Characterization of a *Pseudomonas aeruginosa* Transposon Insertion Mutant with Defective Release of Exoenzymes

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A *Pseudomonas aeruginosa* transposon insertion mutant with defective release of several exoenzymes has been characterized. The Tn5-751 insertion mutation was located in the previously described *xcp-1* locus at 0 min on the chromosomal map and caused several exoenzymes to remain in cell-bound form. At least one of the exoenzymes, elastase, was accumulated in the periplasmic space. The periplasmic elastase had the same M_r as the extracellular enzyme produced by the wild-type strain. The virulence of the mutant was comparable to that of wild-type strains in experimental burn infection in mice. The presence of an easily selectable antibiotic resistance marker in the *xcp-1* locus offers the possibility of cloning the gene(s) involved in exoprotein secretion.

INTRODUCTION

Pseudomonas aeruginosa possesses machinery for the efficient release of exoproteins and is of interest as a host for cloned genes coding for exported products. Six genetic loci (*xcp*) involved in the synthesis or release of exoproteins have been described in *P. aeruginosa* (Wrettlind & Pavlovskis, 1984). Mutations in these *xcp* loci cause pleiotropic effects with reduced activities of several exoenzymes. In some of the Xcp mutant classes (*xcp-1*, *xcp-5* and *xcp-6*) exoproteins are synthesized but are not exported efficiently, indicating that the corresponding loci control some steps in the transport machinery. In order to study the mechanism of protein export further in *P. aeruginosa* we have isolated transposon insertion mutations in those *xcp* loci that affect exoprotein release. In the present work the genetic and enzymic characterization of a mutant with a transposon insertion in the *xcp-1* locus is presented. We have also studied the effect of this mutation on virulence since roles for exotoxin A and elastase in certain types of *Pseudomonas* infections have been postulated (Lui, 1974; Pavlovskis & Wrettlind, 1979; Woods & Iglewski, 1983; Wrettlind & Pavlovskis, 1983).

METHODS

Bacterial strains, plasmids and bacteriophage. The bacterial strains, plasmids and bacteriophage used in this study are listed in Table 1. The insertion mutation was isolated as an elastase deficient mutant after transposon mutagenesis with Tn5-751 (Haas *et al.*, 1984) and transferred to the prototrophic strain PAO1 by transduction. The resulting transductant PAO6204 was protease-negative on skimmed milk agar and exhibited resistance to kanamycin and trimethoprim (Haas *et al.*, 1984). Strain PAO6204 was compared to strain PAO6202 by doing enzyme assays and virulence tests. In strain PAO6202 Tn5-751 is inserted into a *hyu* (hydroxyproline utilization) locus, but otherwise the two strains are isogenic.

Media and growth conditions. For the assays of enzyme activities and for the identification of enzyme antigens the following media were used: (a) minimal medium of Davis & Mingioli (1950) supplemented with glucose (5 g l⁻¹) and yeast extract (2 g l⁻¹) (MYG) for the assay of alkaline proteinase (EC 3.4.24.4); (b) MYG medium

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase.

Table 1. *Bacterial strains, plasmids and bacteriophage used in this study*For genetic symbols see Holloway *et al.* (1979) and Holloway & Matsumoto (1984).

