JOURNAL OF BACTERIOLOGY, Jan. 1984, p. 7–12
0021-9193/84/010007-06$02.00/0

Vol. 157, No. 1

Transposon Mutagenesis of *Pseudomonas aeruginosa* Exoprotease Genes

M. J. STAPLETON,* K. S. JAGGER, AND R. L. WARREN

Microbiology and Immunology, College of Science & Engineering and School of Medicine, Wright State University, Dayton, Ohio 45435

Received 19 July 1983/Accepted 4 October 1983

Transposon Tn*5* was used to generate protease-deficient insertion mutants of *Pseudomonas aeruginosa*. The presence of Tn*5* in the chromosome of *P. aeruginosa* was demonstrated by transduction and DNA-DNA hybridization. The altered protease production and kanamycin resistance were cotransduced into a wild-type *P. aeruginosa* strain. A radiolabeled probe of Tn*5* DNA hybridized to specific *Bam*HI fragments isolated from the insertion mutants. Two independently isolated Tn*5* insertion mutants had reduced protease production, partially impaired elastase activity, and no immunologically reactive alkaline protease.

Pseudomonas aeruginosa proteases have been implicated as virulence factors in several types of *P. aeruginosa* infections (4, 5, 11, 12, 17, 29, 32). The exact number of *P. aeruginosa* proteases is not known (15, 18, 20, 26, 30), but two immunologically distinct metalloproteases have been isolated, purified, and characterized (25, 27). One, alkaline protease, has a molecular weight of 48,000 (26); the other, elastase, has a molecular weight of 39,500 (26) and uses elastin as well as casein for a substrate.

Since the virulence of *P. aeruginosa* is the result of the interaction of multiple factors, the contribution of any single factor to virulence can be determined by comparing isogenic mutants. To insure that the mutants are deficient in a single specific exoproduct, a transposon (Tn*5*) mutagenesis procedure was developed for *P. aeruginosa*. The transposon Tn*5* was used to construct mutants because Tn*5* inserts randomly into the genome of *Rhizobium* spp. (2) and *Caulobacter cresentus* (8). Beringer et al. (2) constructed a plasmid vector, pJB4JI, which harbors both Tn*5* and bacteriophage Mu. The phage interferes with the maintenance of this plasmid in hosts other than *Escherichia coli*. Therefore kanamycin-resistant exconjugants are the result of Tn*5* inserting into the recipient genome. Transposition of Tn*5* from the plasmid to the recipient genome can be scored by its phosphotransferase type II gene, which confers resistance to kanamycin, neomycin, and geneticin.

Transposon mutagenesis has important advantages over chemical mutagenesis. Introduction of Tn*5* into the genome of *P. aeruginosa* provides a positive selection marker for both strain construction and the analysis of the mutant phenotype. The mutations can be transferred to different *P. aeruginosa* strains by either transduction or conjugation simply by selecting for kanamycin resistance. In addition, the region of DNA containing the Tn*5* element can be subcloned into *E. coli* by selecting for kanamycin resistance in the transformants. Once the *P. aeruginosa* DNA containing the Tn*5* element is cloned into *E. coli*, the cloned DNA fragment can be used as a probe for the identification of the wild-type gene in a *P. aeruginosa* gene bank.

In this paper we describe the procedure for construction of exoprotease mutants, and then we characterize two of these mutants. Since there are at least two *P. aeruginosa* exoproteases that act on casein, selecting a specific protease mutant on skim milk medium is virtually impossible. A mutation in one protease could be masked by the proteolytic action of others. Our strategy was to select those colonies with reduced proteolytic activity for further screening on elastin medium and with alkaline protease and elastase antisera. We present genetic and physical evidence for insertion of Tn*5* into the *P. aeruginosa* genome. The pleiotropic nature of these mutants suggested that a common factor is necessary for the production or secretion of both alkaline protease and elastase.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in these studies are given in Table 1. *P. aeruginosa* strain PAO1161 was chosen because it is highly proteolytic.

