

## Adherence of Protease-Deficient Mutants of *Pseudomonas aeruginosa* to a Rabbit Cornea Cell Line (SIRC) Cells

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### ABSTRACT

Seven protease-deficient mutants were isolated from *Pseudomonas aeruginosa* strain IFO 3455 which was mutagenized with nitrosoguanidine. Characterization of these mutants *in vitro* revealed that all mutants showed pleiotropic changes in the production of other extracellular substances. Among the mutants, two were chosen for a bacterial adherence test to Rabbit Cornea Cell Line (SIRC) cells. One mutant (IFO 3455-2) completely lost its protease activity. Another (IFO 3455-3) retained a low protease activity and was relatively similar to the parental strain with respect to extracellular products except for protease. Both mutants gave not a marked but a slight decrease of adherence as compared with the parental strain. This finding suggests that besides protease more factors are involved in the adhesion between *P. aeruginosa* and SIRC cells.

It is well known that *Pseudomonas aeruginosa* produces many extracellular enzymes and toxins which play an important role in the pathogenesis of the infections. Two distinct proteases, alkaline protease and elastase, have been isolated and characterized by Morihara<sup>12)</sup>. *P. aeruginosa* proteases were reported to contribute to the corneal ulcer by several research groups years ago<sup>3,10,11)</sup>. Recently, Howe and Iglewski<sup>6)</sup> investigated the virulence of an alkaline protease-deficient mutant of *P. aeruginosa* and demonstrated that alkaline protease was necessary for the establishment of corneal infection.

Our previous work<sup>9)</sup> showed that protease-producing strains of clinical isolates of *P. aeruginosa* adhered more avidly to Rabbit Cornea Cell Line (SIRC) cells than non-producing ones. The result suggests the possibility that the protease takes part in the adhesion between *P. aeruginosa* and SIRC cells. Subsequently, we isolated protease-deficient mutants of *P. aeruginosa* and investigated the ability of the

mutants to adhere to SIRC cells.

### MATERIALS AND METHODS

**Organisms:** *P. aeruginosa* strain IFO 3455, which produces protease and elastase, was used for the isolation of mutants. Strains PA 103 (P<sup>+</sup>E<sup>-</sup>) and NC 5 (P<sup>-</sup>E<sup>-</sup>) were also used. Strain PA 103 produces a trace of protease but no elastase, whereas strain NC 5 produces neither of the enzymes. All of the organisms were maintained on slopes of trypticase soy agar.

**Isolation of mutants:** Log-phase cultures of strain IFO 3455 grown in nutrient broth containing 0.5% yeast extract (NYB) were mutagenized with 100 µg of N-methyl-N-nitrosoguanidine (NTG; Aldrich Chemical Co., U.S.A.) per ml in tris-maleate buffer, pH 6.0<sup>2)</sup>. Thirty min after incubation with NTG, the bacteria were washed twice with and suspended in NYB, and cultured overnight with shaking. The culture was diluted to yield approximately 50 colonies per plate and inoculated on nutrient

agar plates containing 1% casein (from milk; Nakarai Chemicals, Ltd., Japan). After the incubation of these plates for 24 hr, colonies were examined for clear zones around them. Colonies which produced no clear zone were chosen for further study.

*Characterization of mutants:* Serological typing was done by the slide agglutination using *P. aeruginosa* antisera (Toshiba Chemical Industries, Ltd., Japan). Motility was observed microscopically, and a growth curve was measured spectrophotometrically. Elastase production was determined on nutrient agar containing 1% elastin (Nakarai Chemicals Ltd., Japan), gelatinase on gelatin plate<sup>6)</sup>, lipase on tributyrin agar<sup>7)</sup>, and hyaluronidase on brain heart infusion (BHI) agar containing 0.04% hyaluronic acid (from human umbilical cord, grade III-S; Sigma Chemical Co., U.S.A.)<sup>16)</sup>. Activities of these extracellular enzymes were defined as clear zones produced around the streaks. Esterase production was judged by blue colonies on BHI agar supplemented with 0.1%  $\alpha$ -naphthyl acetate (Sigma Chemical Co., U.S.A.)<sup>5)</sup>, and DNase was demonstrated on DNase test agar (Eiken Chemical Co., Ltd., Japan). Hemolysin production was determined on sheep blood agar (Eiken Chemical Co., Ltd., Japan), and fluorescein production on King A agar (Eiken Chemical Co., Ltd., Japan).

