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D. E. Zoutman
University of Alberta

W. C. Hulbert
University of Alberta

B. L. Pasloske
DNAX Institute of Molecular and Cellular Biology

A. M. Joffe
University of Alberta

K. Volpel
University of Alberta

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THE ROLE OF POLAR PILI IN THE ADHERENCE OF PSEUDOMONAS AERUGINOSA TO INJURED CANINE TRACHEAL CELLS: A SEMIQUANTITATIVE MORPHOLOGIC STUDY

D.E. Zoutman,[1] W.C. Hulbert,[2] B.L.Pasloske,[1,3] A.M.Joffe,[1]
K. Volpel,[1] M.K. Trebilcock,[2] W. Paranchych[1,*]

Departments of Microbiology[1] and Medicine[2],
University of Alberta, Edmonton, Alberta, Canada
[3]Present Address: DNAX Institute of Molecular and Cellular Biology,
901 California Avenue, Palo Alto, CA 94301-1104

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Abstract

Pseudomonas aeruginosa adheres to respiratory epithelial cells in a highly specific fashion. In order to study the role of *P. aeruginosa* polar pili in the adherence process we conducted a quantitative morphological electron microscopic examination of *P. aeruginosa* adherence to SO_2 injured canine tracheal cells *in vitro*. A pilin lacking background strain of *P. aeruginosa* PAK (BLP2) was constructed using a gene replacement and it in turn was engineered to express either the pilin gene of *P. aeruginosa* PAO, PAK, or no pilin gene. After 30 minutes incubation of these bacterial strains with injured canine tracheal rings the *P. aeruginosa* strains expressing pili adhered quantitatively more to the injured tracheal cells than did the pili lacking strains. PAO bearing strains adhered in greater numbers than PAK bearing strains. Healthy tracheal cells did not have any bacteria bound to their surfaces. The bacteria bound to the cilia and lateral edge of the exfoliating tracheal cells. Invasion of tracheal cells by piliated *P. aeruginosa* bacteria and penetration into the submucosa was also demonstrated. These data confirm the role of pili as important adhesins to injured tracheal cells. The difference in the adherence characteristics of pilin types PAK versus PAO may relate to the differences in the primary structure of these two pilin molecules.

Key Words: Canine, trachea, injury, sulfur dioxide, Pseudomonas aeruginosa, pili, adherence

*Address for Correspondence:
W. Paranchych, Department of Microbiology,
M330 Biological Sciences Center,
University of Alberta,
Edmonton, Alberta, Canada T6G 2E9
Telephone No. (403) 492-3277
Fax No. (403) 492-2216

Introduction

Pseudomonas aeruginosa is an important pathogen causing serious systemic and localized infections in patients with thermal injury, complex medical illnesses and immune suppression (19, 27, 59). In children and young adults suffering from Cystic Fibrosis (C.F.), *P. aeruginosa* is an ubiquitous colonizer of the respiratory tract and is believed to play an important role in the progressive pulmonary damage which characterizes C.F. (5, 57, 66-69). Once a susceptible host's upper airway is colonized by *P. aeruginosa*, it is believed that it gains access to the lower respiratory tract by a descending mechanism (27).

The association of *P. aeruginosa* with human respiratory epithelial cells is now established to be a specific bacterial cell to epithelial cell interaction resulting in reversible and ultimately irreversible adherence of *P. aeruginosa* to the host's respiratory epithelium (2, 3, 30, 31, 34). Recent work has shown that *P. aeruginosa* uses both alginate and pili to facilitate its adhesion to mammalian cells (10-12, 35, 43). *P. aeruginosa* produces polar filaments called pili which are composed of 15,000 Dalton protein subunits (7, 15, 62). These pilin subunits are probably arranged in a helix with five subunits per turn and a pitch of 4.1 nm. The assembled pilus organelle has an average length of 2,500 nm, a diameter 5.2 nm and a central channel of 1.2 nm (15, 62). These pili are flexible structures which promote twitching motility and serve as receptors for specific bacteriophages (8, 9, 21). Pili of *P. aeruginosa* strains PAK and PAO have been purified and the complete amino acid sequence of PAK pilin determined (52). The amino terminal amino acid is N-methyl-phenylalanine (NMePhe) (18). This residue is conserved among all *P. aeruginosa* strains, a property it shares with the pilins of other bacteria, namely: *Niesseria gonorrhea, Niesseria meningitidis, Moraxella bovis, Moraxella nonliquifaciens,* and *Bacteriodes nodosus* (17, 22, 36, 38). *P. aeruginosa* pilin is encoded by one gene located on the bacterial chromosome and the structural genes for six unique pilin genotypes have been cloned and sequenced (39, 40).

Bacterial adhesion to mammalian cells is a complex process involving attractive and repulsive forces,

stearic hindrance, surface charge, covalent, hydrogen, ionic and hydrophobic binding. Bacteria initially adsorb to the mammalian cell surface in a reversible fashion and then use specific bacterial surface structures to irreversibly adhere to the host cell (3). Woods *et al.* demonstrated that *P. aeruginosa* pili promote the adherence of the bacteria to human buccal epithelial cells (BEC's) (70). Subsequently Doig *et al.* used a kinetic model to determine the numbers of receptors for *P. aeruginosa* on the epithelial cell surface and the apparent association constants of these pili-receptor interactions for both BEC's and tracheal epithelial cells (TEC's) (11,12). Purified pili can inhibit the adhesion of *P. aeruginosa* to epithelial cells in a concentration dependent fashion (10). Pure pili also bind directly to epithelial cells and can be shown to follow saturation kinetics (12). Fragments of the carboxy terminal end of the native pilin molecule and homologous synthetic peptide analogues also block adhesion (28, 33). The disulfide loop at the carboxy terminal end of the pilin molecule has recently been shown to be the specific binding domain of pilin and binds to surface glycoproteins of epithelial cells (28). However, in order to fully characterize the extent of bacterial virulence attributable to pili it was necessary to construct a pilin-minus *P. aeruginosa* background strain into which two different cloned pilin genes were introduced. The resulting isogenic strains, Pil$^-$, PAO, and PAK, allowed us to assess the importance of pili in promoting the adherence of *P. aeruginosa* to injured canine trachea *in vitro* (32). In the current study we present the results of a quantitative morphologic analysis of isogenic pilin-minus *P. aeruginosa* PAK strains which in turn were engineered to express the pilin types of either PAK or PAO.

Materials and Methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids are listed in Table 1. The media used were Luria-Bertani (LB) medium (50) and Pseudomonas isolation agar (PIA) (Difco, Detroit, Mich). Antibiotic concentrations were as follows: ampicillin (Ap) and chloramphenicol (Cm), 50 μg/ml, carbenicillin (Cb), 100 μg/ml, and mercury chloride ($HgCl_2$), 15 μg/ml.

Plasmid transfer by conjugation and recombinant DNA techniques

Triparental matings were performed as described previously (41). Donor and recipient strains were incubated together with *Escherichia coli* MM294, which carries the mobilizing plasmid, pRK2013. This helper plasmid has all the genes required for conjugation and is able to transfer itself into the donor strain. The donor has a plasmid with an origin of transfer recognized by the transfer genes of pRK2013 such that the donor's plasmid is mobilized into the recipient strain by conjugation (63).

Transformation of *E. coli* with plasmid DNA and the isolation of DNA fragments from 5% polyacrylamide were performed as outlined by Sambrook *et al.* (50).

Figure 1. The lower extreme of adherence for both non-piliated strains of *P. aeruginosa* to exfoliating canine tracheal cells after 30 minutes incubation with 1 x 10^5 cfu/ml. Exfoliation of the tracheal cells was induced by exposing the animals to SO_2 500 ppm. Bar = 5 μm. Inset shows the lateral surface of the exfoliating cell at higher magnification. Bar = 2 μm.

Figure 2. The upper extreme of binding for both non-piliated strains of *P. aeruginosa* to injured canine tracheal epithelial cells following 30 minutes incubation with 1x10^5 cfu/ml. Bar = 2 μm.

Figure 3. The binding of the low affinity piliated strain BLP2/PAK to injured exfoliating tracheal epithelial cells following 30 minutes incubation with 1x10^5 cfu/ml. Bar = 5 μm.

Figure 4. The binding of the high affinity piliated strain BLP2/PAO to injured exfoliating tracheal epithelial cells following 30 minutes incubation with 1x10^5 cfu/ml. Bar = 10 μm. Inset shows the magnitude of bacteria adhering after 240 minutes incubation with 1x10^5 cfu/ml illustrating the inability of visually determining the numbers of individuals attached. Bar = 10 μm.

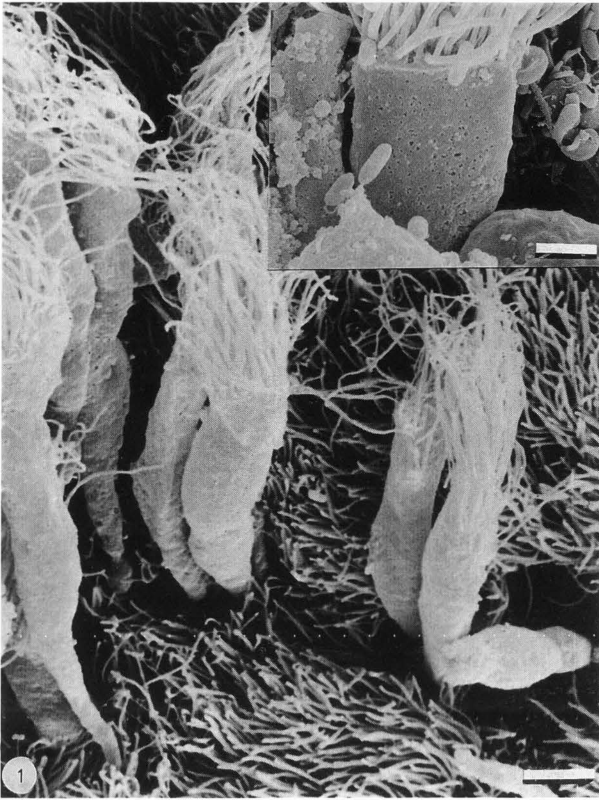
Table 1: Bacterial Strains and Plasmids

Strains or plasmids	Relevant genotype or property	Source or reference
Strains		
E. coli		
MM294 (pRK2013)	Mobilizing plasmid	(49)
JM 83	Δ(lac-proAB), lacZΔM15	(60)
P. aeruginosa		
PAK	Prototroph	ATCC 25102
PAO	Prototroph	ATCC 25247
BLP2	PilA::Tn501 mutant of PAK	(32)
Plasmids		
pUC 19	ColE1 cloning vector, Amp^r	(71)
pKT 210	Broad host range vector, Cm^r	(1)
pBP 400	pT74-pKT210 hybrid control vector	(41)
pBP 500	PAK pilin gene clone	(41)
pBP 161	PAO pilin gene clone	This study
pBP 101	pUC8 clone of PAO pilin gene	(51)

The isolation of plasmid DNA was according to the method of Birnboim and Doly (4).