	Genotype or phenotype and approx. map position of relevant markers	Reference
<i>P. aeruginosa</i> strains		
POA1	Prototroph, <i>chl-2</i>	Holloway <i>et al.</i> (1979)
PAO25	<i>argF10 leu-10</i>	Haas & Holloway (1976)
PAO236	<i>ilv-226</i> (7 min) <i>his-4</i> (15 min) <i>lys-12 met-28 trp-6 proA82 nalA</i>	Haas & Holloway (1976)
PAO6202	<i>hyu::Tn5-751</i>	Haas <i>et al.</i> (1984)
PAO6204	<i>xcp::Tn5-751</i>	Haas <i>et al.</i> (1984)
PAO7520	<i>pur-67</i> (90 min) <i>cys-59</i> (93 min) <i>proB</i> (2 min) Str ^R	Streptomycin resistant mutant of PAO6020 (Soldati <i>et al.</i> , 1984)
KS904	<i>ilv-226 his-4 lys-12 met-28</i> <i>trp-6 proA82 xcp-1</i> (0 min)	Wrettlind & Pavlovskis (1984)
Plasmids		
FP2	Hg Tra Cma IncP-8	Pemberton & Holloway (1972)
R68.45	Cb Nm/Km ^R Tc ^R Tra Cma IncP-1	Haas & Holloway (1976)
Bacteriophage		
E79tv2	General transducing phage	Morgan (1979)

supplemented with Casamino acids (10 g l⁻¹) (MCYG) for the assays of elastase (EC 3.4.24.4) and staphylolytic enzyme; (c) phosphate deficient medium according to Torriani (1968) for the assay of alkaline phosphatase (EC 3.1.3.1); (d) MYG medium supplemented with tryptone (5 g l⁻¹) for the assay of lipase (EC 3.1.1.3); and (e) dialysed tryptic soy broth supplemented with 5 mM-nitritoltriacetate and glycerol (10 g l⁻¹) for the assay of exotoxin A. The strains were grown in baffled shaking flasks at 37 °C for media (a)–(d) and at 30 °C for medium (e) (Wrettlind & Pavlovskis, 1984; Björklind *et al.*, 1985). Enzyme activities were assayed in overnight cultures since the extracellular levels of the different exoenzymes were then found to be maximum in cultures of wild-type bacteria. There were no significant differences in growth yield between the mutants and strain PAO1. The two strains were cultured at the same time and the enzyme assays were then done in parallel.

Genetic techniques. Recombinants were selected on agar plates with the minimal medium of Davis & Mingioli (1950) supplemented with glucose and appropriate growth factors. Trimethoprim resistance (Tp^R) was scored on nutrient agar supplemented with 500 µg trimethoprim ml⁻¹.

The isolation of the mutant strain PAO6204 by transposon insertion mutagenesis has been described previously (Haas *et al.*, 1984). Conjugational crosses with the *Escherichia coli* strain ED8654 (pME9) and the *P. aeruginosa* strain PAO6049 were performed and the transposon phenotype (Km^R Tp^R) was selected for at 43 °C. The donor was counterselected with streptomycin. After purification of the Km^R Tp^R Str^R recombinants it was confirmed that they were sensitive to carbenicillin and tetracycline, i.e. that the vector plasmid was no longer present. The recombinants were then screened for elastase activity on elastin agar plates. One elastase deficient mutant was found among 2500 recombinants carrying the Tn-5-751 transposon from pME9. The transposon insertion mutation causing the elastase deficient phenotype was subsequently transferred to strain PAO1 by transduction and one of three tested transductants had the exoprotein deficient phenotype (Xcp⁻) (Haas *et al.*, 1984). This transductant (PAO6204) was used in the present study.

Conjugational crosses for mapping of the transposon mutation were done by the membrane filter mating technique (Curtis, 1982). Since strain PAO6204 is prototrophic it was counterselected with streptomycin or nalidixic acid (1 mg ml⁻¹) when used as a donor in conjugation. Transduction was done as described previously (Haas *et al.*, 1977; Wrettlind & Pavlovskis, 1984). All recombinants were purified once by picking them onto the same medium as they had been selected on, before scoring for unselected markers. The Xcp phenotype was scored on tryptic soy agar supplemented with 15% (w/v) skimmed milk.

