

## Role of Pili in Adherence of *Pseudomonas aeruginosa* to Mammalian Buccal Epithelial Cells

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Adherence of *Pseudomonas aeruginosa* organisms to the upper respiratory epithelium of seriously ill patients in vitro is correlated with subsequent colonization of the respiratory tract by this opportunistic pathogen. The role of pili in the attachment to epithelial cells of *P. aeruginosa* was studied in an in vitro system employing human buccal epithelial cells and *P. aeruginosa* pretreated by various means. Pretreatment of the bacteria with proteases, heat, or Formalin caused a significant decrease in adherence. A decrease when compared with controls was also noted in the adherence of *P. aeruginosa* organisms to buccal epithelial cells preincubated with purified pili prepared from the strain used for adherence testing; however, pili prepared from a heterologous strain failed to block adherence. Similar results were obtained in serological studies when antisera to purified pili prepared from the strain used for adherence testing decreased adherence, whereas heterologous antiserum to pili did not decrease adherence. From these results it appears that pili mediate the adherence of *P. aeruginosa* organisms to human buccal epithelial cells.

Gram-negative aerobic bacilli are common causes of pneumonia in seriously ill hospitalized patients. The respiratory tracts of healthy individuals are rarely populated with these organisms (9, 18). In contrast, gram-negative bacilli can frequently be isolated from the respiratory tracts of seriously ill patients. These findings suggest that the mechanisms which normally exclude gram-negative bacilli from the oropharyngeal flora of healthy individuals may become impaired in stressed individuals. Increased susceptibility to colonization could possibly explain the repeated observation that certain patient populations tend to become colonized with gram-negative bacilli even in the absence of environmental reservoirs (14) or in the presence of rigorous attempts to prevent colonization (17).

Recently, we have demonstrated a correlation between the in vitro adherence of gram-negative bacilli to upper respiratory epithelium and colonization of the upper respiratory tract by these organisms. This colonization is associated with the adherence of the colonizing species to the epithelium of the upper respiratory tract in vivo (10). Further, gram-negative bacilli adhered in greater numbers to buccal epithelial cells recovered from colonized patients in vitro than to cells from healthy individuals.

For a number of gram-negative bacteria, attention has been directed to surface appendages

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as mediators of adherence. The ability of gonococci to establish infection on the intact mucosal surface of the genitourinary tract has been correlated with the presence of adherence-mediating pili on the surface of virulent organisms (22), and similar observations have been made in different systems with *Moraxella bovis* (16) and *Escherichia coli* (11). Duguid et al. (3) have demonstrated adherence of piliated enteric bacteria to erythrocytes, leukocytes, and epithelial cells, and they found this attachment to be sensitive to inhibition by mannose. Salit and Gotschlich (20) utilized isolated pili to show that *E. coli* adhere to Vero cells via pili which bind to specific mannose-containing receptors on the cell surface.

A number of investigators (6, 24) have reported the presence of pili on different pseudomonads, but no correlation between pili possession and adherence has been demonstrated in these organisms. The present study was undertaken to investigate the role of pili in the adherence of *Pseudomonas aeruginosa* to buccal epithelial cells as a prerequisite to colonization of the upper respiratory tract by these organisms.

### MATERIALS AND METHODS

**Bacteria.** Two strains of *P. aeruginosa* (DG1 and PK) were used in these studies. These strains were isolated from respiratory tract secretions, identified by standard methods (12), and classified serologically as Habs types 7,8 and 5,6, respectively. The organisms were maintained in 1-ml samples of 5% skim milk and