Media and growth conditions. *P. aeruginosa* PAO1161 and PAO1670 and *E. coli* were grown in Luria broth (LB) (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) supplemented with selective agents as required. Bacto-Agar (Difco Laboratories) was added to a final concentration of 2% for solid media. The antibiotic concentration used to select for PAO strains was 500 μg/ml of kanamycin (Sigma Chemical Co.) or geneticin (GIBCO Laboratories). A dose of 50 μg of kanamycin or geneticin per ml was adequate to distinguish between resistance and sensitivity in *E. coli*. Either kanamycin or geneticin was used for selection of transposon insertion mutants. Pseudomonas isolation agar (PIA) (Difco) was used to select against *E. coli* strains. The high salt concentration of PIA eliminated the selective effect of kanamycin and geneticin (unpublished observation), so neither could be added to PIA for mating selection. Skim milk powder (Difco) and powdered elastin or elastin-Congo red (Sigma) were added to PIA or LB plates at a concentration of 0.1% (wt/vol) for screening protease-deficient mutants. When both elastin and skim milk were added to LB agar plates, they were designated LB-SKE.

Bacterial conjugation. Conjugal transfer was performed by the spot-mating procedure of Morgan (24). A lawn of the recipient cells was spread on agar and allowed to dry; then 10-μl drops of donor cells were spotted on the recipient lawn. This procedure resulted in at least 30 independent matings per plate, and one exconjugant was isolated per spot by restreaking for single colonies on PIA.

Transduction. Phage F116L (13) lysates of mutant strains

TABLE 1. Bacterial strains and plasmids

Bacterial strains	Relevant genotype/ phenotype[a]	Source/reference
P. aeruginosa		
PAO1161	*leu-38 hsd-10* FP2$^+$	J. Miller
PAO1670	*pur-136 leu-8 clm-3 rif-1*	9
WS1100	*els-1*	This study
WS1700	*els-2*	This study
E. coli		
1830	*pro met* pJB4JI	2
HB101	*pro leu recA*	7
HB9001	*pro leu* pMS101	This study
HB9001	*pro leu* pMS170	This study
Plasmids		
pBR322	Ap Tc	3
pJM01 (pBR322 + RSF1010 hybrid)	Ap Tc Sm Su	J. Miller
pJB4JI	Gm Nm/Km/Gt	2
pMS007	Ap Tc Km/Gt	pBR322 + KmR(pJB4JI)
pMS101	Ap Km/Gt	pJM01 + WS1100 *Bam*HI fragment
pMS170	Ap Km/Gt	pJM01 + WS1700 *Bam*HI fragment

[a] Genotype abbreviation: *els*, elastase deficient. Phenotype abbreviations: Ap, ampicillin resistance; Gm, gentamicin resistance; Nm/Km/Gt, neomycin, kanamycin, and geneticin resistance; Sm, streptomycin resistance; Su, sulfonamide resistance; Tc, tetracycline resistance.

of PAO1161 and PAO1670 were prepared by the soft agar method (35). The recipient cells were grown to the late log phase in LB. Phage was mixed with these cells at a multiplicity of infection of 1. Each mixture was incubated at 37°C for 30 min and plated in 0.7% soft agar over LB plates containing kanamycin. Drug-resistant colonies were screened on skim milk and elastin plates.

DNA isolation. Genomic DNA from late-exponential-phase cells was isolated by a variation of the procedure of Marmur (22). After the DNA threads were wound onto a glass rod and dissolved in 1.0 ml of 0.1% SSC (8.8 g of NaCl and 4.4 g of sodium citrate per liter, pH 7.0), proteinase K (50 μg/ml) was added, and the mixture was incubated for several hours at 37°C. Then the DNA was extracted with phenol and precipitated with ethanol by standard methods (21). Plasmid DNA from *E. coli* was isolated by the cleared lysate method and purified by equilibrium density centrifugation in CsCl-ethidium bromide (7).

Restriction endonuclease digests. All restriction enzymes were obtained from Bethesda Research Laboratories, and conditions for restriction enzyme reactions were as specified by the manufacturer. Restriction endonuclease-digested DNA samples were electrophoresed through 0.7% agarose gels in Tris-acetate buffer (TEA) (40 mM Tris-hydrochloride, 5 mM sodium acetate, and 1 mM EDTA, adjusted to pH 7.6 with acetic acid) at 80 V for 2 h. DNA fragments were visualized by staining with ethidium bromide (2 μg/ml) and photographed with short-wavelength UV light.

Cloning techniques. Plasmid or chromosomal DNA was digested to completion with restriction endonucleases, mixed together and ligated with T4 ligase (Bethesda Research Laboratories; 1 U/μg of DNA) at 10°C for 18 to 24 h. *E. coli* was transformed with the ligated DNA by standard methods (21).