*Protease assay:* To determine extracellular protease quantitatively, cells were grown in Davis minimal medium supplemented with 2.5 g of yeast extract per liter, 25 mM glucose and 1 mM CaCl<sub>2</sub>. For cell-bound protease, cells were disintegrated by ultrasonic treatment (200W, 5 min; Insonator Model 200M, Kubota Shoji Co., Ltd., Japan). To 0.25 ml of the culture supernatant or the cell extract was added 0.5 ml of 2% casein (nach Hammarsten; E. Merk, Darmstadt, Germany) in 0.1 M sodium phosphate buffer at pH 8.0 and 0.25 ml of the buffer. The mixture was incubated at 37°C for 30 min, followed by the addition of 5 ml of 3% TCA. The amount of TCA-soluble peptides was determined by Folin-Ciocalteu's reagent using tyrosine as a standard<sup>11)</sup>. Protease was expressed as  $\mu$ g of tyrosine released per min per ml.

*Labelling of bacteria:* Bacteria were prepared for isotopic labelling by growing the cells overnight at 37°C in trypticase soy broth. The bac-

teria were washed once with M-9 broth and inoculated to give an optical density of 0.59 at 570 nm (approximately  $2.0 \times 10^9$  organisms/ml) by Coleman Junior Spectrophotometer into M-9 broth supplemented with 0.5  $\mu$ Ci of <sup>14</sup>C-lysine (Amersham Corp., U.S.A.) per ml. After incubation at 37°C for 4 hr, the bacteria were washed three times with and suspended in phosphate-buffered saline (PBS) to contain  $2.0 \times 10^9$  organisms per ml.

*Epithelial cell culture:* SIRC cell cultures were prepared as described previously<sup>9)</sup>. Briefly, the cells were suspended at a density of  $2.0 \times 10^5$  cells per ml of Dulbecco's modified essential medium (Nissui Seiyaku Co., Ltd., Japan) supplemented with 10% calf serum (Grand Island Biological Co., U.S.A.), allowed to settle on coverslips in multi-well tissue culture plates (Falcon 3008; Becton & Dickinson Co., U.S.A.) and incubated for 48 hr in an atmosphere of 5% CO<sub>2</sub> in air at 37°C.

*Adherence assay:* The culture medium of SIRC cells was replaced by the suspension of the isotope labelled bacteria. Thirty min after statical incubation, the coverslips were washed carefully three times with PBS to remove non-adherent bacteria and placed in scintillation vials containing 10 ml of toluene with 0.04 g of 2,5-diphenyloxazole (PPO; Katayama Chemical Industries Ltd., Japan). The vials were counted in a liquid scintillation spectrophotometer (Pakard Model 3320).

The number of bacteria was evaluated by measuring scintillation count. The number of epithelial cells was determined by detaching cells from coverslip with trypsinization and counting with a hemocytometer. Adherence was calculated by dividing the number of adhered bacteria by the number of epithelial cells.

## RESULTS

*Isolation of protease-deficient mutants:* After three independent NTG mutageneses, approximately 21,000 colonies were examined and those colonies which produced no clear zone on casein plates were selected. Seven protease-deficient mutants were isolated and designated IFO 3455-1 to IFO 3455-7.