stored at  $-70^{\circ}\text{C}$ . To prepare cultures for adherence testing, a 1-ml sample was thawed and inoculated into 200 ml of M-9 medium (19) containing 0.2% glucose. This was incubated without shaking for 12 h at  $37^{\circ}\text{C}$ . One milliliter of a 1:100 dilution of this 12-h culture (approximately  $10^6$  organisms) was transferred to 15 ml of fresh M-9 containing 0.2% glucose and 0.2 ml of uniformly labeled [ $^{14}\text{C}$ ]lysine (New England Nuclear Corp.; specific activity,  $50 \mu\text{Ci/ml}$ ) was added. This suspension was incubated at  $37^{\circ}\text{C}$  for 3 h in a shaking water bath at 1,000 rpm. At the end of the incubation period the bacteria were washed twice with phosphate-buffered saline containing 0.001 M  $\text{MgCl}_2$  at pH 7.2 (PBS) and suspended in PBS at a concentration of  $10^6$  organisms per ml. The specific activity per milliliter of bacteria labeled was determined by pipetting 0.1-ml samples into liquid scintillation vials, adding scintillation cocktail, and counting for 1 min on a Searle Mark III scintillation counter equipped with computer conversion of counts per minute to disintegrations per minute.

**Epithelial cells.** Buccal epithelial cells from healthy adults were collected by vigorous swabbing of the buccal mucosa with a sterile, cotton-tipped swab. The cells were dislodged by swirling the swab in 5 ml of PBS and washed three times by centrifugation (10 min at  $150 \times g$ ) to rid them of unattached bacteria. The cells were then suspended in PBS. Since few *P. aeruginosa* attach to the surface of normal human buccal epithelial cells (10), the epithelial cells were exposed to  $2.5 \mu\text{g}$  of trypsin (Sigma) per ml for 10 min at  $37^{\circ}\text{C}$ , washed twice, and suspended in PBS at a concentration of  $5 \times 10^4$  cells per ml, as determined by microscopic count.

**In vitro adherence assay.** The adherence of *P. aeruginosa* to epithelial cells in vitro was examined by mixing 1-ml samples of standardized suspensions of [ $^{14}\text{C}$ ]lysine-labeled bacteria ( $5 \times 10^6/\text{ml}$ ) and epithelial cells ( $5 \times 10^4/\text{ml}$ ) together in a shaking water bath (1,500 rpm) at  $37^{\circ}\text{C}$  for 2 h. The epithelial cells were then washed free of unattached bacteria by continuous washing over filters (10  $\mu\text{m}$  pore size; Gelman Instrument Co.). The filter was then placed in a scintillation vial and solubilized; scintillation cocktail was added, and the mixture was counted for 1 min to obtain total disintegrations per minute per  $10^4$  epithelial cells. From the specific activity measurements of the bacterial suspension obtained previously, the results were expressed finally as total adherent bacteria per epithelial cell.

**Electron microscopy.** One drop of a washed suspension of the material to be examined was placed on a 0.25% Formvar-coated copper grid and allowed to stand for 5 min. Excess fluid was removed with filter paper, and a drop of distilled water was added to the grid for 30 s. After two additional 1-min washes with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, excess fluid was removed, and the preparation was shadowed with platinum. The angle of shadowing was not determined.

**Pretreatment of bacteria.** In some studies bacteria were killed before adherence testing. Washed bacteria were exposed to heat ( $100^{\circ}\text{C}$  for 3 h), 1% Formalin (Fisher Scientific Co.,  $4^{\circ}\text{C}$  for 24 h), and ultraviolet light ( $100 \mu\text{W/cm}^2$  at room temperature for 30 min). Samples of all treated preparations were

plated on nutrient agar (Difco Laboratories) to check for sterility. PBS-washed bacteria were treated for 30 min at  $37^{\circ}\text{C}$  with  $200 \mu\text{g}$  of trypsin (Sigma Chemical Co.) or chymotrypsin (Sigma) per ml in phosphate buffer (pH 8.2). After treatment, the bacteria were washed three times in PBS to remove the enzyme before adherence testing. In adherence testing of enzyme-treated bacteria, chloramphenicol ( $2.5 \mu\text{g/ml}$ ) was added to prevent pilus regeneration (1).