Extraction of DNA from agarose. Restriction endonuclease DNA fragments were separated electrophoretically in 0.7% agarose, cut out of the gel, and eluted into a low-salt buffer as previously described (34). The agarose was removed, and the DNA was concentrated with an ELUTIP-d column (Schleicher & Schuell Co.).

Nick translation and DNA-DNA hybridization. Restriction endonuclease fragments from digested DNA were separated by agarose gel electrophoresis and electrophoretically transferred to a nitrocellulose filter (Schleicher & Schuell) by the method of Stellwag and Dahlberg (33) in a Trans-blot cell (Bio-Rad Laboratories) with TEA buffer at 8 V and 250 mA for 2 h. The DNA was fixed to the nitrocellulose filter by heating in vacuo (80°C, 2 h). The DNA-DNA hybridization was carried out as previously described (37). The *Eco*RI fragment carrying the Tn*5* element was extracted from agarose, digested to completion with *Hin*dIII, and then labeled to high specific activity by DNA polymerase I extension of exonuclease III-generated primer termini (D. James and M. Leffak, submitted for publication).

Immunoprecipitation. Immunoprecipitation was performed in situ as described previously (28). Anti-elastase and anti-alkaline protease were prepared by immunizing New Zealand white rabbits with the purified enzyme (Nagase Ltd., Osaka, Japan). The antisera were assayed for purity by the Ouchterlony method (10) and by enzyme-linked immunoassays (14). Immune titers of 32,000 for anti-elastase and 64,000 for anti-alkaline protease were measured by enzyme-linked immunoassay. Anti-alkaline protease antibody did not cross-react with purified elastase in either the Ouchterlony assay or the enzyme-linked immunoassay. Anti-elastase antibody did not cross-react with purified alkaline protease in the Ouchterlony assay, but it had a cross-reacting titer of less than 1,000 by the enzyme-linked immunoassay method.

RESULTS

Isolation of Tn*5* insertion mutants. The Tn*5* element was introduced into *P. aeruginosa* by conjugal transfer of pJB4JI from *E. coli* strain 1830. The mating experiments were performed on solid medium to facilitate the conjugal transfer of the transposon's vector and to insure that independent mutants were recovered. The solid medium was supplemented with 500 μg of kanamycin per ml to select against the *E. coli* donor and to select for *P. aeruginosa* strains carrying the Tn*5* element.

Colonies from 50 independent spots were restreaked on PIA to isolate single colonies of *P. aeruginosa*. Only 50% of these colonies tested grew on PIA. The inability to isolate *P. aeruginosa* from each spot mating suggested that the *E. coli* strain 1830 grew on kanamycin (500 μg/ml) plates. When an oxidase disk was placed next to colonies arising within the mating spot, most of the colonies were oxidase negative, demonstrating that *E. coli* was capable of growth on medium containing high levels of kanamycin. Kanamycin-resistant *P. aeruginosa* strains isolated on PIA from spot matings were transferred onto medium containing either elastin or skim milk. Some kanamycin-resistant strains had reduced elastolytic activity (Fig. 1A). Several of these colonies were chosen for the following transduction experiments.

Genetic evidence for Tn*5* insertion. Kanamycin-resistant, elastase-deficient exconjugants and a spontaneous kanamycin-resistant mutant were infected with the general transducing phage F116L. The resulting phage were used to infect PAO1161, and kanamycin-resistant transductants were isolated and screened for their elastase phenotype. Elastase-deficient transductants, WS1100 and WS1700 (Fig. 1B),