Construction of pilin-minus isogenic *P. aeruginosa* PAK

The construction of a pilin-minus isogenic *P. aeruginosa* PAK (BLP2) was accomplished by means of a gene replacement as described previously (32).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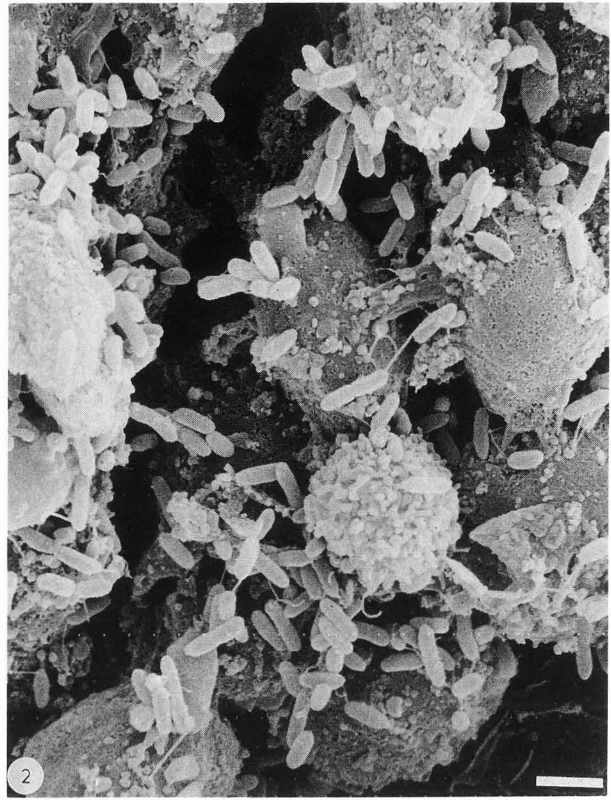
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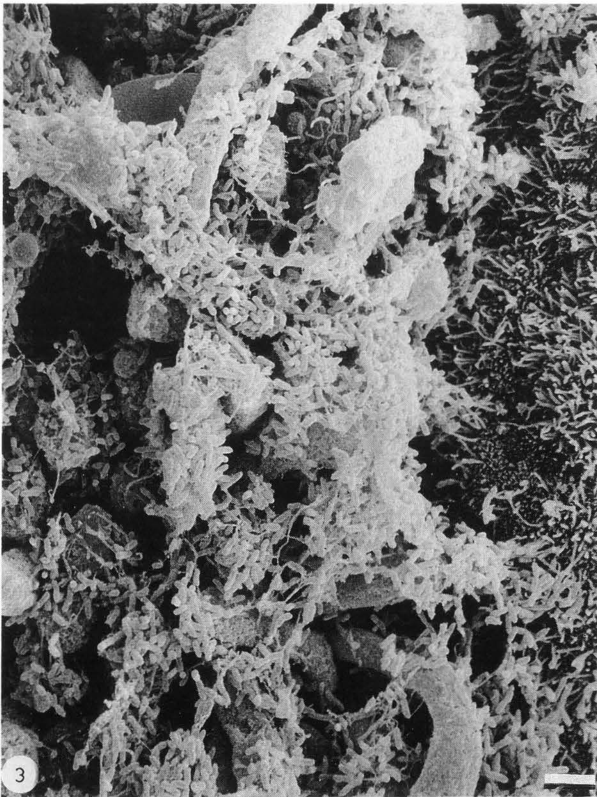
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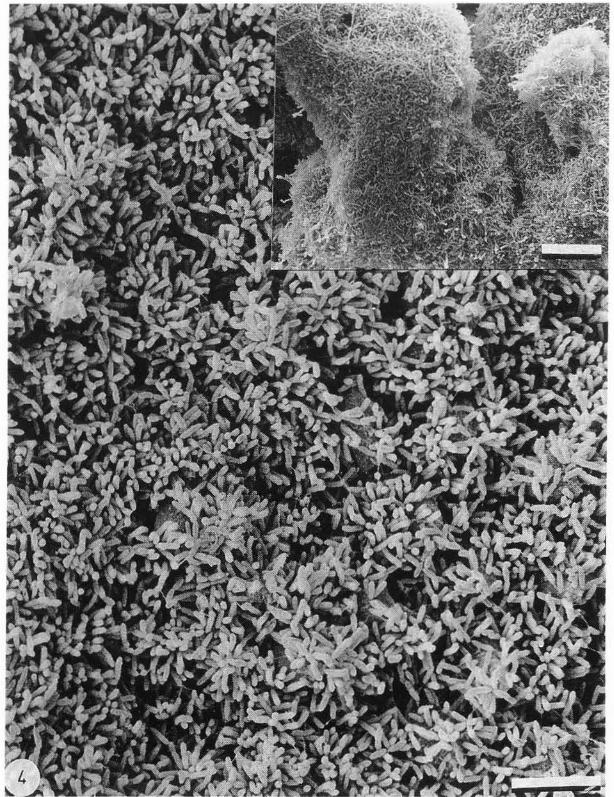
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Construction of broad host range plasmids containing the *P. aeruginosa* PAK and PAO pilin genes

Two broad host range vectors carrying the pilin gene of *P. aeruginosa* PAK or PAO were constructed to express the appropriate pilin type in the pilin minus background strain BLP2. pBP101 is a chimera of the cloning vector pUCl9 and a 1.2 kilobase HindIII fragment encoding the pilin gene from *P. aeruginosa* strain PAO. This plasmid was digested with HindIII and SalI to create a 1.1-kilobase HindIII-SalI fragment containing the PAO pilin gene. The pilin gene fragment was isolated from 5% polyacrylamide, ligated into the HindIII and SalI sites of pUC19 and transformed into *E. coli* JM83. This clone, pBP160, was digested in the presence of the broad host range vector pKT210 with EcoRI. The plasmids were ligated creating the plasmid pBP161 and then transformed into JM83. The recombinants were selected on Ap-Cm-LB agar.

The construction of pBP500 was accomplished in a similar fashion except that the 1.2 kilobase HindIII PAK pilin gene containing fragment was initially ligated into plasmid pT7-4 and then into pKT210. To provide a completely isogenic background strain for comparative purposes a control vector consisting of pT7-4 and pKT210 was also constructed and called pBP400.

Bacterial Growth

Ten ml of each strain of *P. aeruginosa* was grown to 1 x 10^9 cfu/ml at 37°C on a shaking table. Cells were grown in LB broth and cells harboring pBP161, pBP400, or pBP500 were grown in the presence of Cm. The cells were centrifuged and resuspended in 1 ml of LB broth at a concentration of $1x10^{10}$ cfu/ml.

Canine Tracheal Tissue Preparation

Three mongrel dogs weighing between 20 and 30 kg, free of clinically detectable respiratory disease were used in this study. The animals were anesthetized with pentobarbital sodium (30 mg/kg body weight, intravenously) and intubated with the tip of the endotracheal tube at the glottis to maintain the patency of the airway. The dogs were kept anesthetized until death. They were killed by the induction of respiratory arrest with a large intravenous bolus of anesthetic and cardiac arrest with an intravenous injection of saturated KCl. The injury and exfoliation of canine tracheal cells was caused by exposing dogs to SO_2 (500 ppm) for 60 minutes. This causes exfoliation primarily of ciliated cells in approximately 30% of the trachea (26). The trachea was removed immediately after death. Up to 30 pieces of epithelium at least 8 mm diameter were prepared from each trachea ensuring each incubation procedure was conducted in triplicate. The mucosal tissues were mounted in modified Ussing chambers and incubated in oxygenated Krebs-Henseleit solution, at 37°C, pH 7.4.

A suspension of *P. aeruginosa* was added to the chambers to achieve a final concentration of $1x10^5$ cfu/ml in phosphate buffered saline (PBS), pH 7.2. At intervals of 0.5, 1.0, 2.0 and 4.0 hours incubation, the tissues were washed extensively with PBS and fixed in ice cold 4% glutaraldehyde containing 0.1% OsO_4 and 1 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH. 7.4. Colony counts were carried out on the bacterial suspension demonstrating that the number of organisms did not change significantly during the incubation period in PBS (data not shown). Glutaraldehyde and OsO_4 were kept separately on ice and combined immediately before fixation. After primary fixation, the tissues were washed briefly with ice cold buffer, re-fixed in ice-cold 1% OsO_4 in 0.1 M cacodylate buffer. Then, at room temperature, they were washed with distilled water and dehydrated in a continuous ETOH series (i.e., beginning with 30% ETOH and adding small volumes of 100% ETOH to increase its concentration in 2% steps). The tissues were dried to critical-point with CO_2, mounted on aluminum stubs with conductive silver paint, sputter-coated with gold, and examined with a Cambridge Stereoscan 250 scanning electron microscope (SEM). After SEM examination, the tissues were infiltrated with propylene oxide and embedded in LX112 (Ladd Industries). Thin sections were cut on a Porter-Blum MT-2 ultramicrotome using a Diatome diamond knife. They were stained with uranyl acetate and then lead citrate using an LKB automatic grid stainer, and viewed with an Hitachi 6000 transmission electron microscope (TEM). The operator of the electron microscope was blinded as to the strains of the bacteria being tested at all times.