Fractionation of cells. Cell-bound enzyme activities (Table 2) were measured in cell pellets that had been washed once with 0.9% NaCl and then disintegrated by ultrasonic treatment. Spheroplast preparation and shocking procedure were essentially according to the methods of Cheng *et al.* (1971). The cells in a 50 ml overnight culture were washed once with 50 ml 0.01 M-Tris/HCl (pH 7.5) and resuspended in 0.2 M-MgCl₂, 0.01 M-Tris/HCl (pH 8.4), lysozyme (500 µg ml⁻¹). After incubation for 30 min in a 25 °C water bath shaker the cells were centrifuged (13000 g, 15 min), resuspended in cold 0.01 M-MgCl₂, 0.01 M-Tris/HCl (pH 8.4) and incubated at 4 °C for 15 min. During this incubation the majority of the cells became spherical. These spheroplasts were centrifuged (13000 g, 15 min), resuspended in cold 0.2 M-MgCl₂, 0.01 M-Tris/HCl (pH 8.4) and again incubated at 4 °C for

15 min. Finally, the spheroplasts were centrifuged and resuspended in 0.01 M-Tris/HCl (pH 7.5). They were then sonicated and the membrane fraction was pelleted by centrifugation at 105000 *g* for 60 min. During the whole procedure samples were taken from the supernatants and the membrane fraction for assays of elastase and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) activities. Elastase activity was not inhibited by any of the MgCl₂ concentrations used in the shock fluids, while G6PD activity was inhibited by 0.2 M-MgCl₂ but not by 0.01 M.

Enzyme assays. Proteinase activity was measured by a caseinolytic method with azocasein as substrate (Kreger & Gray, 1978) [one unit of enzyme activity (U) corresponds to an increase in A_{440} of 0.1 min⁻¹]. Since the cells were grown in MCYG broth in which little or no alkaline proteinase is formed by wild-type cells, the assay predominantly measures the elastase activity (Jensen *et al.*, 1980*a*, Pavlovskis & Wretling, 1982). Lipase activity was assayed with *p*-nitrophenyl caprylate as substrate (Wretling *et al.*, 1977) and alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate as substrate (Torriani, 1968) (for both assays 1 U causes the hydrolysis of 1 μ mol substrate min⁻¹). Staphylolytic enzyme activity was measured as lysis of a suspension of *Staphylococcus aureus* Copenhagen at 25 °C (Wretling *et al.*, 1977) (1 U corresponds to a decrease in A_{650} of 1.0 min⁻¹). Alkaline proteinase and exotoxin A in cell culture supernatants were assayed by rocket immunoelectrophoresis against rabbit antisera (Laurell, 1966; Björklind *et al.*, 1985). G6PD activity was assayed as described by Malamy & Horecker (1964) (1 U causes the reduction of 1 μ mol NADP min⁻¹). Elastase assays were done twice on culture supernatants and ultrasonic treated cells. The other enzymes were tested once. Although the absolute elastase activity showed a significant variation between experiments, the relative amounts of cell-bound and extracellular activities always showed the same pattern, i.e. no cell-bound forms of exoenzymes could be detected in the wild-type strain under the experimental conditions used here, while the mutant had more than 50% of the total activities of several exoenzymes cell-bound.

SDS-PAGE and immunoblotting. Cells grown in MCYG were washed once with 0.9% saline and disintegrated by ultrasonic treatment. Supernatants from the same cell cultures were concentrated about 20-fold by dialysis against polyethylene glycol 20M. After boiling in SDS-buffer according to Neville (1971), samples of cells, supernatants and an elastase standard were electrophoresed through a stacking gel (6%, polyacrylamide) and a gradient resolving gel (7.5–15%, w/v, polyacrylamide) with the buffer system of Neville (1971). In each experiment two gels were run with the same samples. One gel was stained with Coomassie Brilliant Blue while the separated proteins on the other gel were transferred to a nitrocellulose sheet and submitted to immunoblotting essentially as described by Towbin *et al.* (1979). Elastase antiserum for the immunoblotting procedure was prepared by immunizing rabbits with *P. aeruginosa* elastase as described previously (Pavlovskis & Wretling, 1979). The detecting probe in the immunoblotting procedure was horse-radish peroxidase substrate used according to the directions of the producer.