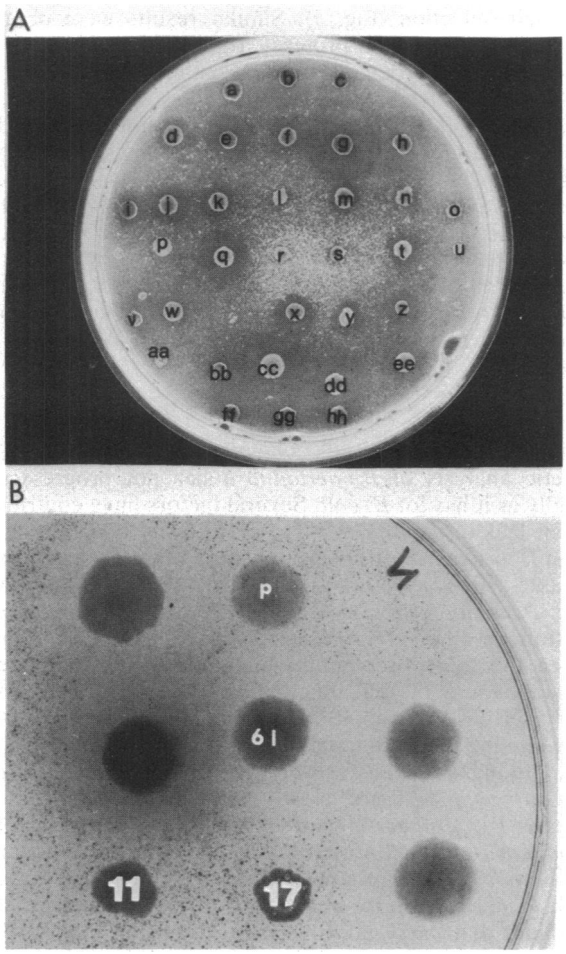

FIG. 1. (A) Differential proteolysis of elastin by exconjugants of *P. aeruginosa* strains PAO1670 (a through o) and PAO1161 (p through hh). Each colony was an isolate from independent matings between *E. coli* 1830 pJB4JI and PAO1670 or PAO1161. The light particulate matter is insoluble elastin. (B) Elastase-deficient transductants. Bacteriophage F1161L was propagated on elastase mutants from exconjugants l and p in A. Kanamycin resistance was cotransduced into PAO1161 along with the elastase-deficient phenotype as represented by two transductants, WS1100 (no. 11) and WS1700 (no. 17). The elastase-deficient, Km^r PAO1161 exconjugant and parent of WS1100 (p in A and B) and one (no. 61) of the transductants of spontaneous kanamycin-resistant PAO1161 mutant DNA, all of which were elastolytic, are indicated. The dark particles in the PIA agar are insoluble elastin-Congo red aggregates.

were chosen for further characterization. The cotransduction of kanamycin resistance and the altered elastase phenotype demonstrated that Tn*5* insertion had occurred near a gene regulating or directing the synthesis of elastase. The phage lysates prepared on spontaneous kanamycin-resistant strains yielded transductants with a proteolytic phenotype identical to that of the wild-type parent.

Additionally, conjugal transfer of the FP2 plasmid from one of the transductant strains, WS1100, to a second *P. aeruginosa* strain resulted in the transfer of Tn*5* at a frequency of less than 10^{-7}. This is the frequency one expects to find if the transposon translocated from the chromosome to the fertility plasmid before transfer (1, 31). These experiments provided genetic evidence for the insertion of Tn*5* into the chromosome of *P. aeruginosa* and showed that the insertion event correlated with the mutant elastase phenotype.

Physical evidence for Tn*5* insertion. Spontaneous kanamycin resistance occurred in *P. aeruginosa* at a relatively high frequency. To distinguish insertion mutants from spontaneous mutants, DNA-DNA hybridization studies were performed. First, an *Eco*RI fragment of pJB4JI carrying the Tn*5* element was ligated into the *Eco*RI site of pBR322. This plasmid, pMS007, was used as a probe for Tn*5* sequences in the *P. aeruginosa* genome. Since there are no reported *Eco*RI sites in Tn*5* (16), pMS007 should contain the entire transposon sequence plus flanking sequences of the parent plasmid pJB4JI. The target DNA for the hybridization studies was prepared by cloning geneticin resistance from WS1100 into *E. coli*. These clones can also be used to probe a *P. aeruginosa* gene bank for the wild-type gene corresponding to each mutant. The total genomic DNA from strain WS1100 was digested with *Bam*HI and ligated into the *Bam*HI site of the plasmid vector pJM01 and used to transform HB101. A geneticin-resistant transformant of HB101 (WS9001) that was ampicillin resistant (100 μg/ml) and tetracycline sensitive (20 μg/ml) was recovered, and the plasmid DNA (pMS101) was isolated for further analysis. Because both the probe DNA, pMS007, and the target DNA, pMS101, possessed pBR322 DNA, the 4.7-megadalton *Eco*RI fragment of pMS007 carrying the Tn*5* element was isolated from agarose gels and then was ^{32}P labeled. The autoradiogram in Fig. 2B demonstrates the presence of Tn*5* in the target DNA of pMS101. No hybridization occurred with the smaller, pBR322 *Eco*RI fragment (Fig. 2A, arrow) from pMS007 (lane 1) or pJM01 (lane 3). The hybridization that occurred with the partial digests of pMS101 (lanes 4, 5 and 6) confirmed the presence of Tn*5* in the cloned region of WS1100. The arrow in lane 6 shows the 2-megadalton internal *Hin*dIII fragment from Tn*5* (16). The hybridization