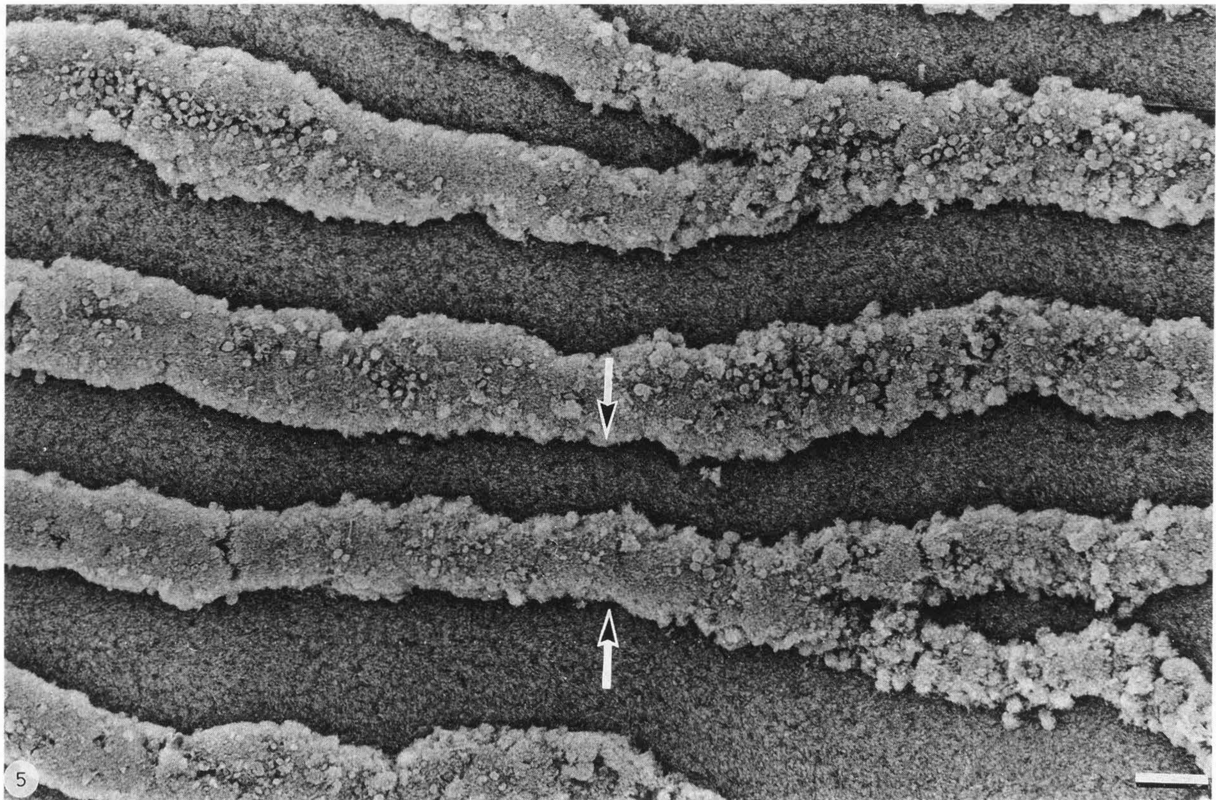
Figure 5. An overview of the canine tracheal surface showing rows of exfoliating cells separated by bands of normal healthy cells. The exfoliating cells exhibit blebbing and some amorphous aggregations that are *P. aeruginosa* bound to exfoliating tracheal cells. This sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 50 μm.

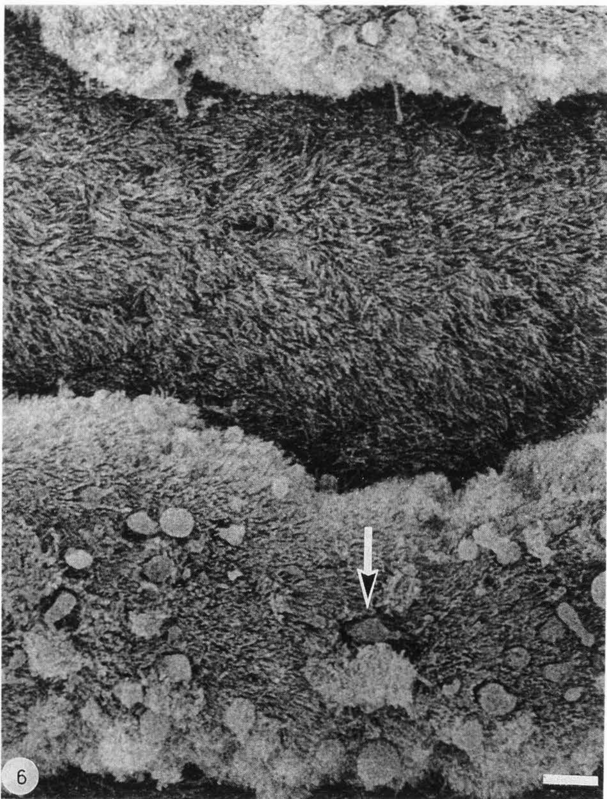
Figure 6. An enlargement of the region between the arrows in Fig. 5 showing the sharp demarcation between the rows of injured and exfoliating cells and the healthy tracheal cells. The blebs and amorphous aggregations on the surface are more evident in this view. This sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 10 μm.

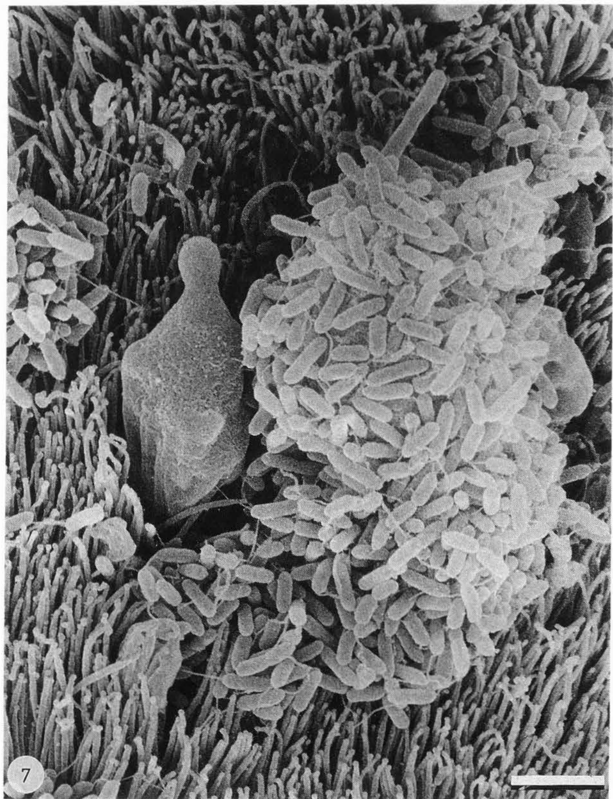
Figure 7. An enlargement of the area (see arrow) in Fig. 6 showing the blebbing of individual non-ciliated cells and that the amorphous objects are aggregations are *P. aeruginosa*. Sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 1 μm.

Results

Table 2 shows the effect of incubation time on adherence to injured tracheal cells by the four strains of *P. aeruginosa* tested. Quantitative observations by SEM revealed few bacteria of strains BLP2 or BLP2/vector adhering to injured TEC's even after 4 hours incubation. Moreover, there was no perceptible difference in the numbers of bacteria bound between the two background strains. Figs. 1 and 2 illustrate the degree of variation in adherence after 30 minutes incubation with these two







strains, the inset in Fig. 1 is an enlargement of the lateral surface of an exfoliated TEC documenting the sparsity of bacteria. For both of these non-piliated strains the number of bacteria adhering increased slightly as the incubation time increased to 4 hours, whereas the increase in adherent bacteria over time was much more obvious with the piliated strains BLP2/PAO and BLP2/ PAK.

By contrast, the expression of PAK and PAO pili by the BLP2 background strain was associated with marked increases in the numbers of bacteria bound to the tracheal epithelial cells compared to the non-piliated strains and the number of adhering bacteria increased proportionally with the incubation time (Table 2). Because of the large increase in bacterial density observed, especially for the BLP2/PAO strain, we selected those tissues fixed after 30 minutes incubation time for comparison because the bacteria did not totally cover the tracheal surface as occurred after 4 hours incubation (inset Fig. 4).

Figs. 3 and 4 show the adherence of BLP2/PAK and BLP2/PAO respectively after 30 minutes incubation time. The inset on Fig. 4 shows an uninterpretable mass of BLP2/PAO after 4 hours incubation time. Note the apparent polarity of the bacterial binding to the TEC's in a seemingly end-on fashion (Fig. 4). These photomicrographs illustrate the difference in binding between the strains BLP2/PAK and BLP2/PAO that have low and high binding affinities respectively (11, 29).

Table 3 shows that all strains of *P. aeruginosa* adhered to the injured mucosal cells, the basement membrane, and mucus strands. No bacteria were detected bound to healthy TEC's or those exhibiting blebs. Figs. 5, 6, and 7 show the cell specificity of binding. Fig. 5 is a low magnification overview of alternating bands of exfoliating cells with normal tissue in between. Fig. 6, an enlargement of the area in Fig. 5 that is delineated by arrows, shows surface blebs on some cells and groups of adherent bacteria. At still higher magnification the specificity of the adherent *P. aeruginosa* for injured TEC's and not those expressing blebs, or healthy cells is apparent (Fig. 7).

Most curious was the observation that the cilia of the cells where bacteria had bound were characterized by a unique structural anomaly consisting of a membranous 'flap' at the ciliary tip (Fig. 8). This was observed on exfoliating TEC's (Fig. 9), but not intact ones (Fig. 10).

Fig. 11 shows the adherence of BLP2/PAO to one exfoliating TEC and not the other. In this instance the bacteria adhere to the lateral surface as well as the ciliary tips of one exfoliating TEC. Why the bacteria are bound to one exfoliating TEC and not to the other is not known. Fig. 12 shows a lack of adherence of *P. aeruginosa* to the lateral sides of exfoliating ciliated cells as well as the surface of reparative cells in a 'micro lesion' on the canine surface. It would be expected that as the lesion is a cavity, there would have been ample opportunity for adherence and that the area would be colonized by bacteria. The fact that it is not raises questions as to the timing of when adherence receptors or specific

Figure 8. The adherence of *P. aeruginosa* to cilia of ciliated cells that are in the process of exfoliation. Sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 2 μm.