**Pilus purification.** *P. aeruginosa* pili were purified according to the method of Frost and Paranchych (5). Briefly, the organisms were grown for 18 h at  $37^{\circ}\text{C}$  on brain heart infusion agar (Difco) in large pans and then harvested by scraping the surface of the agar and suspending the cells in saline citrate buffer (SSC, 0.15 M, pH 8.0) containing 15% (wt/vol) sucrose. After stirring at  $5^{\circ}\text{C}$  for 24 h, the cells were blended in the cold at 2,000 rpm with a Sorvall Omnimixer. The cells were removed by centrifugation, and the supernatant was dialyzed for 72 h at  $4^{\circ}\text{C}$  against several changes of distilled water. Ammonium sulfate was added to bring the dialysate to 50% saturation, and the precipitate was pelleted by centrifugation (30 min at  $20,000 \times g$ ) and dissolved in SSC buffer. After dialysis against SSC, a second ammonium sulfate precipitation (20% saturation) was performed, and the pellet was dissolved in SSC buffer. Two cycles each of sucrose and cesium chloride density gradient centrifugation (20 h at  $20,000 \times g$ ) were performed. The pilus band was retrieved with a syringe, dialyzed against SSC, and then stored at  $-70^{\circ}\text{C}$ .

The ability of purified pili to block the adherence of intact organisms to trypsin-treated buccal epithelial cells was tested by preincubating  $10^4$  epithelial cells with  $250 \mu\text{g}$  of purified pili in 1 ml of PBS for 1 h at  $37^{\circ}\text{C}$ . The epithelial cells were then washed twice in PBS and placed in the adherence assay. As controls, epithelial cells were preincubated in a similar manner with bovine serum albumin (Sigma) and phytohemagglutinin (Microbiological Associates) before placing them in the in vitro adherence assay.

**Antiserum.** Antiserum to purified pili was obtained by injecting female New Zealand white rabbits intradermally with 0.5 mg of purified pili in complete Freund adjuvant. A booster injection was given at 14 days, and the animals were bled out at 28 days. Antibody titer was determined by agglutination of whole bacterial cells (8) with the strains from which the purified pili were obtained.

Antiserum to purified pili was tested for its ability to prevent the adherence of organisms of both homologous and heterologous strains of *P. aeruginosa* to buccal epithelial cells. Samples (1 ml) of suspensions of two different strains (DG1 and PK) containing  $10^6$  organisms per ml were incubated separately for 1 h at  $37^{\circ}\text{C}$  with 1 ml of a 1:2,048 dilution of antiserum to purified pili prepared from DG1 and PK, washed and placed in the in vitro assay for measurement of adherence. The 1:2,048 dilution represented one twofold dilution above the 1:1,024 agglutination titer obtained for the antisera against both DG1 and PK.

## RESULTS

**Effect of heat, Formalin, and ultraviolet light on adherence of *P. aeruginosa* orga-**

nisms to buccal epithelial cells. To determine whether bacterial viability is necessary for adherence, *P. aeruginosa* organisms were killed by various means and examined for the ability to adhere to trypsin-treated buccal epithelial cells in vitro. Untreated organisms adhered to buccal cells at a ratio of  $28.1 \pm 2.7$  bacteria per cell. Heat-killed and Formalin-killed organisms demonstrated a significant ( $P < 0.01$ ) decrease in adherence ( $1.4 \pm 0.6$  and  $1.7 \pm 0.5$  bacteria per cell, respectively) from control organisms, whereas organisms exposed to ultraviolet light adhered to buccal epithelial cells at a rate ( $27.9 \pm 3.1$  bacteria per cell) not significantly different from that of the controls.

**Effect of enzyme treatment on adherence of *P. aeruginosa* organisms to buccal epithelial cells.** The results from the studies on the effects of bacterial killing on adherence suggested that a protein component is involved in the adherence of *P. aeruginosa* organisms to buccal epithelial cells. To test this, bacteria were treated with proteases for 30 min before incubation with epithelial cells. Compared with adherence of non-enzyme-treated, washed bacteria ( $29.4 \pm 3.3$  bacteria per cell), trypsin treatment caused a reduction in *P. aeruginosa* adherence to  $3.2 \pm 1.5$  bacteria per cell ( $P < 0.01$ ), whereas chymotrypsin treatment caused a similar reduction ( $2.6 \pm 1.1$ ).