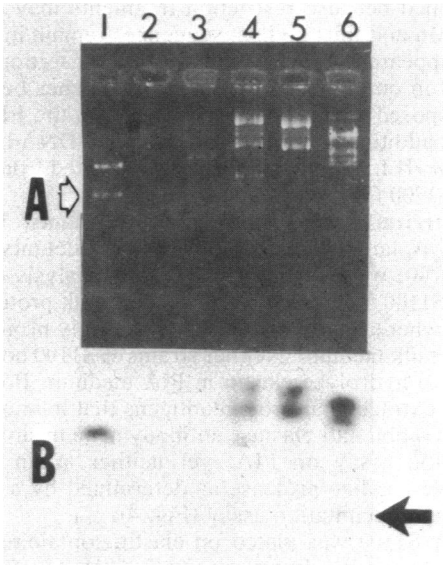

FIG. 2. (A) Restriction endonuclease digests. Lanes: 1, pMS007 (*Eco*RI); 2, no DNA added; 3, pJM01 (*Eco*RI); 4, 5, and 6, pMS101 (*Eco*RI, *Bam*HI, and *Bam*HI-*Hin*dIII, partial digests, respectively). (B) Autoradiogram of the hybridization of the Tn*5* radiolabeled probe to restriction endonuclease fragments of the plasmids in A. The DNA fragments were electrophoretically transferred to nitrocellulose. The hybridization conditions were as indicated in the text. The arrow in A indicates the *Eco*RI fragment of pMS007 and pJM01 that is pBR322 (no hybridization in B). The arrow in B indicates hybridization with the internal, 3-kilobase *Hin*dIII fragment of Tn*5*.

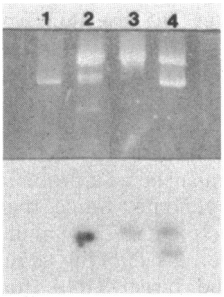

FIG. 3. Hybridization of genomic PAO1161 DNA with *E. coli* clones of elastase mutants WS1100 and WS1700. *Bam*HI fragments of WS1100 and WS1700 were ligated to *Bam*HI digests of vector pJM01. *E. coli* HB101 that was transformed with these ligated fragments produced geneticin-resistant clones. Hybrid plasmids, pMS101 and pMS170, respectively, were isolated and used in this hybridization. Lane 1 contained pJM01 digested with *Eco*RI. The hybrid plasmids, pMS101 (lane 2, *Eco*RI) and pMS 170 (lane 3, *Eco*RI; lane 4, uncut), each contained the Tn*5* insertion and flanking regions of the *P. aeruginosa* genome.

experiments demonstrated that Tn*5* inserted into the chromosome of WS1100.

Although we had evidence for the presence of Tn*5* in the *P. aeruginosa* genome, we wanted to know whether the mutants possessed any additional portion of the vector, pJB4JI, beyond Tn*5*. Transposition of any portion of Mu phage presents potential problems in subsequent conjugation and cloning experiments (23). Mu phage sequences present in pJB4JI are found contiguous with Tn*5* in about 25% of the *Rhizobium meliloti* insertion mutants (23). Meade et al. (22) also found that the strains in which Mu had transposed together with Tn*5* could not be used as Tn*5* donors in conjugation experiments. Another disadvantage is that the mutant gene containing the Mu-Tn*5* transposon may not be easily cloned because restriction fragments may cut in the flanking Mu and thus no host sequences remain in the clone (23). It appeared unlikely that the Tn*5* insertion mutants described in our studies also carried Mu genes because the Tn*5* transposed to a second DNA target in the FP2 experiments. In addition, *P. aeruginosa* genomic DNA hybridized to the *Bam*HI fragments carrying Tn*5* isolated from WS1100 AND WS1700 (Fig. 3).

Characterization of elastase-deficient mutants. Two independently isolated elastase-deficient transductants, WS1100 and WS1700, were selected for further analysis (Fig. 1B). Strain WS1100 failed to hydrolyze skim milk protein in PIA medium, whereas strain WS1700 was weakly proteolytic on PIA skim milk medium. Neither strains WS1100 nor WS1700 was able to hydrolyze elastin in PIA medium. Both strains produced extracellular protein antigens that immunoprecipitated with rabbit anti-elastase antibody in an in situ immunoprecipitation assay on PIA, yet neither strain produced measurable alkaline protease as determined by a similar in situ immunoprecipitation assay (Fig. 4).