Figures 9 -10. Enlargements of the exfoliating cells (**Figure 9**) and cilia (**Figure 10**) in Fig. 8. In **Figure 9** note presence of the entangled flagella and the fact that the cilia possess a membranous flap at their tips. In **Figure 10** the cilia are from cells that are not exfoliating and no bacteria are adhered. Bars = 0.5 μm.

Table 2 Effect of Pilus Type on Adherence of *P. aeruginosa* to Dog Tracheal Preparations

Strain	Relative Adherence[1] at Times Indicated 30 min	60 min	120 min	240 min
BLP2	1+	1+	2+	2+
BLP2/vector	1+	1+	2+	2+
BLP2/PAK	2+	3+	4+	6+
BLP2/PAO	4+	5+	6+	8+

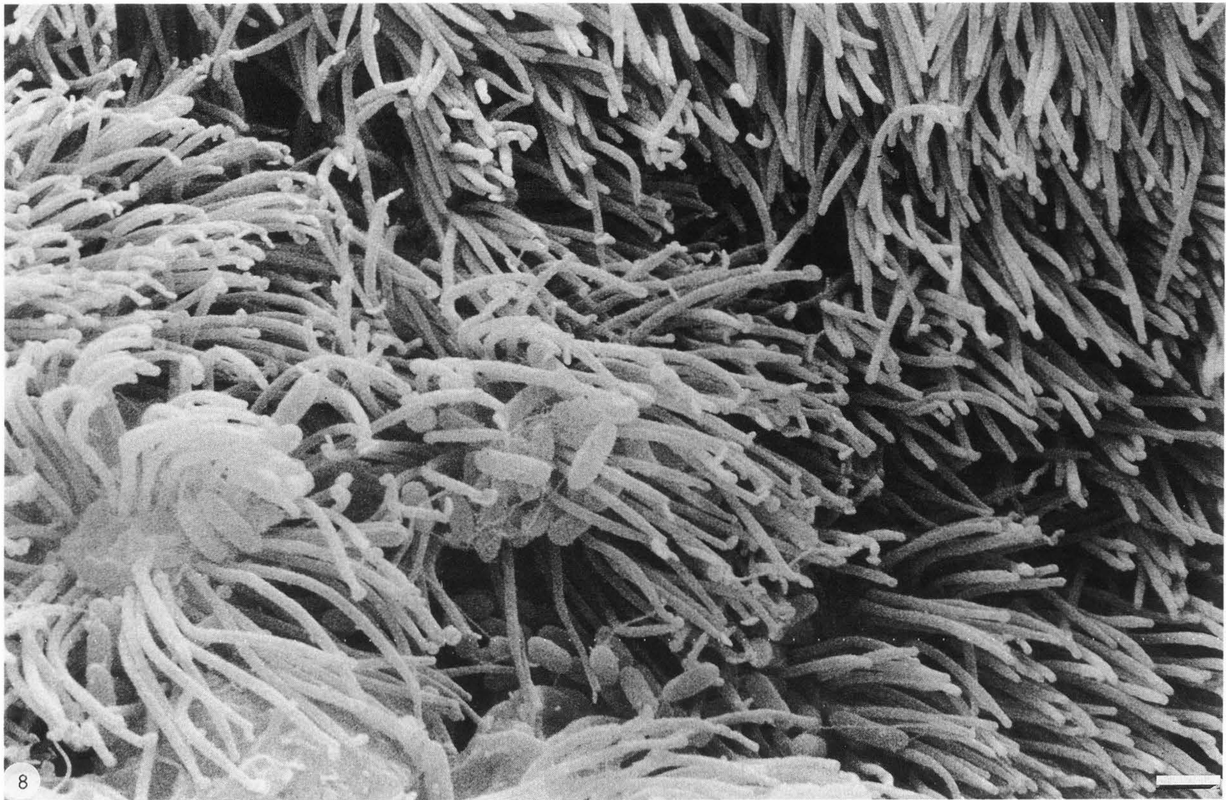
[1] Based on semiquantitative visual assessment by SEM of tissue-attached bacteria. 1+ = few bacteria adhered per cell; 2+ = several bacteria adhered per cell; 3+ = many bacteria adhered per cell; 4+ = cell covered with adhered bacteria; 5+ - 8+ = are moderately high numbers of bacteria adhered per cell, e.g., more than one bacterial cell layer to an indistinguishable number of bacteria adhered per cell (see Figs 1-4).

Table 3 *Pseudomonas aeruginosa* Preference for Adherence Sites in Dog Tracheal Preparations

Adherence Sites	Relative Adherence[1] of *P. aeruginosa* Strains at 30 min incubation BLP2	BLP2/ vector	BLP2/ PAK	BLP2/ PAO
Healthy surface cells	nd[2]	nd	nd	nd
Reparative cells	occ.[3]	occ.	occ.	occ.
Basement membrane	2+	2+	2+	2+
Mucus	1+	1+	2+	2+
Injured mucosal cells	1+	1+	2+	4+
Blebs	nd	nd	nd	nd
Cilia	1+	1+	2+	2+
Lateral surface	1+	1+	2+	4+

[1] Based on semiquantitative visual estimates by SEM of cell- or mucin-attached bacteria (see Figs 1-4; 20-21).

[2]nd: none detected; [3]occ.: occasional bacteria observed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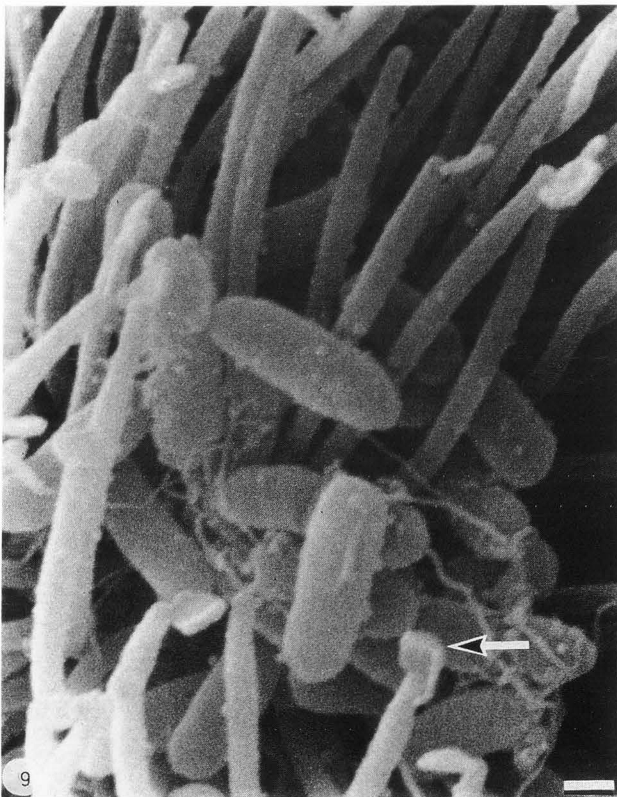
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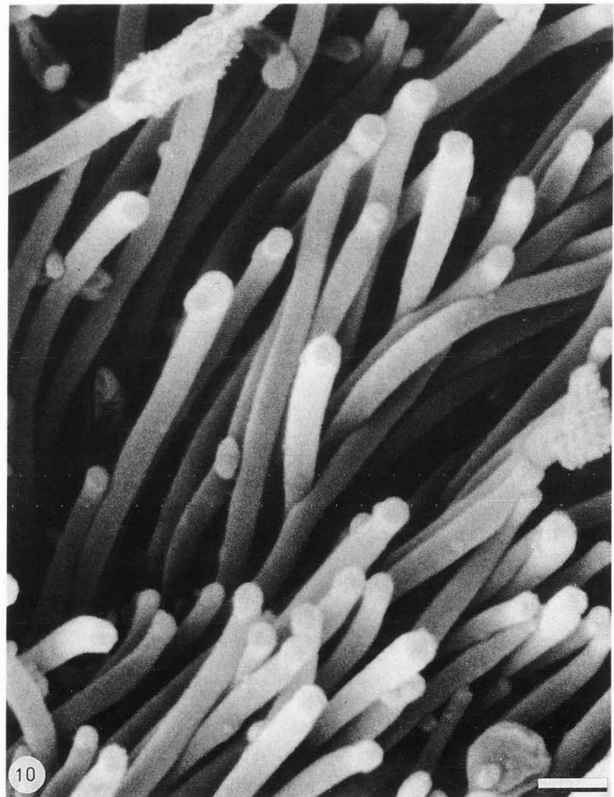
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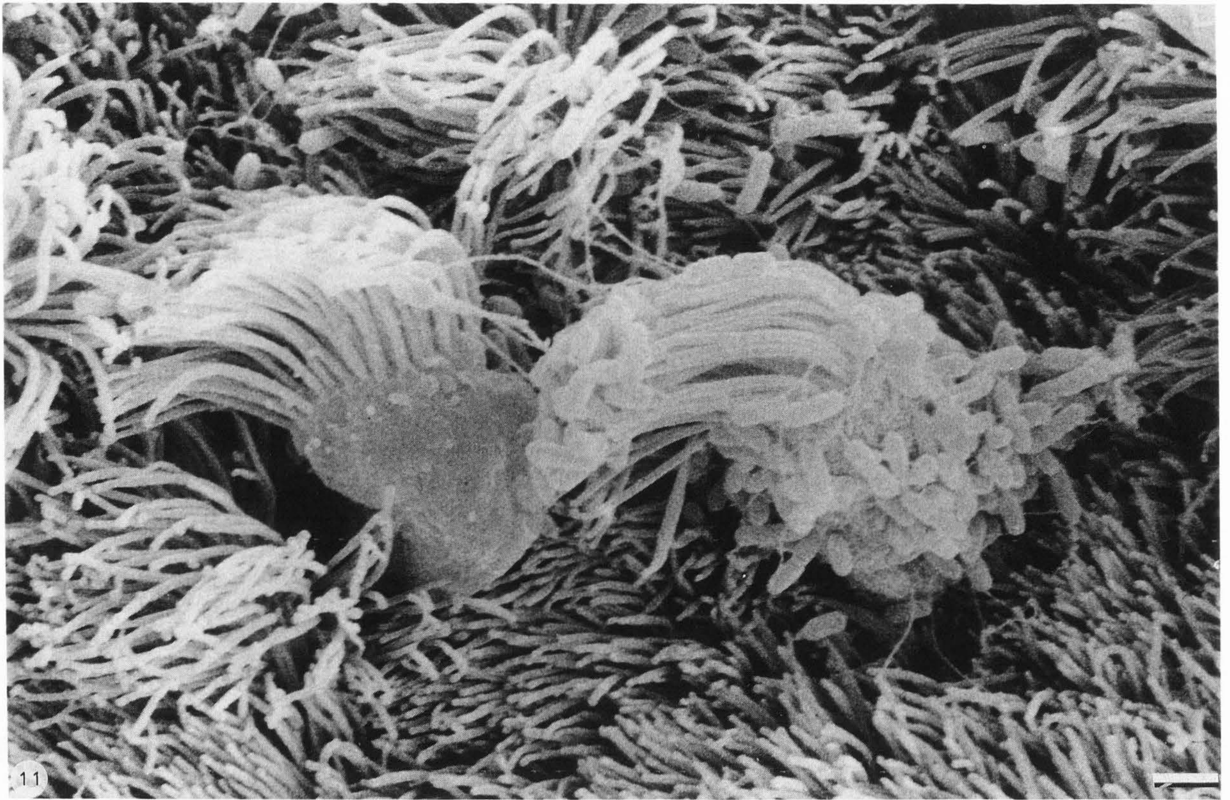

Figure 11. A pair of exfoliating ciliated cells where one cell has *P. aeruginosa* adhering to both cilia and the lateral cell surface and the other cell no adherent bacteria. Bar = 2 μm.