Virulence tests. The bacteria were grown in Brain Heart Infusion broth. They were harvested in the exponential phase of growth, washed once with PBS (0.18 M-NaCl, 0.0036 M-KCl, 0.0086 M-Na₂HPO₄, 0.0020 M-KH₂PO₄, pH 7.4) and then suspended in PBS to approximately 4×10^8 c.f.u. ml⁻¹. From this suspension serial 10-fold dilutions were prepared and inoculated subcutaneously into mice according to the burned mouse model of Stieritz & Holder (1975).

Chemicals. Azocasein, *p*-nitrophenyl caprylate, *p*-nitrophenyl phosphate, glucose 6-phosphate and NADP were from Sigma; *P. aeruginosa* exotoxin A was from the Swiss Serum and Vaccine Institute, Berne, Switzerland; *P. aeruginosa* alkaline proteinase and elastase were from Nagase Biochemicals, Kyoto, Japan; and horse-radish peroxidase, conjugated *S. aureus* protein A and peroxidase substrate were from Bio-Rad.

RESULTS

Biochemical characterization of strain PAO6204

The mutant strain PAO6204 could not efficiently export elastase, lipase, alkaline phosphatase, staphylolytic enzyme or exotoxin A out of the cells. Instead these enzymes were accumulated in cell-bound form (Table 2). Alkaline proteinase was the only exoenzyme which we have found extracellularly in similar amounts as the wild-type strain (Table 2). Thus, phenotypically, strain PAO6204 resembled the previously described exoprotein deficient mutants KS904 (*xcp-1*) and KS902 (*xcp-5*) (Wretling & Pavlovskis, 1984).

The localization in the mutant cells of one of the exoproteins, namely elastase, was further investigated by osmotic shocking of the cells and subsequent disruption by sonication. The major part of the elastase activity was found in the shock fluids. Periplasmic proteins are selectively released by such a shock procedure (Cheng *et al.*, 1971). G6PD was used as an intracellular marker. The activity of this enzyme was inhibited by 0.2 M-MgCl₂ and therefore could be assayed validly only in the membrane fraction, the cell lysate and the 0.01 M-MgCl₂

Table 2. *Localization of exoenzymes in strains PAO6202 (xcp⁺) and PAO6204 (xcp : : Tn5-751)*

Enzyme activities are given as U ml⁻¹ except alkaline protease (µg ml⁻¹) and exotoxin A (ng ml⁻¹). EC, extracellular activity; CB, cell-bound activity.

Strain	Elastase		Lipase		Alkaline phosphatase		Staphylolytic enzyme		Exotoxin A		Alkaline proteinase	
	EC	CB	EC	CB	EC	CB	EC	CB	EC	CB	EC	CB
PAO6202	2.0	0	670	165	180	113	2.2	0.1	400	ND	11	ND
PAO6204	0.5	1.7	67	224	36	960	0.1	3.5	0	ND	10	ND

ND, Not done.

Table 3. *Localization of elastase and G6PD*

Enzyme activities are given as percentages of total activity. Total elastase activity was 2.0 U ml⁻¹ for PAO6202 and 2.4 U ml⁻¹ for PAO6204. Total G6PD activity was 0.171 U ml⁻¹ for PAO6202 and 0.032 U ml⁻¹ for PAO6204.

