavage, ligation of DNA fragments, agarose gel electrophoresis, and in vitro packaging. Restriction digests and ligation reactions were performed according to the manufacturer's recommendations. All enzymes were purchased from New England Biolabs. Agarose gel electrophoresis was carried out in 89 mM Tris base–89 mM boric acid–1 mM EDTA buffer. In vitro packaging was performed as described by Collins and Brüning (4).

Hemolysin and phospholipase assays. Hemolytic titrations were carried out in 2-ml volumes in glass tubes containing 1% (vol/vol) (final concentration) washed, packed sheep erythrocytes as described by deAzavedo and Arbuthnott (6).

Detection of hemolytic plaques on blood agar was accomplished as follows. About 5 × 10^6 C600 cells (grown in the presence of 0.4% maltose) were mixed with about 300 PFU of phage for 15 min in a volume of 200 μl. At this time 4 ml of top agar which had been cooled to 57°C was added to the infection mixture and gently mixed. This was then poured on top a well-dried LB base plate which had been preincubated at 37°C for 1 h. The plates were then allowed to solidify and placed upside down at 37°C, either overnight or until well-developed plaques were visible. At this time a solution of 1% (wt/vol) agarose in phosphate-buffered saline was melted, cooled to 54°C, and made 5% (vol/vol) with packed, washed sheep erythrocytes; plates were then overlaid with 10 ml of sheep erythrocytes. After 10 min at room temperature the plates were incubated at 37°C and read 6 to 8 h later.

Phospholipase activity was also measured by using the specific chromogenic substrate *p*-nitrophenylphosphorylcholine (NPPC; Sigma lot 41F-5074) as described by Kurioka and Matsuda (18) (NPPC was a gift from M. Vasil).

Fractionation of *E. coli* cells. Membrane and cytoplasmic fractions of *E. coli* were prepared by passing late-exponential cells through a French pressure cell at 10,000 lb/in^2 as described by Owen et al. (26). Osmotic shocking was performed as described by Heppel (14).

Minicell analysis, SDS-polyacrylamide gels and autoradiography. Minicell analysis and SDS-polyacrylamide gel electrophoresis and autoradiography were carried out as previously described (7).

RESULTS

Construction of the *P. aeruginosa* gene library. High-molecular-weight chromosomal DNA from *P. aeruginosa* strain PA103 was isolated by a modification of the Marmur procedure (23; see above). The chromosomal DNA was cleaved by using a limited amount of the restriction enzyme *Sau*3a to generate a range of overlapping, linear DNA fragments with a high proportion in the 10- to 15-kilobase (kb) size range.

In initial attempts at producing gene libraries of *P. aeruginosa* DNA the "cosmid" system of Collins and Brüning (4) was used, but the resultant recombinant clones were highly unstable, probably due to the instability of large inserts of *P. aeruginosa* DNA which are needed to fill the cosmid to its requisite size for in vitro packaging. The alternative strategy of using a λ insertion vector which produces plaques rather than transformed cells seemed feasible since the system did not require stable maintenance of foreign DNA within the *E. coli* cell. The insertion vector chosen, λWL47.1 (22), also had the following three additional advantages: (i) a strong promoter, p_L of phage λ, is positioned just downstream from the sites for insertion of foreign DNA; (ii) recombinants could be screened readily since the *E. coli* plating cells ultimately lyse and release the cloned product; and (iii)

recombinants are selected directly by using a P2 lysogen of *E. coli* due to the Spi$^-$ phenotype of vectors which have substituted foreign DNA for their central λ fragment (22).

Approximately 10^5 chimeric recombinants per μg of *P. aeruginosa* DNA were obtained, which is well in excess of the number of phage required to theoretically constitute a complete *P. aeruginosa* gene library (3). Screening of plaques for hemolytic activity was performed on an expanded collection of recombinants produced by passaging 4,000 to 5,000 independent insertions through a P2 lysogen of *E. coli*. This plate lysate (see above) typically contained between 10^8 and 10^9 PFU/ml and was used throughout the work described here. Thereafter, phage platings were carried out with nonlysogenic *E. coli* strains.

Detection of hemolytic plaques. Hemolytic plaques were detected by using an agarose-containing erythrocyte overlay in isotonic solution (Fig. 1). The overlay was placed above a developed plate containing approximately 300 plaques per 9-cm^2 petri dish. Hemolysis was visible 6 to 8 h after overlaying the plates with a frequency of about 1 in 2,500 recombinants. Hemolytic phage was then purified from one such plaque; this phage, called λhlyBC95, was used in subsequent subcloning experiments.

