

Role of Pili in the Adherence of *Pseudomonas aeruginosa* to Mouse Epidermal Cells

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Pili have been demonstrated to be the adhesins of *Pseudomonas aeruginosa* for mouse epidermal cells. The mechanisms of adhesion of *P. aeruginosa* to mouse epidermal cells was studied by using four mutants derived from a single strain: flagellated and piliated (F^+P^+), flagellated and nonpiliated (F^+P^-), nonflagellated and piliated (F^-P^+), and nonflagellated and nonpiliated (F^-P^-) mutants. F^+P^+ and F^-P^+ bacteria efficiently adhered to mouse epidermal cells, while F^+P^- and F^-P^- bacteria hardly adhered to mouse epidermal cells. The number of F^+P^+ bacteria that adhered to mouse epidermal cells was almost the same as that of F^-P^+ bacteria. The number of F^+P^- bacteria that adhered to mouse epidermal cells was almost the same as that of F^-P^- bacteria. The adhesion of P^+ (F^+P^+ and F^-P^+) bacteria was inhibited by antipilus serum, while that of P^- (F^+P^- and F^-P^-) bacteria was not inhibited by antipilus serum. There were no significant differences between the number of bacteria adhering to mouse epidermal cells isolated from normal skin and those adhering to cells isolated from burned skin. Heating of the mouse epidermal cell suspension had no effect on the adhesion of *P. aeruginosa*. These results suggest that pili mediate the adhesion of *P. aeruginosa* to mouse epidermal cells and that *P. aeruginosa* adheres efficiently to mouse epidermal cells despite the loss of cell viability caused by burning.

Pseudomonas aeruginosa is one of the most important bacteria in surgical infection. *Pseudomonas* sepsis is the major cause of death in burn patients (5, 16). Recently, we isolated flagellated and piliated (F^+P^+), flagellated and nonpiliated (F^+P^-), nonflagellated and piliated (F^-P^+), and nonflagellated and nonpiliated (F^-P^-) mutants from a single strain of *P. aeruginosa* and demonstrated that the 50% lethal dose of piliated mutants was at least 10 times (1 order of magnitude) lower than that of nonpiliated mutants (unpublished data). From these results, the enhancement of virulence of *P. aeruginosa* was attributed to the presence of pili. Colonization by *P. aeruginosa* seems to be important in explaining the establishment of infections. It has been demonstrated that adherence of *P. aeruginosa* to buccal epithelial cells is related to the pathogenesis of *P. aeruginosa*-induced lung infection (19) and that adherence of *P. aeruginosa* to upper respiratory cells is related to the host susceptibility to colonization (3). Moreover, it was suggested that pili mediate the adhesion of the nonmucoid strain of *P. aeruginosa* to tracheal cells and that exopolysaccharides mediate the adhesion of the mucoid strain of *P. aeruginosa* to tracheal cells (8, 11). However, the adherence of *P. aeruginosa* to epidermal cells has not been investigated. The present experiments were performed to investigate the role of pili on the colonization of burned skin surfaces by *P. aeruginosa*.

MATERIALS AND METHODS

Microorganisms. The strain used in this study, *P. aeruginosa* TPB-1, was isolated from a patient undergoing surgery at

Teikyo University Hospital, Tokyo, Japan. Its O serotype was B according to the antigenic schema recommended by the Serotyping Committee of the Japan *Pseudomonas aeruginosa* Society, and it was nonmucoid. Four mutants of *P. aeruginosa* TPB-1, F^+P^+ , F^+P^- , F^-P^+ , and F^-P^- bacteria, were obtained in the following ways. The F^- mutant was isolated by the methods of Fujita et al. (2) and McManus et al. (6). A single colony of strain TPB-1 was inoculated into nutrient broth (Nissui) and cultured at 37°C for 5 days. The culture broth was then serially diluted into phosphate-buffered saline (PBS; pH 7.2). Each dilution was mixed with melted (50°C) one-third-strength tryptic soy agar (Difco Laboratories) and cultured at 35°C for 24 h. Nonspreading colonies were examined by electron microscopy for flagellation after agglutination with anti-flagellum serum. Nonflagellated colonies were designated as F^- , and flagellated colonies were designated as F^+ . The P^- mutant was isolated from each of the F^+ and F^- organisms by the methods of Okamoto et al. (7) and Takai et al. (17). The randomly selected colonies of F^+ and F^- organisms were examined by electron microscopy for piliation after agglutination with anti-pilus serum. Nonpiliated colonies were designated as P^- , and piliated colonies were designated as P^+ . The piliation and flagellation of the four mutants were confirmed by electron microscopy with every passage once a month.