	Elastase		G6PD	
	PAO6202	PAO6204	PAO6202	PAO6204
Culture supernatant	> 99.5	23	0	0
0.2 M-MgCl ₂ /lysozyme	0	7	ND	ND
0.01 M-MgCl ₂	0	34	0	3
0.2 M-MgCl ₂	0	20	ND	ND
Membrane fraction of spheroplasts	0	< 0.5	3	0
Cell-free lysate	< 0.5	16	97	97

ND, Not done.

shock fluid. Since no G6PD activity was found in the 0.01 M-MgCl₂ shock fluid, we concluded that there was no extensive cell lysis during the spheroplast formation procedure (Table 3). G6PD activity in the cell lysate of PAO6204 was significantly lower than that of PAO6202 (Table 3). When equal amounts of cell lysate from PAO6202 and PAO6204 were mixed and G6PD activity then assayed, the PAO6202 activity was reduced more than fivefold (data not shown). We suggest that this reduction was due to digestion of G6PD by protease(s) present in the cell lysate from PAO6204, probably as a contamination from the shock fluids (Table 3). From the above experiments we concluded that elastase and probably also the other cell-bound exoproteins were located in the periplasmic space of the mutant cells.

In another mutant (PAKS-18) isolated in our laboratory an inactive elastase precursor is accumulated in the periplasmic space (Wretling *et al.*, 1977; Fecycz & Campbell, 1985). The precursor can be activated by several proteases, including elastase, or by incubation of the sonicated cells at 37 °C. No further activation of elastase could be demonstrated in strain PAO6204 (data not shown).

Exoproteins are generally formed as larger precursors from which the NH₂-terminal signal sequence is cleaved during the transport process (for a review see Silhavy *et al.*, 1983). We therefore compared the molecular size of the cell-bound elastase from strain PAO6204 with that of extracellular elastase from strain PAO6202. The enzymes were analysed by SDS-PAGE combined with immunoblotting. No difference between the elastases from the two strains was found (data not shown).

Mapping of the insertion mutation in strain PAO6204

Conjugational crosses mediated by the plasmids R68.45 and FP2, respectively, were done with strain PAO6204 as a donor and different auxotrophic strains as recipients. Significant linkage was found between the *xcp : : Tn5-751* mutation and the *ilv-226*, *his-4*, *proB* and *cys-59* markers (Table 4). Segregation of trimethoprim resistance (due to the *Tn5-751* insertion) among the Pro⁺ recombinants was controlled in the cross between PAO6204(R68.45) and PAO7520.

Table 4. *Conjugational analysis of the xcp : : Tn5-751 mutation*

Donor strains were PAO6204(R68.45) and PAO6204(FP2), respectively. Recipient strains were PAO236 and PAO7520.

Selected marker	Percentage of Xcp ⁻ recombinants	
	Donor R68.45	Donor FP2
<i>ilv-226</i> (7 min) <i>his-4</i> (15 min)	61	36
<i>proB</i> (2 min)	90	55
<i>cys-59</i> (93 min)	93	65
<i>pur-67</i> (90 min)	7	ND

ND, Not done.

All the Xcp⁻ recombinants were resistant to trimethoprim while all the Xcp⁺ recombinants were sensitive. Thus, trimethoprim resistance segregated together with the Xcp⁻ phenotype, indicating that this phenotype of strain PAO6204 was caused by the insertion of Tn5-751. In transductional crosses mediated by phage E79tv2 no linkage was found between the *xcp* insertion mutation and *proB* or *cys-59* markers (data not shown).

The mapping data from the conjugational crosses indicated that the *xcp : : Tn5-751* mutation was located in the same region as the previously described *xcp-1* mutation near 0 min on the chromosomal map (Wretling & Pavlovskis, 1984). In order to confirm the close vicinity between the *xcp : : Tn5-751* and *xcp-1* mutations, we did two R68.45 mediated crosses with strain KS904 as recipient and with strains PAO6204 and PAO25, respectively, as donors. Ilv⁺ recombinants were selected for and the frequency of Xcp⁺ was then scored. In the cross with PAO25 (which is Xcp⁺) as a donor the co-transfer frequency of Xcp⁺ was 58%. When strain PAO6204 was used as a donor no Xcp⁺ recombinants could be found among 199 scored Ilv⁺ exconjugants. We concluded that *xcp-1* (in strain KS904) and *xcp : : Tn5-751* are located close to each other in the same gene and thus recombination between the two mutations is prevented or is a very rare event.