Subcloning of the *P. aeruginosa* hemolysin determinant onto the *E. coli* plasmid pHC79. DNA was prepared from λhlyBC95 and cleaved with the restriction endonucleases *Hin*dIII, *Eco*RI, and *Bam*HI. Since the *P. aeruginosa* DNA was originally cleaved with *Sau*3a and ligated to the WL47.1 vector cleaved with *Bam*HI, both *Bam*HI sites were not conserved in λhlyBC95. This enzyme was therefore inappropriate for subcloning. *Eco*RI cut λhlyBC95 at the two sites within the λ arms and once within the cloned *P. aeruginosa* DNA fragment about 0.5 kb from one of the fragments. Conveniently, no *Hin*dIII sites were located within the cloned DNA; since WL47.1 has two *Hin*dIII sites which flank the insert, it was possible to subclone a 9.5-kb fragment encoding the hemolysin determinant from λhlyBC95 into the *Hin*dIII site on pHC79 (Fig. 2). One such recombinant plasmid, called pKC95, was used for further studies. DNA of pKC95 was purified and cleaved with restriction enzymes to produce the map shown in Fig. 2. When C600 harboring this plasmid was stabbed onto a blood agar plate and grown overnight at 37°C, virtually no hemolysis occurred around the colonies, and even after 3 days of incubation the amount of hemolysis was still low, although consistently detectable. Lysates of C600 harboring pKC95 or pHC79 were prepared by passing cells through a French pressure cell (see above) and assayed for the presence of phospholipase C activity by using the chromogenic substrate NPPC. High levels of activity were detected in C600 lysates prepared from cells harboring pKC95, but not pHC79, suggesting that hemolysis was due to the cloned gene product.

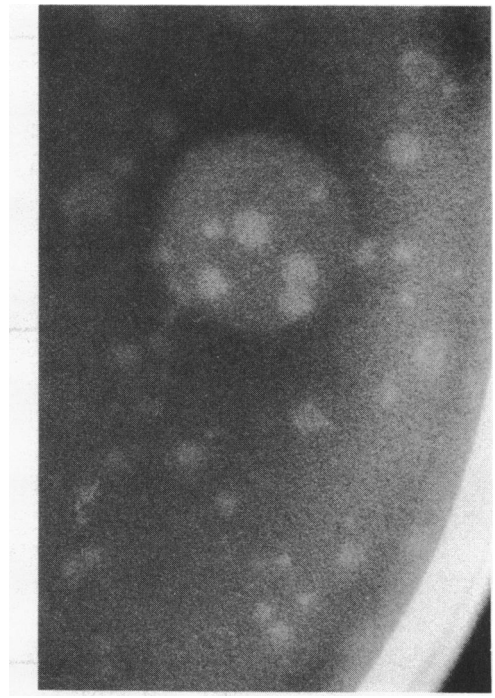

FIG. 1. Detection of hemolytic plaques by erythrocyte overlay. Plates containing developed plaques were overlaid with sheep erythrocytes (as described in the text) and incubated for 24 h at 37°C. A clear zone centering around one of the plaques occurred where erythrocytes lysed over a plaque with hemolytic activity.

Deletions of pKC95 were generated with the restriction enzymes *Eco*RI and *Pvu*II. pKC95 DNA was cleaved with *Eco*RI, ligated, and used to transform C600. All transformants tested retained their hemolytic properties. Plasmid DNA was purified from several transformants, and one plasmid, named pKC97, lacked the small 800-base-pair *Eco*RI fragment of pKC95 (Fig. 3). pKC97 DNA was purified and cleaved in limited amounts with *Pvu*II; after ligation and transformation into C600 ampicillin-resistant colonies were screened for plasmid DNA. Two plasmids, pKC101 (3.8 kb) pKC103 (8.3 kb), were found to contain the deletions shown in Fig. 3. Cells harboring these plasmids failed to produce detectable levels of active hemolysin even after prolonged incubation on blood agar plates. Assays for phospholipase C activity were also negative.