Figure 12. An exfoliating ciliated cell. Note the striking lack of bacteria on its surface or cilia. This photomicrograph is included for comparison to the image in Fig. 13. This sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 2 μm.

Figure 13. An exfoliated ciliated cell covered with a mass of *P. aeruginosa* BLP2/PAO. This sample was viewed under the same conditions as Fig. 12. Bar = 2 μm.

Figure 14. *P. aeruginosa* bacteria burrowing into the lateral side of an exfoliated ciliated cell. Sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 1 μm.

Figure 15. An exfoliating ciliated cell in this thin section demonstrates the burrowing of the *P. aeruginosa* into the nucleus of the cell. Sample was viewed after 30 minutes incubation with $1x10^5$ cfu/ml of BLP2/PAO. Bar = 1 μm.

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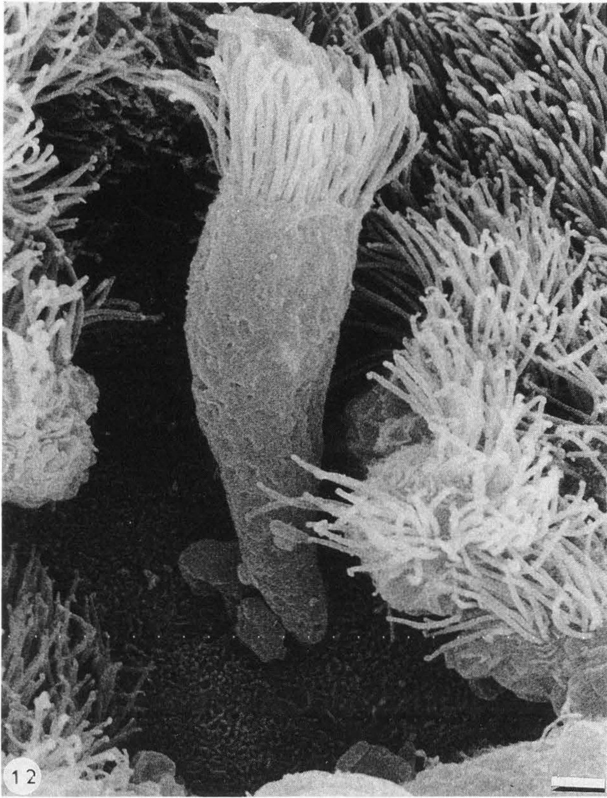
membrane conformations are expressed in the cell death sequence. Fig. 13 demonstrates the unique specificity of adherence when it does occur as there are three aggregations of adhered *P. aeruginosa* in this image and yet each is uniquely separate. Note the similarity in shape between the two cells in Figs. 12 and 13. Figs. 14 and 15 show BLP2/PAO burrowing into the lateral side of an exfoliated TEC. Also note the *P. aeruginosa* positioned within the nucleus of the exfoliating cell.

Figs. 16 and 17 show binding of BLP2/PAO to the basement membrane after 30 and 240 minutes of incubation respectively. As the slightly cuboidal basal cells flattened and spread laterally to cover the exposed basement membrane, the bacteria became aligned between the basal cells in a polar manner relative to the basement membrane (Fig. 18). Figure 19 shows the penetration of BLP2/PAO below the basement membrane into the submucosa. Although not shown, BLP2, BLP2/Vector and BLP2/PAK also demonstrated similar binding to the basement membrane (Table 3).

The adherence of all strains of *P. aeruginosa* to the mucous blanket and mucoid secretions from glands was observed (Figs. 20-23). Little difference in binding was noted between piliated and non-piliated strains (Table 3). An example of this is found in Fig. 20 which is a low magnification view of the background strain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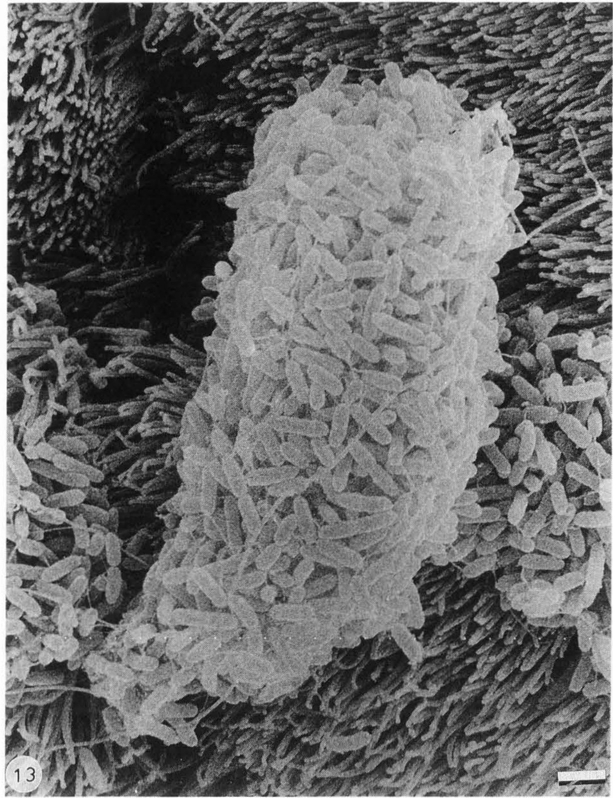
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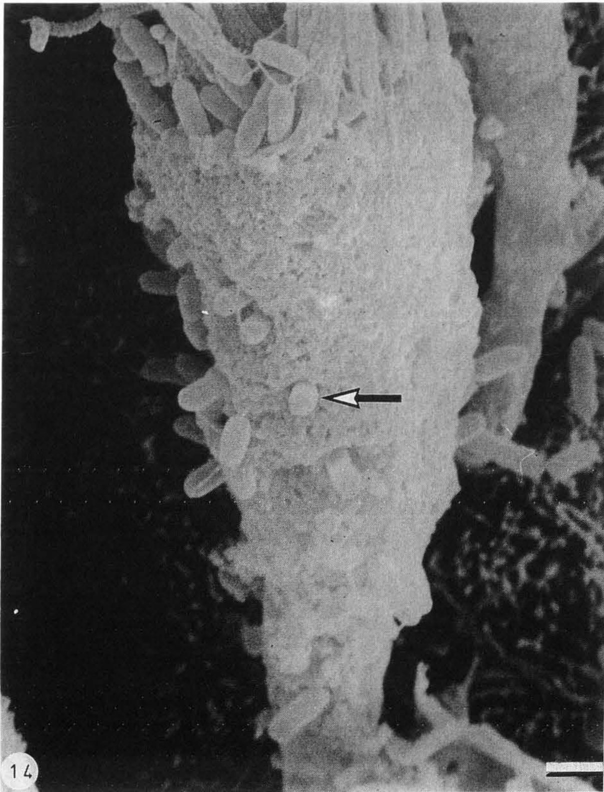
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BLP2/vector adhering to a blanket of tracheobronchial mucin. Fig. 21 is a higher magnification view of the mucus surface in Fig. 20 showing the bacterial cells lying on the surface and enveloped within the mucin strands. Fig. 22 demonstrates a mucosal gland excreting a strand of mucin in an area of otherwise healthy TEC's. In Fig. 23 one observes a mass of bacteria adhering to the mucin but not to the healthy TEC's.

Discussion

In this study we have demonstrated that piliated strains of *P. aeruginosa* adhere to damaged tracheal epithelial cells in a much more specific and quantitative fashion than do their non-piliated counterparts. Even after 4 hours of adherence time very few of the non-piliated bacteria were attached, whereas significant numbers of the piliated strains adhered to injured TEC's. This observation, taken along with previous kinetic data, confirms that pili play an important role in adhesion to damaged respiratory epithelial cells (29). The key to our experimental design was the use of a pilin-minus background strain (BLP2) which was then used to construct isogenic strains carrying plasmids expressing one of the two types of *P. aeruginosa* pilin studied. The resulting experimental bacteria expressed either no pili, PAO pili, or PAK pili. This allowed direct comparison of the effects of piliation on adhesion and the differences in adhesion characteristics of the two pilin types. Our results show that the bacteria appear to adhere at their polar ends to the damaged TEC's, which is consistent with the fact that *P. aeruginosa* pili are polar structures.

Piliated bacteria bind to the cilia as well as to the lateral margins of the luminal aspect of the TEC's (Figs. 9-11, 13-15). This finding correlates well with previous observations which showed that piliated *P. aeruginosa* cells, purified PAK pili and, synthetic peptides of the carboxy terminus of pilin bind preferentially to the cilia of human TEC's (12, 16, 28). Our observation of the morphological change in the cilia tips to form flap-like structures has not been documented before. Whether this is an effect of bacterial adhesion or related to other factors requires further study (6, 23, 24, 56, 64, 65). These are different from the claw-like structures that were observed at the tips of cilia from chicken tracheal epithelial cells by Reissig *et al.* (48).