Isolation of epidermal cells. Epidermal cells were isolated from the skin of female CD-1 mice (each weighing 22 to 24 g; Charles River Japan). The skin of the back of each mouse was soaked in sterile PBS containing 100 IU of penicillin per ml and 100 µg of streptomycin per ml. After being soaked, the skins were cut to lie flat. The subcutaneous tissue and the dermis were removed with scissors. A scalpel was used to scrape the dermis. After being rinsed in PBS, the skins were cut into pieces approximately 1 by 1 mm with curved iris scissors (1, 12). The small pieces of skin were placed in a flask and then trypsinized at 37°C for 20 to 30 min. Epidermal

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cells were washed three times by centrifugation and suspended in PBS at a concentration of 10^6 cells per ml. Most of the cells were nonkeratinized epidermal cells which possessed oval nuclei and a small amount of cytoplasm. Epidermal cells were considered to be viable if they did not stain with trypan blue. More than 90% of epidermal cells were viable.

Adhesion assay. The epidermal cell suspension (0.5 ml) and bacterial suspension (0.5 ml) were mixed and incubated at 37°C for 60 min in a shaking water bath. The influence of cellular and bacterial concentration on adhesion was tested by using various concentrations of epidermal cell (10^5 and 10^6 cells per ml) and bacterial (10^6 , 10^7 , 10^8 , and 10^9 CFU/ml) suspensions. The influence of the incubation period and reaction temperature was tested by using various incubation periods (15, 30, 60, 90, and 120 min) and reaction temperatures (4, 25, and 37°C). The influence of the number of washings of the bacterium-epidermal cell mixture was tested by using one to five washings. The influence of trypsinization of epidermal cells was tested by use of epidermal cells isolated by scraping the epidermis and those isolated by trypsinization of small pieces of skin. After incubation, the bacterium-epidermal cell mixture was washed three times by centrifugation to remove any unattached bacteria. Smears were made, air dried, fixed in methanol, and stained with Giemsa staining solution. The number of bacteria adhering to mouse epidermal cells was counted under a light microscope. In each experiment, the first 50 well-defined epidermal cells were observed. Aggregates or sheets of epidermal cells were disregarded. Three independent trials were used to obtain the mean number of bacteria adhering to cells in each experiment.

Inhibition of adhesion. The bacteria were suspended in PBS containing 0.05, 0.1, 0.2, and 0.4% anti-pilus serum. These suspensions (0.5-ml samples) were preincubated at 37°C for 30 min, 0.5 ml of epidermal cell suspension was then added, and the bacterium-epidermal cell mixtures were incubated at 37°C for 60 min. Bacteria treated with normal rabbit serum instead of anti-pilus serum were used as a control.

Antiserum. Anti-pilus and anti-flagellum antibodies were induced in female Japanese White rabbits by intradermal immunization. Rabbits were injected with 500 μ g of either pilus or flagellum preparation with complete Freund adjuvant (Difco). At 1 week later, each rabbit was injected with 100 μ g of either pilus or flagellum preparation without adjuvant. This procedure was repeated three times at 1-week intervals. Each rabbit was bled out 1 week after the final immunization.

The agglutination titers of anti-pilus and anti-flagellum sera were 1:1,024 and 1:2,048, respectively.

