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Role of Pili in the Adherence of *Pseudomonas aeruginosa* to Injured Tracheal Epithelium

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Pili have been demonstrated to be the adhesins of nonmucoid *Pseudomonas aeruginosa* for buccal cells. In this study, we examined their role in the adherence of both mucoid and nonmucoid strains to injured tracheal cells. Pili incubated with tracheal cells inhibited the adherence of a nonmucoid strain in a dose-dependent manner. Both homologous and heterologous pili inhibited this nonmucoid strain. Antibody against pili from the nonmucoid strain inhibited adherence of the homologous but not a heterologous strain. Pili failed to inhibit two mucoid strains, but inhibited nonmucoid variants derived from mucoid strains. These studies suggest that pili mediate the adherence of nonmucoid strains to injured tracheal cells but that they are not the final mediators of adherence of mucoid strains. It is also inferred that there are differences in the receptor for mucoid and nonmucoid strains.

The mechanisms by which *Pseudomonas aeruginosa* cells adhere to the cells of the lower respiratory tract have not been fully elucidated. However, we have learned from studies with human buccal cells that pili mediate the adherence of nonmucoid strains (10). Little is known about the adherence of mucoid strains, since they adhere poorly to buccal cells (9). Using a recently described model of adherence to injured tracheal cells to which both varieties of *P. aeruginosa* adhere (4), we were also able to partially characterize the tracheal tissue receptor (5) and could thus study the role of pili in the adherence of both types of strains to this tissue. In this study we examined the ability of pili to inhibit homologous and heterologous strains of nonmucoid *P. aeruginosa*, the role of antipilus antibody in inhibiting homologous and heterologous strains, and the ability of pili to inhibit mucoid strains and nonmucoid variants of mucoid strains. The results of this study suggest that pili mediate the adherence of nonmucoid strains but not mucoid strains.

MATERIALS AND METHODS

Bacteria. The strains of *P. aeruginosa* used in this study are clinical isolates from a variety of sources. The mucoid strains are isolates from patients with cystic fibrosis; M35, a stable mucoid isolate, is a gift from Lee Boyd, University of Texas, San Antonio; M307 is a clinical mucoid isolate from a hospitalized patient; R5M was originally a mucoid strain which progressively lost its mucoid characteristics; R307 is a nonmucoid variant derived from M307; 12-4-4 is a nonmucoid strain provided by A. T. McManus, U.S. Army Institute of Surgical Research, San Antonio, Tex.; R_1 is a nonmucoid strain isolated from the sputum of a hospitalized patient; and strains 686B and T2A were nonmucoid clinical isolates from the urinary tract. Strains were kept on MacConkey agar plates at room temperature and were subcultured monthly. Bactria were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C under static growth conditions. After 19 to 24 h of incubation, the organisms were centrifuged at 10,000 × *g* for 15 min, washed with phosphate-buffered saline, pH 7.2 (PBS), and suspended as per the experimental protocol. Inocula were approximated by optical density measurement and confirmed by viable counts. Between 10^7 and 10^8 organisms per ml were used in the experiments.

Pili and antibody. Pili from strains 12-4-4, T2A, and 686B were the kind gifts of Charles Brinton, University of Pittsburgh, Pa. They gave a single band on discontinuous sodium dodecylsulfate-polyacrylamide gels. The molecular weights of pili from strains 12-4-4 and 686B were ca. 18,500 each, and the molecular weight of pili from strain T2A was about 16,500 (J. D. Silipigni, A. M. Levine, J. Sadoff, and C. C. Brinton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B18, p. 17). Antibody against pili from strain 12-4-4 was prepared by vaccinating rabbits with 500 μg of pilus protein subcutaneously, followed by intravenous injections on days 3, 4, 7, 8, and 21. The animals were bled on day 28. Antibody levels against pili were determined by a quantitative solid-phase radioimmunoassay (11). Comparisons between levels of antibody against pili and lipopolysaccharide from strain 12-4-4 were made in an enzyme-linked immunosorbent assay (ELISA) (8). Conditions were identical to those for the radioimmunoassay, except alkaline phosphatase-labeled, affinity-purified goat anti-rabbit immunoglobulin G (Kierkegaard-Perry, Gaithersburg, Md.) was used as a secondary antibody. Reactions were stopped at 30 min and read with an automatic ELISA reader (Dynatech Laboratories Inc., Alexandria, Va.) As determined by solid-phase radioimmunoassay, the preimmune serum contained 0.16 μg of antibody against the homologous pili per ml, and the immune serum contained 2.9 μg/ml. As determined by ELISA, the preimmune serum titer to pili was 4.5 U, and the immune serum titer was 47.1 U. Both the preimmune serum and the immune serum had low antilipopolysaccharide (strain 12-4-4) titers of 7.1 and 4.5 U, respectively. Neither the preimmune serum nor the immune serum diluted of 1/100 caused macroscopic or microscopic agglutination of test strains.