The effects of time on the adhesion process were striking (Table 2). Kinetic studies have shown that bacterial adhesion tends to saturate at about 60-120 minutes (11). The SEM showed masses of piliated bacteria covering the injured TEC's at 4 hours and it was only at the 30 minute interval that we could detect differences in the adherence characteristics of the different piliated strains. This contrasts the results for the non-piliated strains which showed only a slight increase in adherence to injured TEC's over the four hour interval.

P. aeruginosa strains BLP2/PAO and BLP2/PAK are quite different in their binding characteristics to damaged tracheal epithelium. The PAO pilin gene conferred the ability to adhere much more avidly to the

Figure 16. A view of the tracheal mucosa with the superficial ciliated and non-ciliated cells stripped off leaving the basal cells and basement membrane exposed 30 minutes after exposure to SO_2 500 ppm. *P. aeruginosa* BLP2/PAO are adhered to the basement membrane and not the cell surfaces. Bar = 5 μm.

Figure 17. A view of the tracheal mucosa with the superficial ciliated and non-ciliated cells stripped off leaving the basal cells and basement membrane exposed 240 minutes after exposure to SO_2 500 ppm. The basal cells have flattened and spread laterally and the BLP2/PAO bacteria are aggregated at the cell-cell boundaries. Bar = 5 μm.

Figure 18. High magnification of *P. aeruginosa* from Fig. 17 illustrating the polarity of adhesion. Note also the filamentous web of flagella. Bar = 1 μm.

Figure 19. Thin section of exfoliating ciliated cells showing the invasion of *P. aeruginosa* below the basement membrane in tissue examined 30 minutes after incubation with the strain BLP2/PAO. Bar = 5 μm.

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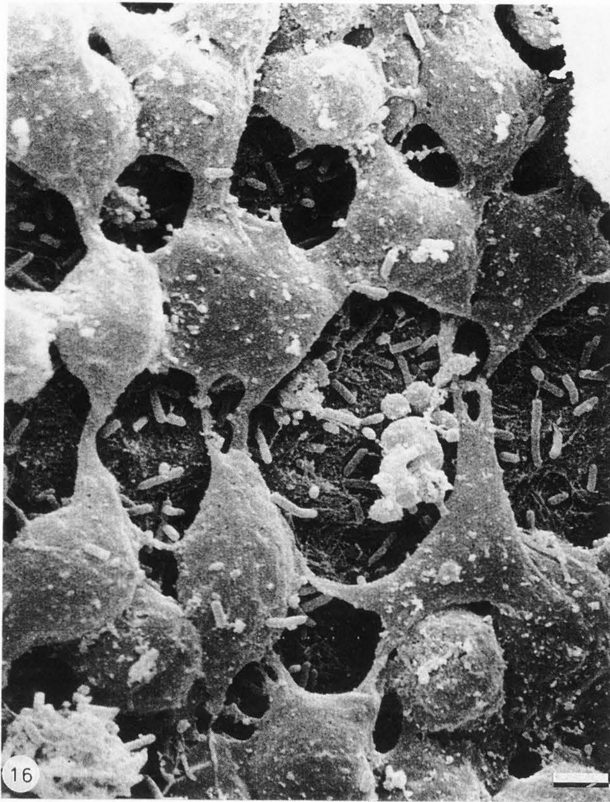
tracheal cells than did the PAK gene. This is consistent with an earlier observation that *P. aeruginosa* PAO has a ten fold greater affinity for BEC's than *P. aeruginosa* PAK, and that PAO and PAK strains compete for the same receptor sites (11, 29).

Consistent with our observations are studies which indicate that piliated *P. aeruginosa* are more virulent than non-piliated strains. Sato *et al.* demonstrated that piliated strains bind to mouse epidermal cells ten times more efficiently than non-piliated strains (53). In a mouse infection model, piliated strains were ten fold more virulent than non-piliated bacteria (54, 55).

P. aeruginosa capsular alginate is a complex heteropolymer of D-mannuronic and L-guluronic acids (13). It is rarely expressed in wild type strains in nature. However, *P. aeruginosa* isolates from C.F. patients frequently produce large amounts of mucoid capsule. The biosynthetic pathway of alginate production has been partly deduced and the genetic control of its expression is beginning to be understood and studied in more detail (48). Alginate has been shown to bind to and agglutinate human buccal and tracheal epithelial cells in a concentration dependent fashion (11). Alginates from different *P. aeruginosa* isolates do not behave similarly, which implies a variability of alginate structure from strain to strain. Alginate has been shown to be an integral part of microcolony formation and to protect the bacteria from the hosts immune system and antimicrobial agents (20). The *P. aeruginosa* strains that initially colonize patients with C.F. are non-mucoid and only later in the course of the disease do mucoid strains appear (42). Since pili are found on most *P. aeruginosa* strains from clinical isolates, it may be that pili play a dominant role in the earliest stages of colonization of the respiratory tract of C.F. patients whereas alginate aids in the establishment of chronic infection by microcolony formation. The strains used in this study were all non-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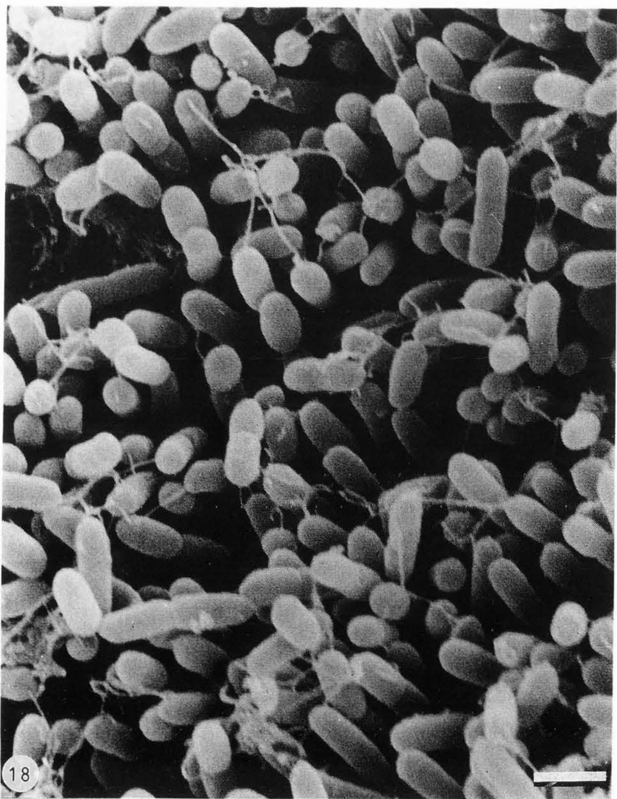
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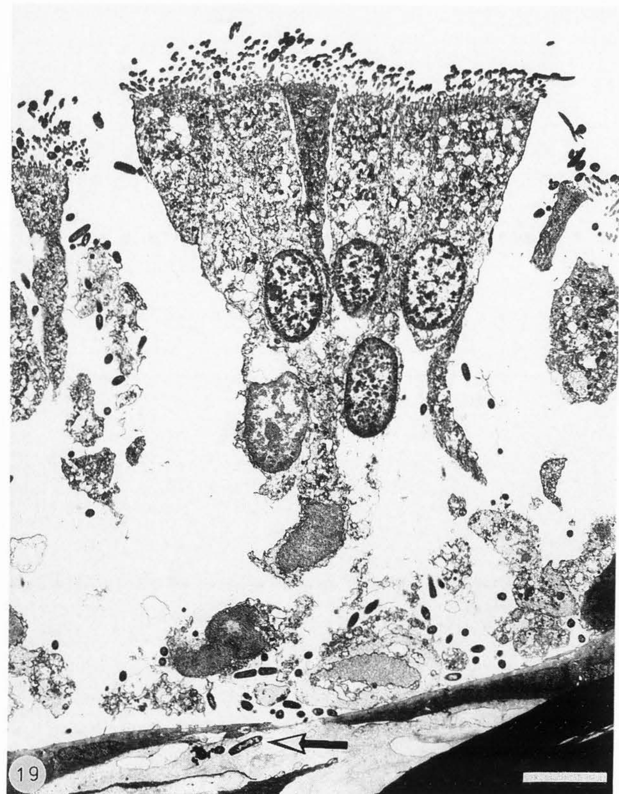
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mucoid so that alginate would not influence the adhesion to the canine trachea.

Tracheobronchial mucin has been demonstrated to possess aminosugar receptors for *P. aeruginosa* cells and to act as a site of colonization of the respiratory tract (25, 43-46, 61). *P. aeruginosa* alginate and pili have been shown to bind to purified mucin and antibodies to alginate block its attachment to tracheobronchial mucin (43, 47). Purified pili of a non-mucoid strain inhibits the adhesion of non-mucoid strains to tracheobronchial mucin but has no effect on the adhesion of mucoid *P. aeruginosa* (43). In our study we saw the same number of non-piliated bacteria adhering to mucin as piliated bacteria. There was no difference in the adhesion to mucin between the two pilin types PAK or PAO. In our model it appears that tracheobronchial mucin is binding both the pili lacking and the pili expressing bacteria equally in its role as a host defence mechanism. In the case of C.F. where the physicochemical properties of mucin prevents its proper clearance, mucin may fail to act as a defence mechanism to remove bacteria, but rather may promote their prolonged and intimate contact with the respiratory epithelium.

To our knowledge the invasion of mammalian cells by *P. aeruginosa* has not been demonstrated before. We saw many examples of piliated *P. aeruginosa* penetrating the damaged tracheal cells (Figs. 14, 15). Bacterial invasion of mammalian cells has been demonstrated for several other genera of bacteria to be an important step in the penetration of the host organism. The same may also be true for *P. aeruginosa*, although the mechanisms of invasion are probably different from those of invasive bacteria such as *Salmonella* (14).