**Effect of purified pili on adherence of *P. aeruginosa* organisms to buccal epithelial cells.** The purity of pili obtained from strain DG1 (Fig. 1) at various stages of the purification procedure was monitored by means of electron microscopy and sodium dodecyl sulfate-discontinuous gel electrophoresis (23). The purified pili migrated as a single band with an estimated molecular weight of 17,500 (Fig. 2) on 12.5% SDS-disc gels and appeared homogeneous when

examined by transmission electron microscopy (Fig. 3). Chemical analysis of the purified pili yielded results of 99.1% protein content as measured by the Lowry procedure (15), 0.6% carbohydrate content as determined by the anthrone reaction (13), and a lipopolysaccharide level of 2 ng per mg of protein by the *Limulus* lysate procedure (21).

The effects on *P. aeruginosa* adherence of preincubation of buccal cells with various protein preparations before incubating these cells with bacteria are shown in Table 1. Adherence of *P. aeruginosa* organisms to epithelial cells previously exposed to bovine serum albumin or phytohemagglutinin was  $30.2 \pm 2.8$  or  $29.5 \pm 2.7$  bacteria per cell, respectively. These bacteria/cell ratios were not significantly different from the control value of  $30.6 \pm 2.8$ . When epithelial cells were preincubated with purified pili, however, a significant ( $P < 0.01$ ) decrease in adherence of intact organisms to  $5.7 \pm 1.9$  bacteria per cell was noted. The ability of purified pili to block the adherence of *P. aeruginosa* organisms was found to be a dose-related phenomenon with maximal blocking activity occurring at a protein concentration of  $250 \mu\text{g}$  per  $10^4$  epithelial cells (Fig. 4).

**Effect of antiserum to pili on adherence of *P. aeruginosa* organisms to buccal epithelial cells.** Antiserum to purified *P. aeruginosa* pili blocked the adherence of homologous intact organisms to trypsin-treated buccal epithelial cells, but it did not block adherence of a heterologous strain. As shown in Table 2, when antiserum to DG1 pili was preincubated with  $10^6$  organisms before washing and placing these organisms in the in vitro adherence assay, a significant ( $P < 0.01$ ) decrease in adherence from the control value of  $24.8 \pm 1.9$  to  $1.4 \pm 0.3$  bacteria per cell was noted. Similar results were obtained with strain PK ( $25.3 \pm 2.0$  reduced to  $2.4 \pm 0.7$  bacteria per cell ( $P < 0.01$ ). Antisera to pili from the heterologous strain did not cause a significant reduction in in vitro adherence. When examined by double immunodiffusion in agar gel, the antisera against the two pili preparations did not cross-react (Fig. 5).

## DISCUSSION

Selective bacterial adherence to mucosal surfaces of the respiratory tract seems to be a major determinant of the composition of the indigenous bacterial flora. Organisms which are unable to adhere to these surfaces are removed by secretions and thus fail to maintain colonization (7). Colonization of mucosal surfaces by newly acquired pathogenic bacteria may also be mediated by bacterial adherence, a process involving both host and microbial factors. Numerous

TABLE 1. Effect of purified *Pseudomonas* pili, bovine serum albumin (BSA), and phytohemagglutinin (PHA) on adherence of *P. aeruginosa* to buccal epithelial cells<sup>a</sup>

Epithelial cells	Bacteria per cell <sup>b</sup>
Control	$30.6 \pm 2.8$
Preincubated with pili <sup>c</sup>	$5.7 \pm 1.9^d$
Preincubated with BSA <sup>c</sup>	$30.2 \pm 2.8$
Preincubated with PHA <sup>c</sup>	$29.5 \pm 2.7$

<sup>a</sup> Buccal epithelial cells previously treated for 10 min at 37°C with 2.5  $\mu\text{g}$  of trypsin per ml.