Tracheal organ culture preparation. Tracheal organ cultures were prepared from 6- to 8-week-old CD-1 mice as described previously (4). In brief, the tracheas were injured by the exposure of the luminal surface to 0.1 N hydrochloric acid (pH 1.48) for 15 min; the tracheas were then sectioned into pieces consisting of two to four rings.

Adherence testing. Three or four pieces of tracheal tissue

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TABLE 1. Effect of homologous and heterologous pili[a] on the adherence of *P. aeruginosa* to injured tracheal cells

Test strain	Pilus type	Adherence[b] Control[d]	Adherence[b] Test	P value[c]
12-4-4	12-4-4	42.5 ± 4.4	14.5 ± 4.4	<0.001
12-4-4	686B	14.9 ± 7.7	3.1 ± 2.2	<0.001
	T2A		5.2 ± 3.5	<0.01

[a] Pili used at a concentration of 250 μg/ml.
[b] Data expressed as bacteria per 1,000 μm^2.
[c] Student's *t* test.
[d] Strain 12-4-4; different inocula in separate experiments.

were placed in a small plastic petri dish, to which 1 ml of the test bacterial suspension had been added. The liquid always covered the tissues. The petri dish was incubated at 37°C for 1 h. After the incubation period, each piece of tissue was rinsed gently in three changes of PBS and then put in 3% glutaraldehyde for fixation. Aftr a minimum of 24 h of fixation, the tracheal samples were mounted, critical point dried, and coated with gold-palladium for examination by scanning electron microscopy as previously described (6).

Quantitation of adherence. Adherence was quantitated by means of direct counting with the scanning electron microscope as described previously (4). Briefly, we counted the number of bacteria in a microscopic field of a fixed size. The size of the field was fixed by setting the working distance from the sample to the bottom pole piece of the objective lens (10 mm), fixing the angle from which the sample was viewed (30°), and using the same magnification (ca. ×3,600) each time. The number of bacteria within a field was counted, and the field was randomly changed. Six randomly selected fields were counted on each of three pieces of tracheal tissue, and 7 fields were counted on the fourth piece, for a total of 25 fields. The data are reported as a mean number of bacteria ± one standard deviation adherent to 1,000 μm^2 of the tracheal surface.

Inhibitory studies with pili. Tracheal tissue prepared as described above was preincubated with purified pili for 1 h. The tracheal pieces were then rinsed to remove nonadherent pili and incubated with whole bacteria for 1 h. Control tracheal tissue was incubated with PBS for 1 h and then with the test bacterium. With this basic assay system, the effects of homologous and heterologous pili on adherence, the dose

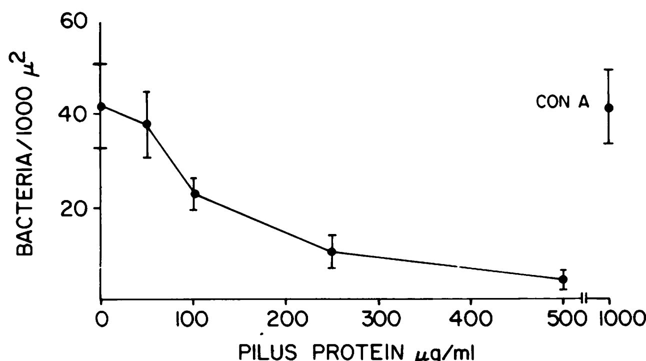

FIG. 1. Effect of increasing concentrations of pilus protein from strain 12-4-4 on the adherence of strain 12-4-4. The inoculum consisted of 8×10^7 organisms per ml. Tracheas were preincubated with pili, rinsed, and then added to the bacterial suspension. CON A, Concanavalin A.

responsiveness of inhibition by pili, and the effects of pili on the adherence of mucoid strains and nonmucoid variants of mucoid strains were examined.