Mouse burn model. The mouse burn model described by Holder and Jorgan (4) and Stieritz and Holder (15) was used in this study. After being given anesthesia, each mouse received a 10-s ethanol flame burn on the back, involving approximately 30% of the total body surface.

Isolation of epidermal cells from burned skin. Epidermal cells were isolated from the skin of burned mice by the method described above for isolation from normal skin and were suspended in PBS at a concentration of 10^6 cells per ml.

Isolation of bacteria from burned skin. Sixteen mice were divided into four groups. Either 10^2 CFU of F^+P^+ or F^+P^- bacteria or 10^6 CFU of F^-P^+ or F^-P^- bacteria were spread over the burned surfaces. Two mice from each group were sacrificed 24 h after inoculation, and two mice were sacrificed 48 h after inoculation for bacterial quantitation. A

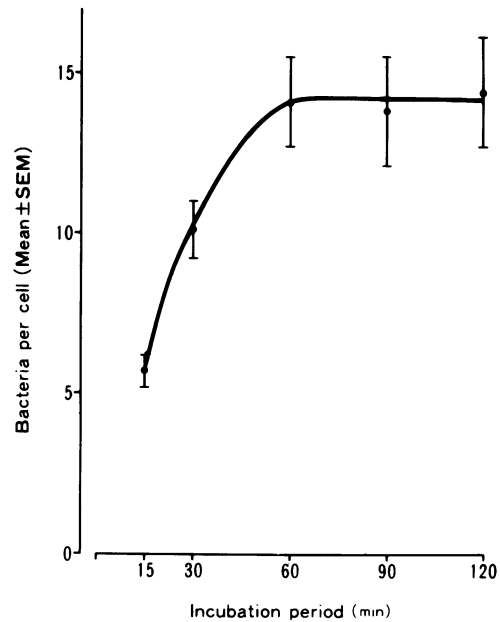


FIG. 1. Influence of incubation period on the adhesion of *P. aeruginosa* F^+P^+ bacteria to mouse epidermal cells. Each point indicates the mean number of bacteria adhering to epidermal cells. Each bar indicates the standard error of the mean (SEM). The adhesion test was done with 10^8 CFU of bacteria per ml and 10^6 epidermal cells per ml. The reaction temperature was 37°C.

full-thickness skin specimen was obtained from the burned area, weighed, and homogenized in 5 ml of PBS. Skin samples were placed on ice diluted with PBS. A 0.2-ml sample of each diluent was plated on NAC agar (Eiken) plates and cultured at 37°C for 24 h.

Effect of heat treatment of cells on adhesion. Cells were heat treated as follows. Epidermal cell suspensions were heated at 100°C for 10, 30, or 60 s. After heat treatment, the percentage of viable cell were counted, and then adhesion test were carried out.

Statistical analysis. Statistical evaluations of differences in the number of bacteria adhering to mouse epidermal cells were done by using Student's *t* test. *P* values of 0.05 were considered to be significant.

RESULTS

Influence of incubation period, bacterial and cellular concentrations, reaction temperature, number of washings, and trypsinization of cells on adhesion. The adhesion of F^+P^+ bacteria to mouse epidermal cells increased with time until 60 min of incubation (Fig. 1). The adherence of F^+P^+ bacteria to mouse epidermal cells increased with the increase of bacterial concentration. The number of bacteria adhering to mouse epidermal cells when a suspension of 10^5 cells per ml was used in the adhesion assay was larger than the number adhering to mouse epidermal cells when a suspension of 10^6 cells per ml was used (Fig. 2). The number of F^+P^+ bacteria adhering to mouse epidermal cells could be well defined when a bacterial suspension of 10^8 CFU/ml and an epidermal cell suspension of 10^6 cells per ml were used in the adhesion assay. The numbers of bacteria adhering to mouse epidermal cells at the various reaction temperatures