When WS1100 was plated on elastin-containing medium without kanamycin, elastase-producing colonies from different subclones appeared at a frequency that ranged from no revertants to 100% reversion. We observed subsequently that colonies of wild-type *P. aeruginosa* produced a diffusible substance that induced exoprotease activity from WS1100 on LB. This was indicated by hydrolysis of skim milk protein or elastin around colonies of WS1100. The cell-free supernatants of wild-type strains also contained this protease-activating factor and induced WS1100 colonies to hydrolyze skim milk and elastin even when grown under geneticin selection (Fig. 5). Similar results were obtained with purified alkaline protease (data not shown). In the absence of wild-type cells or supernatant fluids, colonies of WS1100 do not secrete active proteases.

Mutant strain WS1700, however, could not be induced for protease activity under the same conditions used to activate WS1100. When WS1700 was grown on LB, we were unable to detect any elastase by the in situ immunoprecipitation assay. This is in contrast to the assay on PIA, in which strain WS1700 secreted an inactive elastase. This proelastase can be activated by wild-type cells or culture supernatant fluid. It is not known why medium composition changes protein secretion in WS1700 or why it differs from WS1100 in this respect.

DISCUSSION

The application of molecular genetic techniques to the genetic analysis of *P. aeruginosa* has not progressed as rapidly as it has for *E. coli*. Several factors have contributed to this lack of progress. A system for transposon mutagenesis in *P. aeruginosa* has lagged behind other genera because suitable delivery vectors for the transposons were not available. *P. aeruginosa* is spontaneously resistant to high concentrations of several antibiotics so that discrimination between the transposon antibiotic marker and spontaneous resistance may be difficult. Regional specificity of transposon insertion was reported for Tn*1* in *P. aeruginosa* (19), thus limiting its usefulness in transposon mutagenesis.

Additionally, the analysis of *P. aeruginosa* genes cloned in *E. coli* has progressed slowly because *E. coli* does not express *P. aeruginosa* genes efficiently. The study of *P. aeruginosa*-secreted proteins in *E. coli* is hindered because *E. coli* does not secrete proteins that are normally secreted from *P. aeruginosa* (6, 36). *E. coli* vector systems such as cosmids, that carry large segments (greater than 10 kilobases) of the PAO chromosome are unstable in *E. coli* (6).

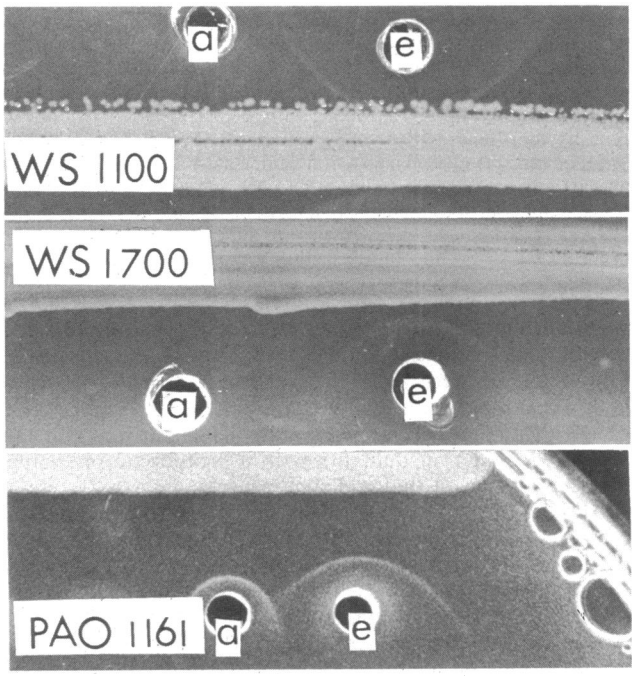

FIG. 4. In situ immunoprecipitation of exoproducts with rabbit anti-alkaline protease (a) and anti-elastase (e).