The dog tracheas used in this study were exposed to the noxious gas SO_2 to produce a predictable injury to the ciliated tracheal cells. This exposure produces exfoliation of some of the TEC's and not of others (26). The observed differences of the adhesion of the piliated *P. aeruginosa* strains to damaged versus healthy TEC's were quite striking. Only the damaged TEC's had bacteria bound to them. In areas where healthy and exfoliating TEC's could be seen side by side, the healthy TEC's were free of bacteria. In experiments where dog tracheas were not exposed to SO_2, very few if any of the bacterial strains studied in this report adhered to the epithelial surface (data not shown). An analysis of the mechanism of exfoliation yielded some conceptual clues that may predispose bacterial adherence to the baso-lateral surface. However, it is not yet clear why some exfoliating cells were seen to be free of bacteria while others were covered extensively.

The event of exfoliation requires de-regulation of the interaction between the cytoskeletal elements and the plasma membrane. Tank *et al.* (71) reports that when the underlying cytoskeleton-membrane interaction is lost on muscle cells, the diffusion coefficient for acetylcholine and concanavalin A receptors approaches 10^{-9} cm^2/s, which is close to the value predicted for hydrodynamic drag in a lipid membrane. This is compared with a diffusion coefficient of between 10^{-10} and 10^{-12} cm^2/s under restrained conditions where the receptors are virtually non-diffusible (58). Thus the event of exfoliation creates the appropriate membrane conditions for the expression of different molecular species that normally are not expressed. We speculate that the basolateral membrane of exfoliating cells expresses a receptor that is recognized by *P. aeruginosa* in a manner related to the binding affinities of the pili. It is therefore possible that the cytoskeletal system of injured exfoliating cells becomes modified in such a manner as to result in a redistribution and/or increase in pili receptors in the tracheal cell membrane and cilia.

Figure 20. Overview of adherence of non-piliated strain of *P. aeruginosa* BLP2 to mucus. The adherence is not different from that observed to the lateral surface of the exfoliating cells for this non-piliated strain. Bar = 5 μm.

Figure 21. Higher magnification of the mucous surface shown in Fig. 20 illustrating bacteria on the mucous surface as well as with in the mucin matrix. Bar = 0.5 μm.

Figure 22. Glandular secretion in a focal area without cellular exfoliation. Tissue from an animal exposed to SO_2 500 ppm. Bar = 1 μm.

Figure 23. High magnification of the glandular secretion in Fig. 22 showing the specificity of adherence of the *P. aeruginosa* for the mucus and not the healthy ciliated cells. Tissue from an animal exposed to SO_2 500 ppm. Bar = 20 μm.

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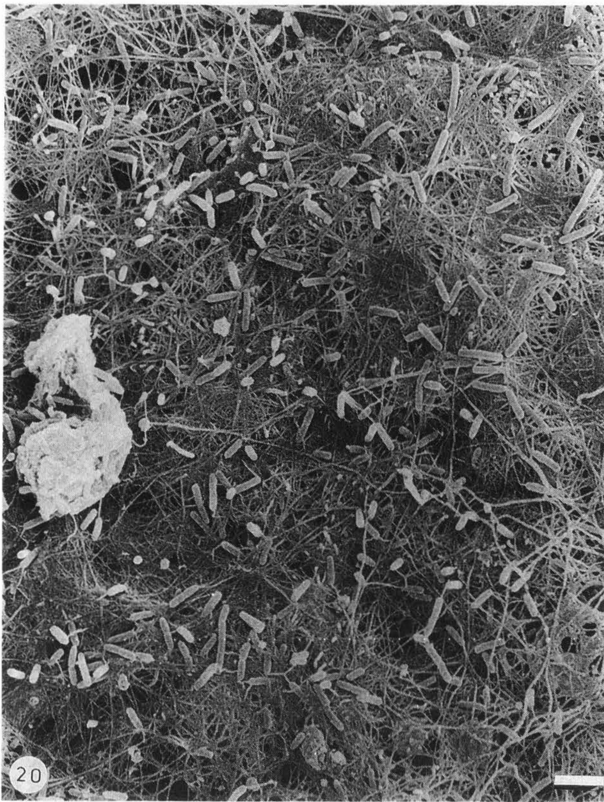
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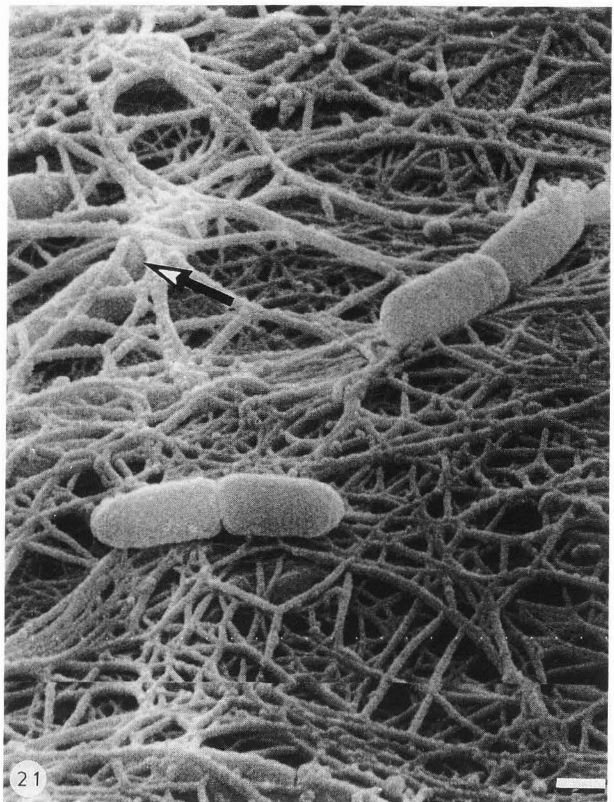
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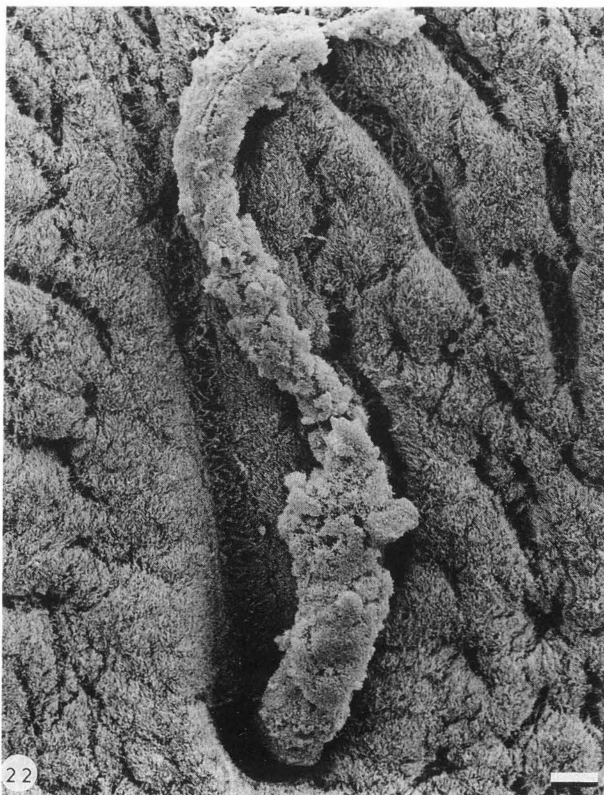
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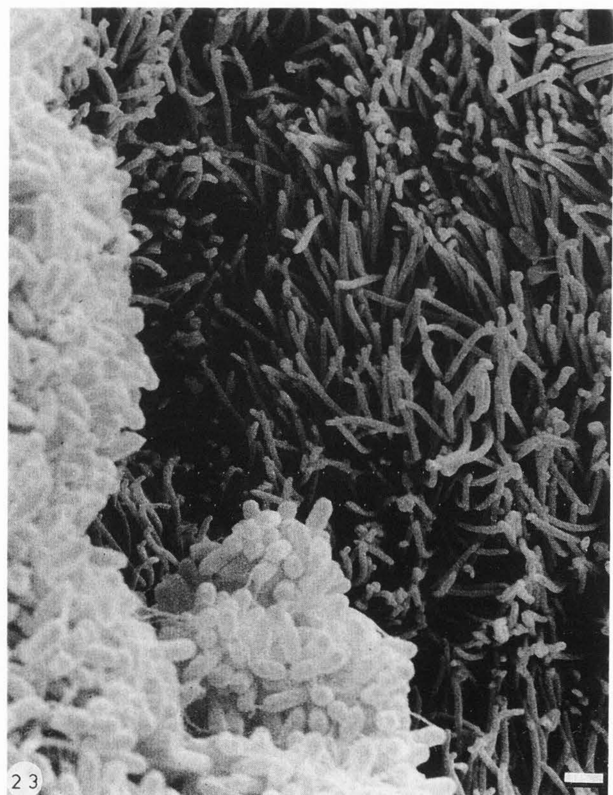
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Discussion with Reviewers

D.E. Woods: Were the numbers of PAO pili expressed in BLP2 the same as the numbers of PAK pili expressed in BLP2? If more PAO pili were expressed, this might explain the increased adherence of the BLP2/PAO over that of BLP2/PAK.

Authors: Both BLP2/PAO and BLP2/PAK were examined by TEM and found to bear the same number of pili. Therefore, we do not feel that any observed differences in the relative adherence of the two strains reflects different degrees of piliation. Furthermore Irvin *et al.* have shown that the affinity constant of wild type *P. aeruginosa* PAO to buccal epithelial cells is ten times greater than that of wild type *P. aeruginosa* PAK and both of these strains are piliated to a similar degree. On the basis of these data we feel that the observed differences in the adherence of *P. aeruginosa* bacteria expressing PAK or PAO types of pili reflects the differences in the primary structure of their respective epithelial cell binding domains.

D.E. Woods: Did the presence of pili change the protease production levels of BLP2? Evidence is mounting that proteolytic activity dramatically influences the adherence of *P. aeruginosa* to eucaryotic cells. Thus, it would be of interest to examine BLP2/PAO over that of BLP2/PAK.

Authors: We agree that the level of protease activity expressed by *P. aeruginosa* has an effect on its adherence to epithelial cells. Saiman *et al.* (Saiman L, Ishimoto K, Lory S, Prince A (1990) The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. J Infect Dis, 161, 541-548) have looked at the contribution of extracellular proteases to the adherence of *P. aeruginosa* P1 strains rendered protease minus by Tn9 mutagenesis to bovine respiratory epithelial cell monolayers and found that it was one-third that of the corresponding protease producing strains. Our purpose here however was to use isogenic *P. aeruginosa* strains so as to determine with as much precision as possible the relative importance of pili alone as an adhesin. To study the relative contributions of extracellular proteases and pili to *P. aeruginosa* adherence would require the construction of protease expressing and protease lacking, as well as pili expressing and pili lacking isogenic bacteria, something we did not endeavor to do.