Polypeptides expressed from pKC97 and pKC95 deletions in *E. coli* K-12 minicells. Poly-

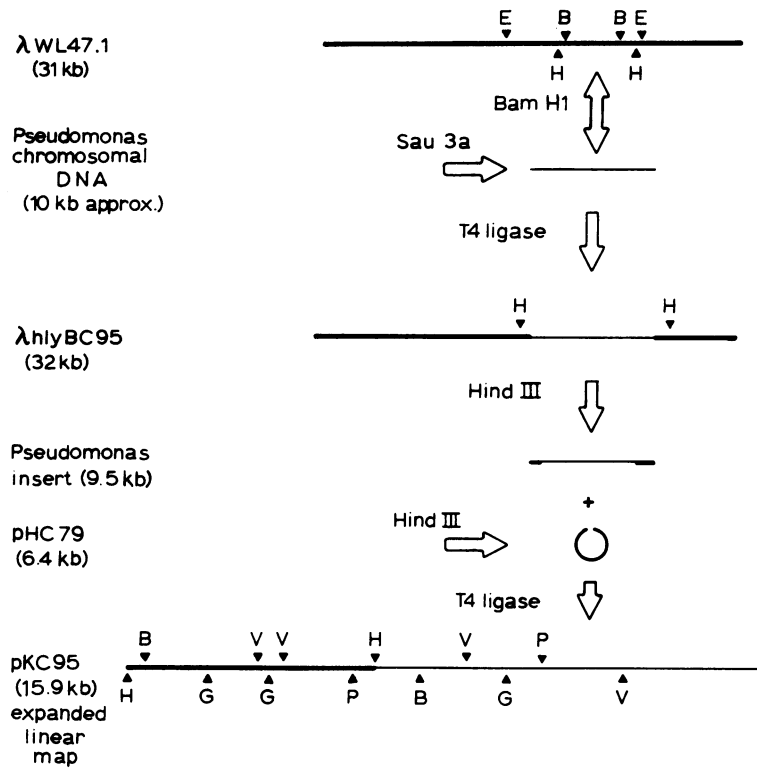

FIG. 2. Cloning strategy and restriction map of pKC95. Arrows and letters indicate restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; G, *Bgl*II; V, *Pvu*II; P, *Pst*I. Heavy lines indicate vector DNA.

peptides expressed from pKC95 and deletions derived from this plasmid were examined by using the *E. coli* K-12 minicell system. pHC79 expressed several polypeptides in minicells, including those associated with ampicillin resistance (Fig. 4). pKC95 directed the expression of several novel polypeptides, including one of 78,000 daltons. This polypeptide was not expressed by pKC101 or pKC103. *P. aeruginosa* phospholipase C activity as measured by NPPC was stable in solutions containing up to 4% SDS (unpublished results). Thus the phospholipase C

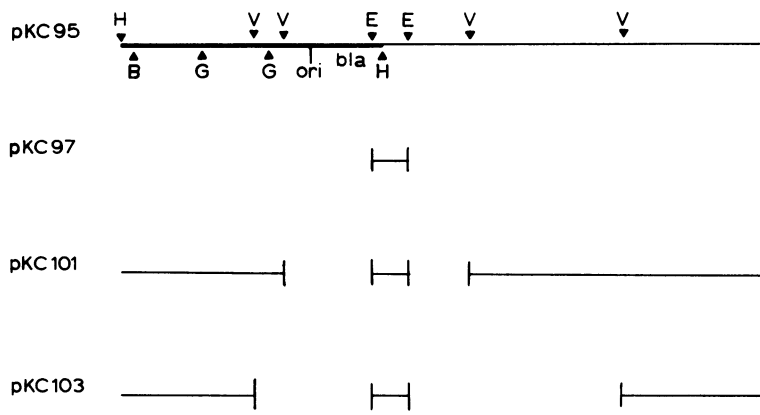

FIG. 3. Map of deletion mutants of pKC95. Heavy lines indicate pHC79 sequences. bla, Position of the β-lactamase gene; ori, origin of replication. Restriction endonuclease cleavage sites: V, *Pvu*II; H, *Hin*dIII; E, *Eco*RI; G, *Bgl*II. Distances are in kilobase pairs. Bar lines delineate pKC95 DNA sequences deleted in each plasmid.

polypeptide could be expected to retain enzymatic activity after SDS-polyacrylamide gel electrophoresis. Minicell samples were suspended in SDS-polyacrylamide gel sample buffer containing 1% SDS and loaded onto SDS-polyacrylamide gels without boiling. After electrophoresis the gels were washed briefly in distilled water and were overlaid with strips of filter paper soaked in a solution of NPPC substrate. A pale yellow chromogenic reaction was detected on the filter paper overlaying the gels in the region of the 78,000-dalton polypeptide with samples prepared from minicells harboring pKC95, but not with samples prepared from minicells harboring pKC103, pKC101, or pHC79. Thus the 78,000-dalton polypeptide is likely to be the hemolytic factor. Several other polypeptides were expressed in minicells harboring pKC95, but it is not yet known whether they are involved in the expression of the *P. aeruginosa* hemolysin.