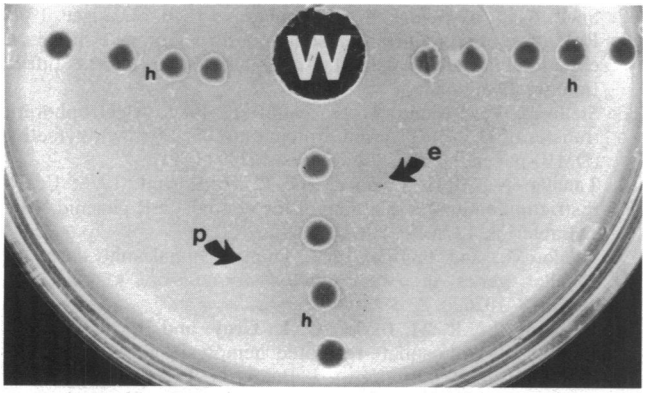

FIG. 5. WS1100 activation on LB Gt500-SKE medium. Isolates of WS1100 became proteolytic or elastolytic when wild-type culture supernatant fluid (W) was placed in the center well. The arrow (e) represents the edge of elastin hydrolysis by elastase diffusing from W. The arrow (p) represents the extent of skim milk hydrolysis by protease from W. Additional clearing (h) appeared around induced WS1100 colonies. The opacity in the SKE medium was due to skim milk, and the particulate matter was elastin particles.

Finally, the *P. aeruginosa* genome lacks the large operon organization found in other gram-negative organisms; instead, related functions can be disseminated over the entire chromosome (13). Thus, all genes controlling a secreted protein in *P. aeruginosa* are not likely to be isolated on a single vector.

In spite of the difficulties inherent in this *P. aeruginosa* system, we have generated Tn*5* insertion mutants. Two independent, elastase-deficient Tn*5* insertion mutants were chosen for extensive characterization. Both strains WS1100 and WS1700 produced a proelastase that could be activated by wild-type culture supernatant fluid and purified alkaline protease. Since alkaline protease cannot be detected in either mutant and purified alkaline protease can convert inactive proelastase to active elastase, the pleiotropic property of these mutants may be due to an insertion into a gene for the synthesis or secretion of alkaline protease. The ability of both strains to secrete proelastase is dependent upon the medium composition and is different for the two mutants. The regulation of proelastase secretion may reflect the difference in the original parents.

ACKNOWLEDGMENTS

This research was supported by grants-in-aid from Eli Lilly & Co., Indianapolis, Ind. Additional support was provided by grants from the Wright State University Research Council and the School of Medicine.