<sup>b</sup> Mean  $\pm$  standard error of the mean for five determinations.

<sup>c</sup> Epithelial cells were incubated with 250  $\mu\text{g}$  of protein per ml for 1 h at 37°C, washed, and placed in adherence assay.

<sup>d</sup> Significantly different from control ( $P < 0.01$ ) by Student's *t* test.

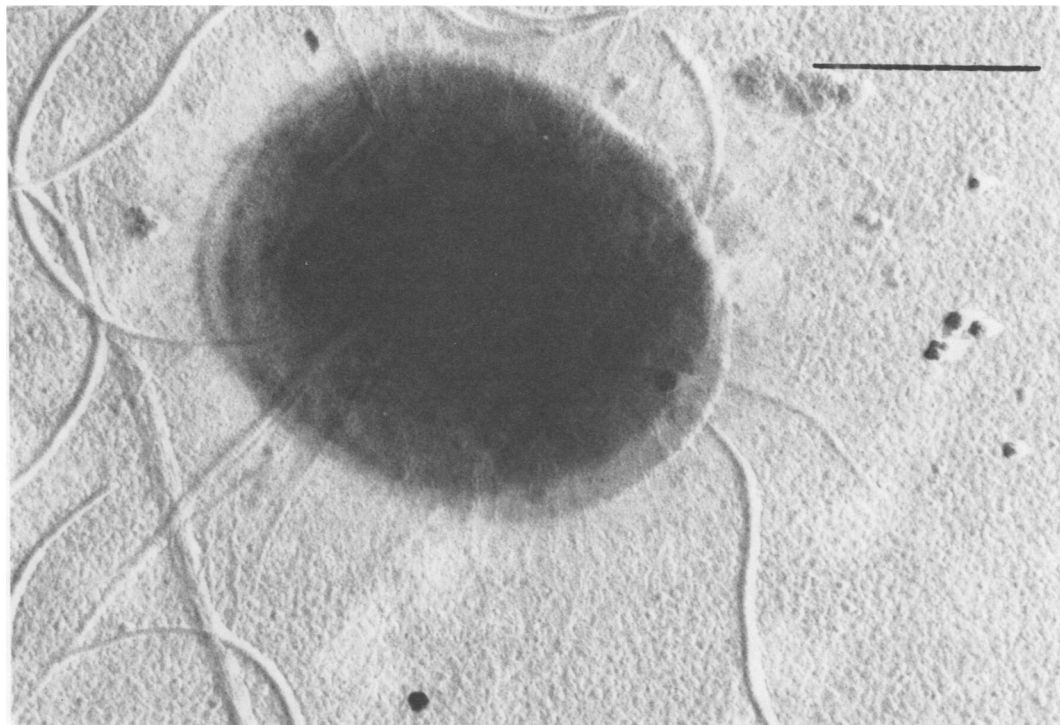


FIG. 1. Electron micrograph of *P. aeruginosa* DG1. Bar, 1  $\mu$ m.

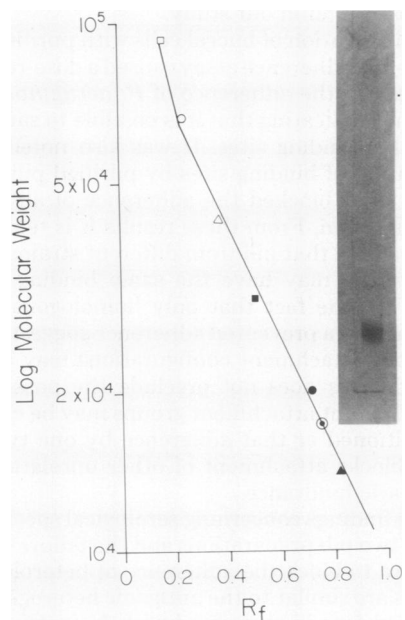


FIG. 2. Molecular weight estimation of *P. aeruginosa* DG1 pili. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pili (○) along with the following standards: □, phosphorylase b; ○, albumin; △, ovalbumin; ■, carbonic anhydrase; ●, trypsin inhibitor; ▲,  $\alpha$ -lactalbumin.