*Characterization of protease-deficient mutants:* Among the parental and 7 mutant strains, no difference was observed regarding serotype, mo-

**Table 1.** Extracellular products of *P. aeruginosa* IFO 3455 and protease mutants<sup>a</sup>

Strain	Protease activity ( $\mu\text{g}$ of tyrosine/min/ml)	(% parent) <sup>b</sup>	Elastase (mm) <sup>c</sup>	Gelatinase (mm)	Lipase (mm)	Esterase (+/-)	Hemolysin (+/-)	Fluorescein (+/-)
IFO 3455	36.9	100	2.0	9.5	1.0	+	+	+
IFO 3455-1	12.8	34.7	0.5	7.0	<0.5	+	+	+
IFO 3455-2	0	0	0	6.5	0	+	-	+
IFO 3455-3	2.14	5.8	1.0	7.0	0.5	+	+	+
IFO 3455-4	7.79	21.1	<0.5 <sup>d</sup>	7.5	0	+	+	+
IFO 3455-5	12.7	34.4	<0.5	7.5	0	+	+	+
IFO 3455-6	13.1	35.5	0.5	7.0	0	+	-	+
IFO 3455-7	6.14	16.6	<0.5	6.5	0	+	+	+

a. None of the strains produced detectable levels of DNase and hyaluronidase.

b. Percent of protease activity as compared with that of the parental strain IFO 3455.

c. Clear zone produced around a streak of growth was measured.

d. Clear zone was not detected around but was underneath a streak.

tivity and growth rate (data not shown). Extracellular products of these strains are shown in Table 1. Although all the mutants gave no clear zone around isolated colonies on the casein plate, the majority were found to retain low protease activity by a quantitative assay. Only one mutant, IFO 3455-2, produced no protease. In addition, this mutant had no detectable levels of elastase, lipase and hemolysin, whereas the other strains showed variable levels of these extracellular products. Among the mutants, IFO 3455-3 was relatively similar to the parental strain with respect to the extracellular products except proteases. The strains IFO 3455-2 ( $P^-E^-$ ) and -3 ( $P^+E^+$ ) were chosen for further examination. Protease activity in the cell extracts of these two mutants and the parental strain was negligible.

*Adherence of the mutants to SIRC cells:* Ad-

herence of the mutants was compared with those of the parental strain IFO 3455 ( $P^+E^+$ ) as well as strains PA 103 ( $P^+E^-$ ) and NC 5 ( $P^-E^-$ ). As shown in Table 2, the mutant IFO 3455-2 ( $P^-E^-$ ) gave the adherence level at 75% and the mutant IFO 3455-3 ( $P^+E^+$ ) at 72% of that of the parental strain, indicating a slight decrease of adherence as compared with the parental strain. On the other hand, strains PA 103 and NC 5 adhered to SIRC cells much less than the strain IFO 3455.

## DISCUSSION

Although a lot of studies on experimental corneal infections with *P. aeruginosa* have been made<sup>3,10,11,13</sup>, there are few reports concerning the adherence of *P. aeruginosa*<sup>17,19</sup>. It has been recognized that the adherence of bacteria to epithelial cell surface is important to the initial

**Table 2.** Adherence of different strains of *P. aeruginosa* to SIRC cells

Strain	Enzyme production		Adherence <sup>a</sup>	
	Protease	Elastase	Exp. 1	Exp. 2
IFO 3455	+++	+++	99.8 $\pm$ 18.5	76.3 $\pm$ 19.4
IFO 3455-2	-	-	75.2 $\pm$ 2.9(75.4)	ND
IFO 3455-3	+	++	ND <sup>b</sup>	54.9 $\pm$ 17.0(72.0)
PA 103	+	-	15.4 $\pm$ 2.2(15.4)	ND
NC 5	-	-	7.0 $\pm$ 0.7(7.0)	ND

a. Mean number of bacteria per cell  $\pm$  standard deviation.

b. Not determined.

Parentheses indicate percent of adherence of the mutants as compared with that of the parental strain IFO 3455.

step in bacterial infection. We<sup>9)</sup> previously examined the adherence of clinical isolates of *P. aeruginosa* to SIRC cells and investigated the correlation between the adherence of the organism and production of extracellular bacterial protease. The conclusion from the experiment was that protease production could not be referred strictly to the pathogenicity, because clinical isolates possessed various properties for the pathogenicity besides protease. Genetic techniques were considered to be necessary for determining the role of protease of *P. aeruginosa* in the adherence.