Inhibition of adherence by antipilus antibody. Homologous and heterologous bacteria were preincubated for 1 h with a 1/100 dilution of specific antipilus antibody prepared as described above. Tracheal pieces were then added to this mixture, incubated for 1 h, rinsed, and prepared for quantitation. Preimmune rabbit serum, also diluted 1/100, was preincubated with bacteria to serve as controls. The agglutinating ability of the specific antibody was tested by culturing samples of homologous and heterologous bacteria in PBS and comparing the count to that of the bacteria incubated in a 1/100 dilution of the specific antibody. A reduction in the inoculum caused by agglutination of bacteria would thus be detected in this fashion.

RESULTS

Effects of homologous and heterologous pili. Pili prepared from strains 12-4-4, 686B, and T2A were used at a concentration of 250 μg/ml to study their effect on the adherence of strain 12-4-4. All three types of pili inhibited the adherence of strain 12-4-4 to various degrees (Table 1). Since the molecular weights of pili from strain T2A were less than those of the other pili (16,500 versus 18,500), the T2A preparation probably contained more pilus fragments. Pili from strain 12-4-4 were used in various doses to determine whether the inhibition was dose responsive. Concanavalin A, a lectin which binds to mannose, was included as a control at 1,000 μg/ml because of its pilus-like activity in some systems (2). The results of this experiment are shown in Fig. 1. Pilus protein showed significant inhibition of adherence at concentrations of 100 μg/ml or higher. At 500 μg/ml the inhibition was about 80% (42.4 ± 7.8 organisms per 1,000 μm^2 for the control versus 4.1 ± 1.8 organisms per 1,000 μm^2). The pretreatment of tracheas with concanavalin A had no effect on adherence; values were almost identical to those for the control (42.8 ± 7.8 bacteria per 1,000 μm^2).

Inhibition of adherence by antipilus antibody. Antibody against pilus protein from strain 12-4-4 was tested for its ability to inhibit the adherence of strains 12-4-4 and R_1. The antibody preparation contained 2.9 μg of antibody per ml in the ELISA assay, and the control serum contained 0.16 μg/ml. The antibody against pili from strain 12-4-4 was inhibitory against strain 12-4-4 at a dilution of 1/100 but did not inhibit strain R_1 (Table 2). To exclude the possibility that the differences in adherence could have been due to bacterial agglutination, samples of the organisms were incubated in PBS, the diluted preimmune serum, and the diluted antibody for 1 h at 37°C. Neither the rabbit serum nor the antibody caused a reduction in CFU when compared with the PBS control.

TABLE 2. Effect of antipilus antibody on the adherence of *P. aeruginosa*

Condition	Adherence (bacteria per 1,000 μm^2) Homologous strain (12-4-4)	Adherence (bacteria per 1,000 μm^2) Heterologous strain (R_1)
Control (PBS)	15.9 ± 4.0[a]	43.9 ± 6.6[b]
Control serum[c]	15.1 ± 3.9	49.2 ± 7.0
Antiserum[c]	4.7 ± 2.1[a]	33.9 ± 9.6[b]

[a] Control versus antiserum, $P < 0.01$.
[b] Control versus antiserum, not significant.
[c] Sera diluted 1/100.

TABLE 3. Effect of pili[a] on the adherence of mucoid strains

Strain	Adherence[b]		P value
	Control	Test	
12-4-4	45.0 ± 4.4	17.2 ± 4.6	<0.001
M307[c]	28.0 ± 7.7	27.4 ± 6.4	NS
M35[c]	39.4 ± 7.0	37.8 ± 11.5	NS
R307[d]	22.0 ± 8.9	3.2 ± 1.7	<0.001
R5M[d]	33.4 ± 5.0	10.7 ± 3.3	<0.001

[a] Pili from strain 12-4-4 used at 250 μg/ml.
[b] Data expressed as bacteria per 1,000 μm^2.
[c] Mucoid strains.
[d] Nonmucoid strains derived from mucoid strains.

Effect of pili on the adherence of mucoid strains. Since pili from heterologous strains inhibited the adherence of strain 12-4-4, we next studied whether pili would inhibit mucoid strains and nonmucoid strains derived from mucoid strains (Table 3). Pili from strain 12-4-4 demonstrated a marked ability to inhibit the nonmucoid variants but not the two mucoid strains, despite the fact that strain R307 was derived from strain M307.