studies have demonstrated that pili play an important role in bacterial adherence and colonization of mucosal epithelium (3, 16, 22); however, the correlation between possession of pili and adherence has not been previously investigated for *P. aeruginosa*. The results of this study provide strong evidence that the adherence of *P. aeruginosa* to upper respiratory epithelium is mediated through pili present on the bacterial surface.

Heat- or Formalin-killed *P. aeruginosa* organisms lost the ability to adhere to buccal epithelial cells, whereas organisms killed by ultraviolet light retained the ability to adhere to these cells in vitro. This is probably explained by the loss of pili after heat and Formalin treatment and the retention of pili after ultraviolet light treatment, as judged by electron microscopy. These results are in agreement with those obtained by Fader et al. in their studies of the role of pili in the adherence of *Klebsiella pneumoniae* organisms to rat bladder epithelial cells (4). The removal of *P. aeruginosa* pili by enzymatic digestion also led to a decreased ability to adhere to buccal epithelial cells. Chloramphenicol was necessarily incorporated into the adherence mixture as pili were rapidly regenerated after enzyme removal, a finding reported by others (1).

The purified pili obtained from *P. aeruginosa*

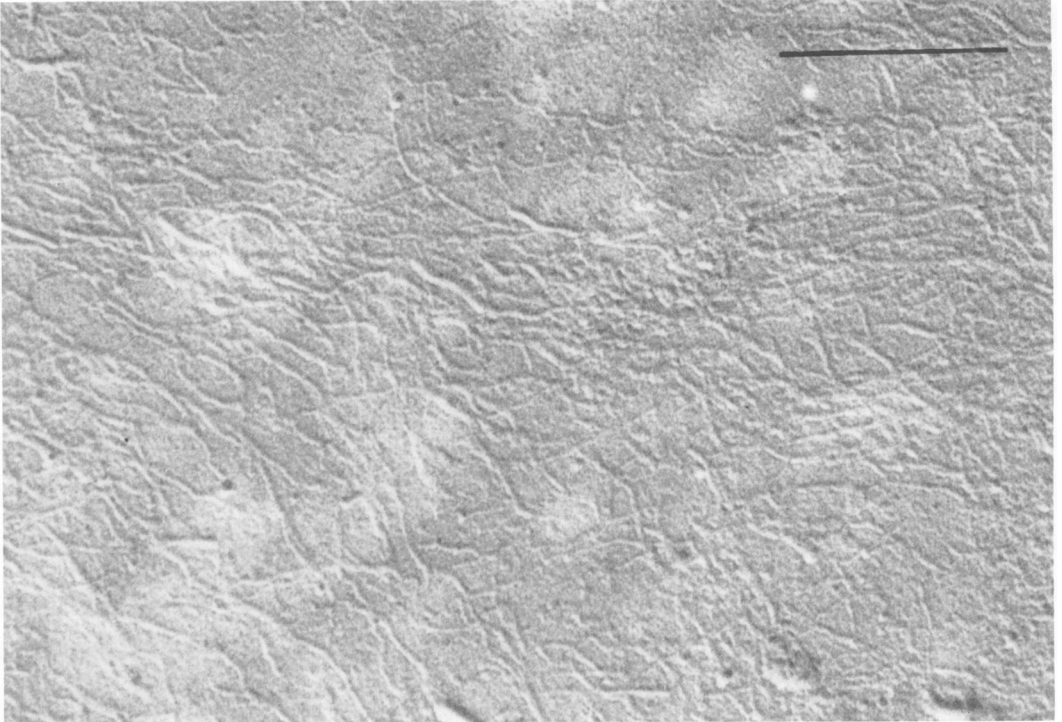


FIG. 3. Electron micrograph of a preparation of purified pili from *P. aeruginosa* DG1. Bar, 1  $\mu$ m.