Location of phospholipase C activity in *E. coli* K-12 strains harboring pKC95. The hemolysin of *P. aeruginosa* is a secreted protein, passing through both the cytoplasmic and outer membrane systems (28). In contrast, *E. coli* secretes few proteins, although some may be exported through the cytoplasmic membrane to ultimately reside in the periplasm or outer membrane (11, 12). It was of interest, therefore, to examine the fate of the cloned *P. aeruginosa* exoprotein in *E. coli* cells. To test this, various fractions were prepared from *E. coli* K-12 strains and isogenic derivatives harboring pKC95. No extracellular phospholipase C (or hemolytic) activity was detected in cell-free culture supernatants of *E. coli* K-12 C600 or *E. coli* K-12 C600 harboring pKC95 when the cells were grown into the late logarithimic or early stationary phase of growth. However, when the cells were left incubating for up to 4 days, some activity was detected in supernatant fluids of *E. coli* K-12 C600 carrying pKC95. This activity appeared concomitantly with the cytoplasmic marker enzyme β-galactosidase, suggesting that lysis of cells was required for the release of the hemolysin. To test whether the phospholipase was being produced, even at early times in the growth cycle, midexponential phase (approximately 2×10^8 cells per ml) cells were lysed with SDS and lysozyme, a treatment to which the phospholipase is resistant. Only lysates of *E. coli* K-12 C600 carrying pKC95 showed activity toward the phospholipase-specific substrate NPPC (data not shown).

E. coli K-12 C600 cells containing pKC95 were disrupted in a French pressure cell, and the membrane and supernatant fractions were tested for phospholipase C activity. Ninety-eight percent of the activity was located in the soluble fraction, indicating that the phospholipase was probably located within the cytoplasm (Fig. 5). Washes obtained after osmotic shocking of *E. coli* K-12 C600 cells carrying pKC95 contained less than 10% of the total phospholipase C activity of the culture.

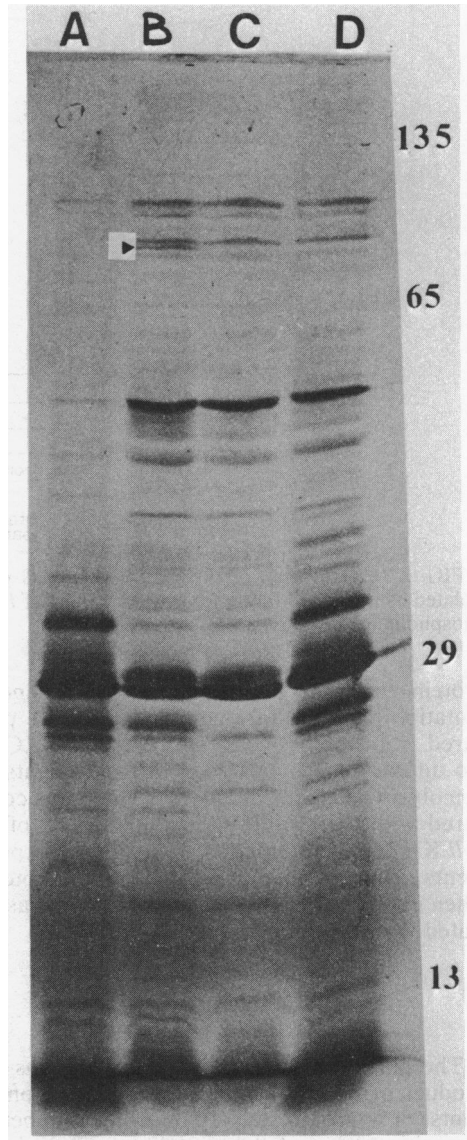

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled polypeptide labeled in minicells harboring (A) pHC79, (B) pKC95, (C) pKC101, and (D) pKC103. Arrow points to the 78,000-dalton polypeptide showing phospholipase C activity. Molecular mass markers are in kilodaltons. Electrophoresis was on a 12.5% SDS-polyacrylamide gel.