LITERATURE CITED

1. **Berg, D. E., C. Egner, B. J. Hirschel, J. Howard, L. Johnsrud, R. A. Jorgensen, and T. D. Tlsty.** 1981. Insertion, excision and inversion of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. **45**:118.
2. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276**:633–634.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95.
4. **Carey, S. A., R. E. Dyster, and R. J. Jones.** 1973. The invasion of burned skin by *Pseudomonas aeruginosa*. Br. J. Dermatol. **88**:539–545.
5. **Cicmanec, J. F., and I. A. Holder.** 1979. Growth of *Pseudomonas aeruginosa* in normal and burned skin extract: role of extracellular proteases. Infect. Immun. **25**:477–483.
6. **Coleman, K., G. Dougan, and J. P. Arbuthnott.** 1983. Cloning and expression in *E. coli* K-12, of the chromosomal hemolysin (phospholipase C) determinant of *Pseudomonas aeruginosa*. J. Bacteriol. **153**:909–915.
7. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 116–117. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Ely, B., and R. H. Croft.** 1982. Transposon mutagenesis in *Caulobacter crescentus*. J. Bacteriol. **149**:620–625.
9. **Finger, J., and V. Krishnapillai.** 1980. Host range, entry exclusion and incompatibility of *Pseudomonas aeruginosa* FP plasmids. Plasmid **3**:322–342.
10. **Garvey, J., N. Cremer, and D. Sussdorf.** 1977. Methods in immunology. W. A. Benjamin, Inc., Reading, Mass.
11. **Hirao, Y., and J. Y. Homma.** 1978. Therapeutic effect of immunization with OEP, protease toxoid and elastase toxoid on corneal ulcers in mice due to *Pseudomonas aeruginosa* infection. Jpn. J. Exp. Med. **48**:41–51.
12. **Holder, I. A., and C. G. Haidaris.** 1979. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: extracellular protease and elastase as in vivo virulence factors. Can. J. Microbiol. **25**:593–599.
13. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43**:73–102.
14. **Jagger, K. S., D. L. Robinson, M. N. Franz, and R. L. Warren.** 1982. Detection by enzyme-linked immunosorbent assays of antibody specific for *Pseudomonas* proteases and exotoxin A in sera from cystic fibrosis patients. J. Clin. Microbiol. **15**:1054–1058.
15. **Jensen, S. E., L. Phillippe, J. T. Tseng, G. W. Stemke, and J. N. Campbell.** 1980. Purification and characterization of exocellular proteases produced by a clinical isolate and a laboratory strain of *Pseudomonas aeruginosa*. Can. J. Microbiol. **26**:77–86.
16. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
17. **Kawaharajo, K., C. Abe, J. Y. Homma, M. Kawano, E. Gotoh, N. Tanaka, and K. Morihara.** 1974. Corneal ulcers caused by protease and elastase from *Pseudomonas aeruginosa*. Jpn. J. Exp. Med. **44**:435–442.
18. **Kessler, E. H., E. Kennah, and S. I. Brown.** 1977. *Pseudomonas* protease. Purification, partial characterization and its effect on collagen, proteoglycan and rabbit corneas. Invest. Ophthalmol. Vis. Sci. **16**:488–497.
19. **Krishnapillai, V., P. Royle, and J. Lehrer.** 1981. Insertions of the transposon Tn*1* into the *Pseudomonas aeruginosa* chromosome. Genetics **97**:495–511.
20. **Liu, P. V.** 1979. Toxins of *Pseudomonas aeruginosa*, p. 63–68. *In* R. G. Doggett (ed.), *Pseudomonas aeruginosa*: clinical manifestations of infection and current therapy. Academic Press, Inc., New York.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
23. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
24. **Morgan, A. F.** 1982. Isolation and characterization of *Pseudomonas aeruginosa* R′ plasmids constructed by interspecific mating. J. Bacteriol. **149**:654–661.
25. **Morihara, K.** 1963. *Pseudomonas aeruginosa* proteinase. I. Purification and general properties. Biochim. Biophys. Acta **73**:113–124.
26. **Morihara, K., and H. Tsuzuki.** 1977. Production of protease and

elastase by *Pseudomonas aeruginosa* strains isolated from patients. Infect. Immun. **15**:679–685.

27. **Morihara, K., H. Tsuzuki, T. Oda, H. Inoue, and M. Ebata.** 1965. *Pseudomonas aeruginosa* elastase. Isolation, crystallization and preliminary characterization. J. Biol. Chem. **240**:3295–3304.
28. **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.
29. **Pavlovskis, O. R., and B. Wretlind.** 1979. Assessment of protease (elastase) as a *Pseudomonas aeruginosa* virulence factor in experimental mouse burn infection. Infect. Immun. **24**:181–187.
30. **Pulverer, G., Z. Wegrzynowicz, H. L. Ko, and J. Jeljaszewacz.** 1980. Influence of *Pseudomonas aeruginosa* proteases on prothrombin, plasminogen and fibrinogen. Zentralbl. Bakteriol. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **248**:99–109.
31. **Sinclair, M. I., and B. W. Holloway.** 1982. A chromosomally located transposon in *Pseudomonas aeruginosa*. J. Bacteriol. **151**:569–579.
32. **Snell, K., I. A. Holder, S. A. Leppla, and C. B. Saelinger.** 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with *Pseudomonas aeruginosa*. Infect. Immun. **19**:839–845.
33. **Stellwag, E. J., and A. E. Dahlberg.** 1980. Electrophoretic transfer of DNA, RNA and protein onto diazobenzyloxymethyl (DMB)-paper. Nucleic Acids Res. **8**:299–317.
34. **Tanaka, N., J. H. Cramer, and R. H. Rownd.** 1976. EcoRI restriction endonuclease map of the composite R plasmid NR1. J. Bacteriol. **127**:619–636.
35. **Tsuda, M., and T. Iino.** 1983. Transductional analysis of the flagellar genes in *Pseudomonas aeruginosa*. J. Bacteriol. **153**:1018–1026.
36. **Vasil, M. L., R. M. Berka, G. L. Gray, and H. Nakai.** 1982. Cloning of a phosphate-regulated hemolysin gene (phospholipase C) from *P. aeruginosa*. J. Bacteriol. **152**:431–440.
37. **Warren, R. L.** 1980. Exfoliative toxin plasmids of bacteriophage group 2 *Staphylococcus aureus*: sequence homology. Infect. Immun. **30**:601–606.