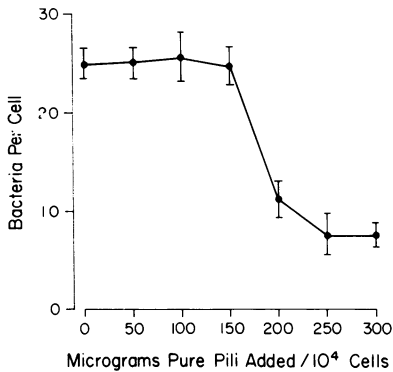


FIG. 4. Dose-response curve reflecting the ability of purified pili to block the adherence of intact *P. aeruginosa*. Buccal epithelial cells exposed to increasing amounts (0 to 250  $\mu$ g/ml) of purified pili for 1 h at 37°C before being tested for intact *P. aeruginosa* adherence.

strains DG1 and PK used in the present study were judged to have a molecular weight of approximately 17,500. This figure is similar to that of Frost and Paranchych, who reported a molecular weight of 17,800 for the pili of *P. aeruginosa* strain PAK (5). These authors also reported chemical composition data which were similar

to those found in our study.

Preincubation of buccal cells with purified pili before the adherence assay caused a dose-related decrease in the adherence of *P. aeruginosa* organisms, indicating that it is possible to saturate available binding sites. It was also noted that saturation of binding sites by purified pili from strain DG1 blocked the adherence of all other strains tested. From these results it is tempting to speculate that pili from different strains of *P. aeruginosa* may have the same binding sites. However, the fact that only homologous anti-pilus antisera prevented adherence suggests that different attachment configurations may be involved. This does not preclude the possibility that different attachment groups may be closely appositioned or that adherence by one type of pilus blocks attachment of other unrelated pili by steric hindrance.

The findings concerning serological specificity of the two pili preparations and the failure of the antisera to block the adherence of heterologous strains are similar to the antigenic heterogeneity reported for pili associated with *Neisseria gonorrhoeae* (2); however, additional studies must be performed to enlarge upon these findings. The studies reported here lend further support to the thesis that bacterial adherence to and

TABLE 2. Effect of antisera to purified pili on *P. aeruginosa* adherence to buccal cells<sup>a</sup>

Source of antiserum	Strain used <sup>b</sup>	In vitro adherence
—	DG1	24.8 ± 1.9
DG1	DG1	1.4 ± 0.3 <sup>c</sup>
DG1	PK	21.9 ± 1.4
—	PK	25.3 ± 2.0
PK	PK	2.4 ± 0.7 <sup>c</sup>
PK	DG1	23.7 ± 1.9

<sup>a</sup> Buccal epithelial cells previously treated for 10 min at 37°C with 2.5 µg of trypsin per ml.

<sup>b</sup> Organisms (10<sup>6</sup>) incubated for 1 h at 37°C with normal rabbit serum (—) or antibody to pili, washed, and placed in in vitro assay.

<sup>c</sup> Significantly different from control ( $P < 0.01$ ) by Student's *t* test.

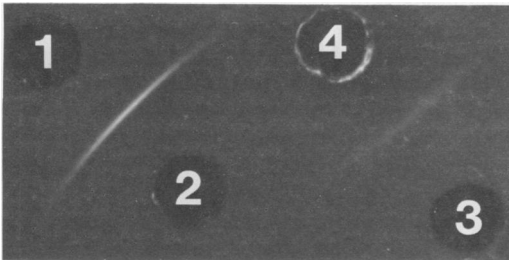


FIG. 5. Double immunodiffusion in agar gel of pili versus antiserum to pili. Well 1, DG1 pili; well 2, antiserum to DG1 pili; well 3, PK pili; Well 4, antiserum to PK pili.

possibly colonization of mucosal surfaces may be prevented by immunological intervention against a specific surface structure of the colonizing organism.

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