In another approach, two mutants of *E. coli* were used, one which leaks periplamic enzymes (the lipoprotein-less strain [Lpo$^-$] JE 5505 [15]) and one which shows a decreased export of

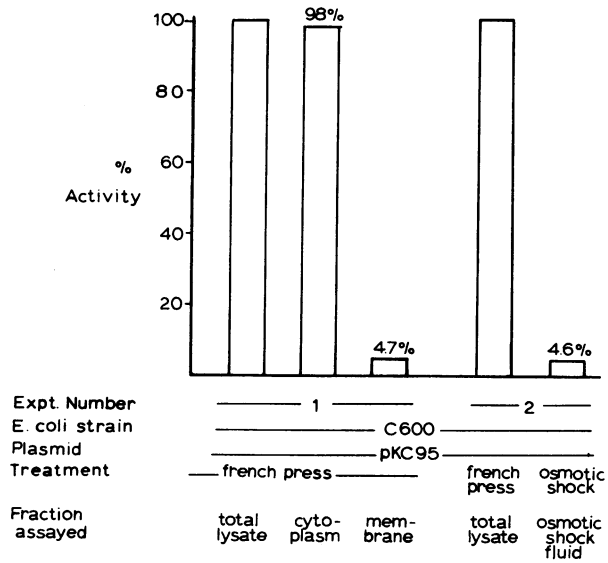

FIG. 5. Localization of the phospholipase C produced in *E. coli*. Various fractions of *E. coli* cells were isolated as described in the text. All fractions of *E. coli* C600 cells (harboring no plasmid) were negative in the phospholipase C assay.

proteins into the periplasm (due to the *perA* mutation [29]). Culture supernatants were prepared from the above strains carrying pKC95. No difference was observed in the amounts of hemolysin produced in any of the mutants compared with their wild-type parent strains of *E. coli* K-12 C600 (data not shown). These experiments show that the *P. aeruginosa* exoprotein, when expressed from a plasmid *E. coli*, is associated with the soluble fraction of the cell.

DISCUSSION

The phage library used in this work was the product of about 80,000 independent recombinants, or approximately 50 times the number of recombinants needed to produce a gene bank with 99% probability of finding any one gene (3). The library was propagated as a collection of plaques, making detection of interesting recombinants less tedious than screening large number of colonies which often must by lysed before the application of a detection system (27).

The dearth of published information on intergenic transfer of *P. aeruginosa* DNA to *E. coli* may reflect some potential problems in using *E. coli* as the host in plasmid cloning experiments involving *P. aeruginosa* DNA. In our first attempts at producing gene banks of *P. aeruginosa* with the cosmid packaging (16) system, we found all recombinant clones to be highly unstable; in contrast, the same vector (pHC79) produced stable clones in *E. coli* when 10-kb fragments were inserted. Since the cosmid system requires about 40 kb or DNA for successful phage packaging in vitro (4) and our subcloned haemolysin determinant contained a fragment of about 10 kb, the critical limit of *P. aeruginosa* extrachromosomal DNA in an *E. coli* plasmid may lie somewhere within these limits. Other workers have also found instability in cosmids and plasmids containing large inserts of *P. aeruginosa* DNA (S. Falkow, personal communication, 10, 13).

In the *E. coli* minicell system, a polypeptide of 78,000 daltons, expressed from the cloned *P. aeruginosa* DNA sequences, was identified and shown to possess phospholipase C activity. This activity was only slightly inhibited in the presence of 1% SDS.

The hemolysin is one of the several secreted exoproteins of *P. aeruginosa* which is able to cross both the inner and outer membrane systems during secretion (28). By contrast, the active hemolysin produced in *E. coli* remained cell associated, apparently unable to cross even the cytoplasmic membrane. Although the possibility that only a portion of the gene lacking "signal" sequences was cloned cannot be excluded, this seems unlikely as the fragment that was cloned was large and should have included such a closely linked function. The *E. coli* α-hemolysin determinant is known to encode additional polypeptides involved in processing and secretion of the active α-hemolysin protein (25); if such a complex situation exists for the transport of the *P. aeruginosa* hemolysin, it may require additional activities to be exported from *E. coli* cells.

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