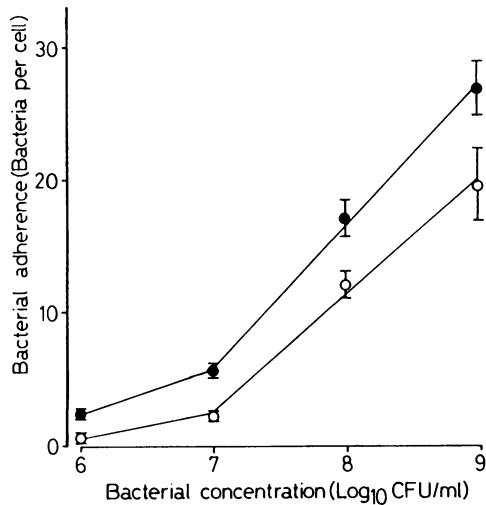


FIG. 2. Influence of bacterial and cellular concentration on the adhesion of *P. aeruginosa* F⁺P⁺ bacteria to epidermal cells. Each point indicates the mean number of bacteria adhering to epidermal cells at 10⁵/ml (●) and 10⁶/ml (○). Each bar indicates the standard error of the mean. The incubation period was 60 min, and the reaction temperature was 37°C.

(4, 25, and 37°C) were not significantly different. The adhesion of F⁺P⁺ bacteria to mouse epidermal cells was not influenced by temperature. Bacterial adherence after one washing of the bacterium-cell mixture was significantly different from that after two washings. However, more than two washings of the bacterium-cell mixture did not change the number of bacteria adhering to mouse epidermal cells. The number of F⁺P⁺ bacteria adhering to mouse epidermal cells isolated by scraping was not significantly different from that adhering to cells isolated by trypsinization. On the basis of the above results, incubation was done at 37°C for 60 min with 10⁸ CFU of bacteria per ml and 10⁶ mouse epidermal cells per ml isolated by trypsinization of the skin. After incubation, the bacterium-cell mixture was washed three times by centrifugation.

Adhesion of four mutants to mouse epidermal cells. The number of piliated (F⁺P⁺ and F⁻P⁺) bacteria adhering to mouse epidermal cells was significantly greater than that of nonpiliated (F⁺P⁻ and F⁻P⁻) bacteria. There were no significant differences between the number of F⁺P⁺ and F⁻P⁺ bacteria adhering to the epidermal cells and between the number of F⁺P⁻ and F⁻P⁻ bacteria adhering to the cells (Table 1). These results indicate that piliated bacteria adhere more efficiently than nonpiliated bacteria to mouse epider-

TABLE 1. Adhesion of four mutant bacteria to epidermal cells^a

<i>P. aeruginosa</i> mutant	No. of bacteria/cell (mean ± SEM)
F ⁺ P ⁺	13.6 ± 1.2
F ⁺ P ⁻	1.4 ± 0.3 ^b
F ⁻ P ⁺	15.0 ± 1.7
F ⁻ P ⁻	1.4 ± 0.3 ^c

^a The bacteria were used at 10⁸ CFU/ml; the cells were used at 10⁶/ml. Incubation was for 60 min at 37°C.

^b Significantly different from the value for F⁺P⁺ bacteria.

^c Significantly different from the value for F⁻P⁺ bacteria.

TABLE 2. Effect of anti-pilus serum on adhesion of mutant bacteria to epidermal cells^a

Pretreatment of cells	No. of bacteria/cell (mean ± SEM)			
	F ⁺ P ⁺	F ⁺ P ⁻	F ⁻ P ⁺	F ⁻ P ⁻
Normal serum	10.6 ± 1.5	1.9 ± 0.3	10.3 ± 1.3	2.0 ± 0.4
Anti-pilus serum				
0.05%	9.8 ± 1.1	1.7 ± 0.2	10.6 ± 1.4	2.1 ± 0.5
0.10%	4.7 ± 1.2 ^b	1.9 ± 0.4	5.1 ± 0.8 ^b	2.2 ± 0.4
0.20%	3.7 ± 0.8 ^b	2.1 ± 0.4	3.6 ± 0.7 ^b	2.0 ± 0.4
0.40%	2.0 ± 0.5 ^b	1.8 ± 0.4	2.4 ± 0.6 ^b	2.0 ± 0.5

^a For reaction conditions, see Table 1, footnote^a.