Virulence of the xcp transposon insertion mutant

Virulence of strains PAO6202 and PAO6204 was compared using the burned mouse model (Stieritz & Holder, 1975; Pavlovskis & Wretling, 1979). No significant difference between LD₅₀ values for the two strains was found in three separate experiments (data not shown).

DISCUSSION

We have studied a transposon insertion mutation in *P. aeruginosa* causing defective release of several exoenzymes. The mutation is located in the previously described *xcp-1* locus. The results of this study indicate that the exoenzymes accumulate in the periplasmic space of the mutant cells. In both wild-type and mutants of certain strains of *P. aeruginosa* an inactive elastase precursor, with the same *M_r* as mature elastase, has been demonstrated in the periplasmic space (Jensen *et al.*, 1980b; Fecycz & Campbell, 1985). Evidence was presented suggesting that activation occurs by removal of a non-covalently bound inhibitory component during transport through the outer membrane. In the mutant strain PAO6204 the periplasmic elastase appears to be enzymically active. However, in some *P. aeruginosa* strains, e.g. PAO strains, proelastase is much more readily activated than in other strains. Thus, elastase in the PAO6204 mutant might have been spontaneously activated during sonication of the cells.

Proteins destined for export are generally cleaved at the NH₂-terminus during the transport process, probably during release from the cytoplasmic membrane (Blobel & Dobberstein, 1975a, b; Silhavy *et al.*, 1983). The molecular size of the periplasmic elastase from PAO6204 is the same as that of extracellular wild-type elastase, suggesting that export is blocked at a later stage in this mutant. The transposon insertion mutation with an easily selectable antibiotic resistance marker (Tp^R) in the *xcp-1* locus offers the possibility of cloning the gene(s) within this locus. The *xcp-1* gene product then could be identified which should be helpful in elucidating the mechanism for protein export in *P. aeruginosa*.

P. aeruginosa mutants with similar properties as those of PAO6204 but mapping in the 55 min region of the chromosome have recently been described (Wretling & Pavlovskis, 1983; A. Filloux *et al.*, unpublished observation). In these mutants only active elastase and no proelastase could be detected in the periplasmic space. In some pleiotropic export mutants of *Aeromonas hydrophila* two outer membrane proteins are missing, while in others the outer membrane protein profile is normal (Howard & Buckley, 1983). It will therefore be of interest to compare the outer membrane composition of the different *P. aeruginosa* mutants with that of the *A. hydrophila* mutants.

The only exoenzyme which we have found to be exported normally by the *xcp-1* mutant is alkaline proteinase. Another transposon insertion mutant unable to produce extracellular alkaline proteinase has been described (Stapleton *et al.*, 1984). The presence of cell-bound alkaline proteinase was not reported and neither was the genetic locus of the mutation in this case. It is clear however that the export of alkaline proteinase is different from that of other exoproteins in *P. aeruginosa* (Wretling & Pavlovskis, 1984).

PAO6202 and PAO6204 are isogenic except for the transposon insertion in *xcp-1* in PAO6204 and in *hyu* in PAO6202. Thus the effect of the *xcp-1* mutation on virulence can be studied without the interference of other mutations. We could not find any significant difference in virulence between the two strains. This is in agreement with the results of Cryz *et al.* (1983) who found that antisera against elastase and exotoxin A did not offer any protection against *P. aeruginosa* infection in a burned mouse model system. However, other investigators have found a small but significant role for exoproteins in this experimental infection model (Pavlovskis *et al.*, 1977; Pavlovskis & Wretling, 1979). Our results concerning virulence must be interpreted with caution, since the mutant PAO6204 accumulates considerable exoenzyme activity in cell-bound form. Cell-bound exoenzyme fractions may be released from dying cells and subsequently contribute to the virulence of the living mutant bacteria.

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