Toxin A<sup>-13,15,18)</sup>, elastase<sup>-13,14)</sup> and alkaline protease-deficient<sup>6)</sup> mutants of *P. aeruginosa* have been isolated by several workers. The first two were obtained by the use of ethyl methanesulfonate as a mutagen, and the last by the use of NTG. The mutations in all the mutants were reported to be specific for each toxin or enzyme production. Our protease-deficient mutants after NTG mutagenesis, however, lost not only the ability to produce protease but also other extracellular substances. The mutants IFO 3455-1 and IFO 3455-3 were relatively similar to the parental strain as to the production of extracellular substances besides protease. Other mutants were apparently pleiotropic. Wretling et al<sup>20)</sup> reported that protease-deficient mutants of *P. aeruginosa* demonstrated pleiotropic changes in activities of other extracellular enzymes, and suggested that the mutations might affect a common regulatory mechanism in the synthesis or release of these proteins.

In our study, the mutants IFO 3455-2 (P<sup>-</sup>E<sup>-</sup>) and IFO 3455-3 (P<sup>+</sup>E<sup>+</sup>) were chosen for an examination of the adherence to SIRC cells. IFO 3455-2 produced neither protease nor elastase, whereas IFO 3455-3 showed low activities of these two enzymes. Protease which we term in this paper represents the protein which hydrolyzes casein so that the protein may have both activities of alkaline protease and elastase. In this sense, IFO 3455-2 is considered to produce none of alkaline protease and elastase, whereas it remains unclear whether or not IFO 3455-3 completely lacks the activity of alkaline protease.

Woods et al<sup>19)</sup> described that protease enhanced the adherence of *P. aeruginosa* to buccal epithelial cells. Our previous work<sup>9)</sup>

demonstrated that *P. aeruginosa* strains producing protease adhered more avidly to SIRC cells than non-producing ones, suggesting that protease production might be closely related to the adherence of *P. aeruginosa*. In the present study, our protease-deficient mutants showed a slight but not marked decrease of adherence as compared with the parental strain. It seems likely from the results that more factors other than protease are involved in the adherence of *P. aeruginosa* to SIRC cells.

Infection of traumatized cornea by *P. aeruginosa* often results in permanent corneal damage. Purified toxin A<sup>8)</sup>, alkaline protease or elastase<sup>10)</sup> of *P. aeruginosa* cause necrosis or opacity in animal eyes, and all were thought to be potential virulence factors in the pathogenesis of *P. aeruginosa* eye infection. The data presented by Ohman et al<sup>13)</sup> indicated that elastase activity did not appear to be essential for corneal disease due to *P. aeruginosa* and that toxin A did not appear to be required to initiate infections in traumatized corneas but did appear to perform at least two functions in corneal infections; corneal tissue destruction and inhibition of the host's bacterial clearance systems. Since the toxin A-deficient mutants they used produced alkaline protease, the production of this enzyme might be part of establishment of corneal infection. Howe and Iglewski<sup>9)</sup> demonstrated that alkaline protease-deficient mutants could not colonize traumatized cornea and did not produce corneal damage. They also observed that the virulence of the mutants was restored by adding not only alkaline protease but also elastase purified from *P. aeruginosa*. The mechanism that allow *P. aeruginosa* to establish infections in traumatized cornea is not yet defined, but proteolysis by alkaline protease or elastase and direct tissue damage by toxin A may contribute to the establishment and progress of corneal infections. Adherence of pathogens to cell surface is the first step in infection. However, our alkaline protease-deficient mutant gave only a slightly lower level of adherence to SIRC cells than the parental strain. Physiological differences between traumatized cornea *in vivo* and intact SIRC cells *in vitro* may be part of colonization of this organism.

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