^b Significantly different from the values for normal serum.

mal cells and that flagella do not influence the adhesion of *P. aeruginosa* to the cells.

Effect of anti-pilus serum on adhesion. Anti-pilus serum (0.1%) inhibited the adhesion of piliated bacteria (F⁺P⁺ and F⁻P⁺) to the epidermal cells. The inhibition of adhesion increased as the concentration of anti-pilus serum increased. However, anti-pilus serum had no effect on the adhesion of nonpiliated (F⁺P⁻ and F⁻P⁻) bacteria to the epidermal cells (Table 2). Anti-pilus serum (0.05 to 0.4%) did not cause clumping of P⁺ and P⁻ bacteria during the adhesion assay. These results indicate that pili were the major mediators of piliated *P. aeruginosa* organisms to mouse epidermal cells.

Adhesion of four mutants to mouse epidermal cells derived from burned skin. The number of piliated (F⁺P⁺ and F⁻P⁺) bacteria adhering to the epidermal cells isolated from the burned skin was not significantly different from that isolated from the normal skin. There were no significant differences between the number of nonpiliated (F⁺P⁻ and F⁻P⁻) bacteria adhering to the epidermal cells isolated from burned skin and the number adhering to cells isolated from normal skin (Table 3). These results indicate that pili mediate the adhesion of *P. aeruginosa* to epidermal cells derived from the burned skin.

Effect of heat treatment of cells on adhesion. After the epidermal cell suspension was heated at 100°C for 30 s, viable cells were approximately 50% compared with the unheated control. After the cell suspension was heated at 100°C for 60 s, the cells became nonviable. There were no significant differences between the number of piliated (F⁺P⁺ and F⁻P⁺) bacteria adhering to the heated epidermal cells and the number adhering to the unheated cells. The number of nonpiliated bacteria (F⁺P⁻ and F⁻P⁻) adhering to the heated epidermal cells was not significantly different from the number adhering to the unheated cells. The number of piliated bacteria adhering to the heated cells was significantly greater than the number of nonpiliated bacteria adhering to the heated cells (Table 4).

Bacterial isolation from burned skin. Each mouse inocu-

TABLE 3. Comparison of the number of bacteria adhering to mouse epidermal cells derived from normal skin and burned skin^a

Skin type	No. of bacteria/cell (mean ± SEM)			
	F ⁺ P ⁺	F ⁺ P ⁻	F ⁻ P ⁺	F ⁻ P ⁻
Normal	10.2 ± 2.9	1.3 ± 0.3 ^b	8.0 ± 1.2	1.1 ± 0.3 ^c
Burned	10.9 ± 1.6	0.9 ± 0.2 ^b	8.4 ± 1.1	1.0 ± 0.3 ^c

^a For reaction conditions, see Table 1, footnote^a.

^b Significantly different from the value for F⁺P⁺ bacteria.

^c Significantly different from the value for F⁻P⁺ bacteria.

TABLE 4. Effect of heat treatment of cells on adhesion of bacteria to mouse epidermal cells^a

Heating time (s)	% Viable cells	No. of bacteria/cell (mean \pm SEM)			
		F ⁺ P ⁺	F ⁺ P ⁻	F ⁻ P ⁺	F ⁻ P ⁻
0	97	11.2 \pm 1.4	0.8 \pm 0.2 ^b	10.2 \pm 2.1	0.6 \pm 0.2 ^c
10	90	11.4 \pm 2.0	1.0 \pm 0.2 ^b	9.9 \pm 2.1	0.8 \pm 0.2 ^c
30	48	9.0 \pm 1.2	1.3 \pm 0.4 ^b	8.4 \pm 1.5	1.0 \pm 0.4 ^c
60	4	10.0 \pm 1.8	1.2 \pm 0.3 ^b	8.1 \pm 1.2	1.0 \pm 0.3 ^c

^a For reaction conditions, see Table 1, footnote a.