DISCUSSION

The important role played by pili in promoting the adherence of bacteria to a variety of tissues has been the subject of much discussion (3). The present study adds to the list of tissues to which pili may mediate adherence. This work confirms that of Woods et al. (10), which suggests that pili mediate the adherence of nonmucoid *P. aeruginosa*, but we extend it to tracheal cells, the target tissues for *P. aeruginosa* colonization of the airways. We also demonstrate that pili may not be the final mediators of adherence for strains which have the mucoid exopolysaccharide coat, although pili may mediate the adherence of nonmucoid variants derived from these strains.

The observation that heterologous pili inhibited strain 12-4-4 lends itself to the interpretation that nonmucoid *P. aeruginosa* strains share either a common receptor site or closely positioned receptor sites. The former interpretation is supported by the fact that sialic acid is a part of the tissue receptor for all strains tested (5). The fact that antibody against one strain does not inhibit binding of a heterologous strain suggests that there is antigenic heterogeneity between pili. It is not known whether a common binding domain is present on these pili, as suggested for gonococcal pili (7), but it follows from our observation and that of Woods et al. (10) that if there is a common binding site, it is not recognized by antipilus antibody raised in rabbits. However, it does appear that the antibody recognizes some component of the pilus which must be close to the binding site and which must not be present on heterologous pili. We also tested a single monoclonal antibody against pili from strain 12-4-4 (data not shown), which, although binding to these pili, did not inhibit the adherence of the parent strain. Clearly, not every antibody that recognizes an antigenic site on a pilus is capable of inhibiting adherence.

Another aspect of this work which requires discussion is the observation that pili inhibited nonmucoid derivatives of mucoid strains but not mucoid strains. This finding was unexpected since we have previously reported that sialic acid is involved in the adherence of both types of strains (5). Implicit to this observation was the assumption that there was a common receptor for both types of strains. If this is true, then an explanation is required for the observation that pili did not inhibit the adherence of mucoid strains by occupying the receptor. One possibility is that pili mediate the first stage of adherence and that an additional factor found on mucoid strains mediates a second, more permanent bond, as suggested by Costerton et al. for the adherence of *Escherichia coli* to the gut (1). Another possibility is that the adhesin of mucoid strains displaces pili from a common receptor for pili and the mucoid adhesin. Alternatively, the receptors may be quite distinct even though both appear to contain sialic acid.

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LITERATURE CITED

1. **Costerton J. W., R. T. Irvin, and K. J. Cheng.** 1981. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol. **8**:303–338.
2. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature (London) **265**:623–625.
3. **Pearce, W. A., and T. M. Buchanan.** 1980. Structure and cell membrane binding properties of bacterial funbriae, p. 291–344. *In* E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.
4. **Ramphal, R., and M. Pyle.** 1983. Adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to acid-injured tracheal epithelium. Infect. Immun. **41**:345–351.
5. **Ramphal, R., and M. Pyle.** 1983. Evidence for mucins and sialic acid as receptors for *Pseudomonas aeruginosa* in the lower respiratory tract. Infect. Immun. **41**:339–344.
6. **Ramphal, R., P. A. Small, J. W. Shands, Jr., W. Fischlschweiger, and P. A. Small, Jr.** 1980. Adherence of *Pseudomonas aeruginosa* to tracheal cells injured by influenza virus or by endotracheal intubation. Infect. Immun. **27**:614–419.
7. **Schoolnik, G. K., J. Y. Tai, and E. C. Gotschlich.** 1982. Receptor binding and antigenic domains of gonococcal pili, p. 312–316. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
8. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme-linked immunosorbent assay, p. 359–371. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.
9. **Woods, D. E., J. A. Bass, W. G. Johanson, Jr., and D. C. Straus.** 1980. Role of adherence in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. Infect. Immun. **30**:694–699.
10. **Woods, D. E., D. C. Strauss, W. G. Johanson, Jr., V. K. Berry and J. A. Bass.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29**:1146–1151.
11. **Zollinger, W. D., J. M. Dalrymple, and M. S. Artenstein.** 1976. Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. J. Immunol. **117**:1788–1798.