^b Significantly different from the value for F⁺P⁺ bacteria.

^c Significantly different from the value for F⁻P⁺ bacteria.

lated with 10² CFU of F⁺P⁺ and F⁺P⁻ mutants was sacrificed. Burned skin was removed at 24 and 48 h after infection. At 24 h after infection, 10 times more bacteria were recovered from burned skin taken from mice inoculated with F⁺P⁺ bacteria than from those inoculated with F⁺P⁻ bacteria. At 48 h after infection, 100 times more bacteria were recovered from burned skin taken from mice inoculated with F⁺P⁺ bacteria than from those inoculated with F⁺P⁻ bacteria (Table 5). Burned skin was taken at 24 and 48 h after inoculation of 10⁶CFU of F⁻P⁺ and F⁻P⁻ bacteria. At 24 h after infection, 10 times more bacteria were recovered from burned skin taken from mice inoculated with F⁻P⁺ bacteria than from those inoculated with F⁻P⁻ bacteria. After 48 h of infection, 100 times more bacteria were recovered from burned skin taken from mice inoculated with F⁻P⁺ bacteria than from those inoculated with F⁻P⁻ bacteria (Table 5).

DISCUSSION

The present study demonstrates that pili mediate the adhesion of *P. aeruginosa* to mouse epidermal cells and that flagella do not influence this adhesion.

It has been reported that pili mediate the adhesion of *P. aeruginosa* to mammalian buccal epithelial cells (20) and to the tracheal epithelium (11). Pilus-mediated adhesion of bacteria to erythrocytes and epithelial cells has been found for *Escherichia coli* (18), *Neisseria meningitidis* (14), and *Corynebacterium renale* (17). Ramphal et al. recently reported that mucoid exopolysaccharides mediate the adhesion of the mucoid strain of *P. aeruginosa* to tracheal cells (8). The present study indicates that adhesion of the nonmucoid strain of *P. aeruginosa* to mouse epidermal cells is mediated by pili.

Ramphal et al. also demonstrated that *P. aeruginosa* adheres efficiently to acid-injured tracheal cells, probably owing to changes in the binding sites or the physiological state of the cell membrane as a result of the acid treatment (10). For other bacteria, such as *C. renale*, it was shown that the bacteria adhered efficiently to aged and differentiated epithelial cells to disclose masked cellular receptors or

increase cellular receptors by the aging of cells (13). On the contrary, our results indicate that the number of *P. aeruginosa* cells adhering to heat-treated epidermal cells is nearly equal to the number adhering to nontreated cells. These results suggest that exposure or closure of the cellular receptor and increase or decrease of the number of cellular receptors were not affected by heat treatment.

Recently, we demonstrated that the 50% lethal dose of piliated mutants was at least 10 times lower than that of nonpiliated mutants (unpublished data). In the present study, we demonstrated that pili mediate the colonization of burned skin by *P. aeruginosa*. Therefore, it was suggested that the pathogenesis of *P. aeruginosa* burn infection is enhanced by an increase in the number of bacteria colonizing burned skin.

The present study suggested that pili are important for the adhesion of *P. aeruginosa* to normal and heat-treated mouse epidermal cells and that pilus-mediated adhesion of *P. aeruginosa* to epidermal cells plays an important role in the establishment of *P. aeruginosa* infection of burned skin in mice.

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TABLE 5. Bacterial isolation from burned skins

<i>P. aeruginosa</i> mutant	No. of bacteria (log ₁₀ CFU/g) isolated at:	
	24 h postinfection	48 h postinfection
F ⁺ P ⁺ ^a	4.3 \pm 0.1	7.4 \pm 0.2
F ⁺ P ⁻ ^a	3.1 \pm 0.1	5.6 \pm 0.1
F ⁻ P ⁺ ^b	4.5 \pm 0.1	7.3 \pm 0.1
F ⁻ P ⁻ ^b	3.3 \pm 0.1	5.2 \pm 0.2

^a Challenge dose, 10² CFU.

^b Challenge dose, 10⁶ CFU.

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