D.E. Woods: Were the fibronectin levels on the exfoliated cells examined?

Authors: We did not look for fibronectin levels on the exfoliated cells.

J.L. Carson: What were the criteria for identification of "healthy surface cells, reparative cells and injured mucosal cells" (Table 3). For example, Fig. 11 illustrates two extruded cells which appear essentially identical, except that one exhibits bacterial attachment and the other does not. Which one is injured? Can these criteria be established by morphology alone?

Authors: Our criteria for healthy cells was that they were functionally intact within the epithelium, they exhibited a completely normal cellular ultrastructure as determined from TEM evaluation and normal morphological features on SEM examination (e.g., no blebs or other membrane related anomalies). The reparative cells have been shown to be non-ciliated and basal cells that spread laterally to accommodate the epithelial space vacated by exfoliating ciliated cells.

The point of showing the two cells exfoliating in Fig. 11, one with bacteria adhered to it and the other without is to illustrate that there are some as yet unknown factors that are involved in the cell injury-receptor expression sequence that we do not understand. Both cells are injured as indicated by their exfoliation from the epithelium; however, only one had *P. aeruginosa* adhered to it. The reason remains obscure but we speculate that one cell was expressing 'receptors/membrane conformations' that the other was not. Whether both cells exfoliated at the same time is an important question that implies that there may be a programmed sequence of surface membrane alteration synchronized with cell death. Whether this is the case is speculative.

It is indeed possible to establish criteria for the three stages of epithelial cell expression using only, and simply, morphological means. Combining SEM with TEM, and if necessary immunochemistry or cryo-techniques is definitive in this matter. Readers are directed to the voluminous works of Dr. B. Trump, for one example, for extensive quantitative correlations between ion fluxes, metabolic biochemistry and morphology in cell death.

J.L. Carson: Is it possible that the membranous "flap"

(Figs. 8-10) in SO_2 injured cells represents a processing "artifact"? A possible scenario might be that the integrity of the ciliary membrane is compromised in some way by the SO_2 exposure so that subsequent processing causes extrusion of the microtubular bundle. Fig. 10 suggests that the terminus of the ciliary shaft is somewhat bulbous and "different" from the remainder of the cilium.

Authors: It is extremely unlikely that the membranous "flap" on some SO_2 injured cells represents a processing "artifact". Only those cells that expressed that ciliary abnormality exhibited *P. aeruginosa* adhered to their apical surface. *P. aeruginosa* only adhered to injured cells in the canine model. If there was extrusion of the microtubular bundle, then the structure would protrude out the tip of the cilium like a needle extending beyond the end of a surrounding sheath. The "flap" structure is different and its formation is unknown. The bulbous nature of the canine cilium is curious and does indeed suggest that the terminus of the ciliary shaft is somehow different from the remainder of the cilium. We have made this observation consistently over the past 8 years of the canine tracheal epithelium and have, as yet, no explanation for it.

J.L. Carson: Are you suggesting that Figs. 14 and 15 document true invasiveness of the host cell by *P. aeruginosa*? Is it possible that the images represent indentation and gradual opportunistic entry by the bacterial cell into a dead or dying host whose membrane integrity has been breached by SO_2 exposure?

Authors: Yes, we are suggesting that these figures represent true invasiveness of the host cells and tissues by P. *aeruginosa*. The bacteria shown in Fig. 15 penetrating into the nucleus of the cell might be interpreted as the reviewer suggests as penetration of *P. aeruginosa* below the basement membrane. Fig. 19. however demonstrates invasiveness, not opportunistic burrowing into dead or dying cells and tissues because immediately following an acute inhalation injury the basal cells spread laterally to preform the new epithelial barrier these areas then are composed of healthy tissues in the process of regeneration and/or repair, not necrotic material.

E.A. Worobec: How would you predict a multipiliated variant such as *P. aeruginosa* 2Pfs would adhere under the same conditions used in this study? How about strains with related pilus types *(Moraxella* or *Neisseria)* or E. *coli* expressing one of these pilus types?

Authors: *Pseudomonas aeruginosa* 2Pfs is a multipiliated strain whose pili do not retract normally. We have not looked at the adherence of this strain in this system or in other work that we have done previously. Saiman *et al.* did look at the adherence of a related strain *P. aeruginosa* DB2 which is a multipiliated mutant of *P. aeruginosa* PAO. *P. aeruginosa* DB2 binds about 20% less than the parent PAO strain to bovine tracheal epithelial cell monolayers. Whether or not this decreased adhesion is due to stearic inhibition of adhesion due to the masses of pili on the surface of the DB2 mutant or other factors remains to be seen. Studies of *P. aeruginosa* pili expressed in *Neisseria sp* or *Moraxella sp* have not been done. Given that different species of the genus *Neisseria* and *Moraxella* do colonize the upper respiratory tract in healthy persons from time to time and that these pathogens are not generally seen as pathogens in the face of mucosal injury or disease one could speculate that these bacteria would adhere in small numbers to the healthy respiratory epithelial cells and not in any greater number to the damaged epithelial cells.

E.A. Worobec: Please explain how you can compare your tissue system (injured canine tracheal cells) to what may be found in the human Cystic Fibrosis patient. Have you considered repeating your experiments with human tissues (biopsies or tissue culture derived from tracheal samples)?

Authors: It is difficult to extend any observations between species, however, having said that, the canine tracheal model has served extremely well as a model for the human tracheal tissue. Transport of ions, measurements of bioelectrical properties and the cell composition are not significantly different between these species. Thus, we speculate that the correlation between events observed on the canine tracheal surface and the human trachea may be applicable.

Cystic Fibrosis, in addition to being characterized by abnormal Cl^- transport, is also characterized by the colonization of respiratory tissues by *P. aeruginosa*. Two factors interplay to facilitate the adherence and colonization by *P. aeruginosa* of the respiratory tissues: cell injury and elaboration of mucous secretions. A major problem for those with C.F. is the inappropriate hydration of the mucous secretions that is related to the abnormality in Cl^- secretion. This reduces the effectiveness of the mucociliary escalator in clearance, promotes the retention of inhaled toxins and increases the time necessary for the clearance of exfoliating cells when injuries do occur. We speculate that as exfoliated cells would be present on the airway surface for a longer period of time than in normals, they will present a surface for adherence of *P. aeruginosa* before the injured cells are cleared from the airway surface. This manuscript shows that *P. aeruginosa* adheres to the lateral sides and cilia of injured tracheal cells, not healthy cells nor reparative cells. Thus, exfoliating cells in patients with C.F. will likely provide a surface for adherence of *P. aeruginosa* in addition to tracheobronchial mucin.

E.A. Worobec: Can you speculate as to why you think *P. aeruginosa* adheres to some but no other cells in the same preparation?

Authors: As we know that adherence is receptor mediated we speculate that the lack of adherence reflects either the lack of expression of the appropriate receptors or membrane conformations. As to how this could occur in two cells exfoliating side-by-side is not known. However, this observation implies that either not all cells

express the appropriate domains for recognition, or that their expression may be related to some preset biochemical events that translate with appropriate molecular expression that are triggered by the cell-death sequence. Because not all cells that exfoliate are at the same stage of cell death, the expression of the components involved in the adherence of *P. aeruginosa* may follow different temporal sequences.

E.A. Worobec: In your speculation of how exfoliation enhances the ability of *P. aeruginosa* to bind you mention certain receptors for pili may become available upon exfoliation. Please expand on this theory including your speculation as to the origin and chemical composition of these putative pilus receptors.
Authors: The search for the receptor on mammalian cells surfaces which permit binding of *P. aeruginosa* bacteria is still in its beginnings. The evidence to date suggests that in the case of buccal epithelial cells the receptor is a glycoprotein or a group of glycoproteins on the cell surface. We have previously shown that purified PAK pili bind in vitro to various proteins such as aldolase much more than to mono- and oligosaccharides. At this point in time it is impossible to be certain what the receptors are on the SO_2 injured tracheal cells. We believe it is likely that during the process of dying these cells express different surface proteins and/or glycoproteins but their nature has not been determined.

E.A. Worobec: A very exciting observation has arisen from your experiments in which you have shown, for the first time, the ability of *P. aeruginosa* to invade host tissue. Do you have any plans to further this phenomenon? Do you feel that now knowing that *P. aeruginosa* has invasive properties will affect how these infections are treated?
Authors: We found the demonstration of "invasion" of injured tracheal epithelial cells by *P. aeruginosa* interesting as well. This invasion process is likely to differ in many important ways from that of other classical invasive organisms such as *Salmonella sp. P. aeruginosa* appears to have an absolute requirement for prior damage to the tracheal epithelial cell before it can either adhere or invade. However, the bacteria did not invade the basal reparative cells which are not injured by the SO_2 treatment. The invasion process may be mediated by the release of proteolytic enzymes by the bacteria causing disruption of the cell membrane culminating in penetration by the *P. aeruginosa* cell. We do not know, as is the case in *Salmonella sp.*, if new invasion proteins are expressed by *P. aeruginosa* during this process. We plan to follow up on these observations in the near future using both the SO_2 injured canine tracheal model and an *in vitro* cell monolayer system. We do not believe that *P. aeruginosa* is an intracellular pathogen and therefore treatment need not, and currently does not, have to be directed at killing intracellular organisms. The most promising approach still is preventing the adherence in the first place with an effective vaccine.

J.W. Costerton: The pilus genes were certainly cloned into the strains used here but it would be good to prove that the gene product (the pili) was actually produced *in vitro* (ELISA) and *in vivo*, by the use of monoclonal antibodies to the pili which will thicken these structures so that they can be visualized by TEM. The K99 pili of *E. coli* have been visualized, on an infected tissue surface, by this method, and these data would support the polar association interpretations in this paper. The direct demonstration that these pili are actually produced by bacteria in the tracheal ring preparations would complete this link to Randy Irvin's nice purified pilus work.
Authors: The isogenic strains used in this study have been examined for the production of the corresponding type of pilin by Western blots using specific polyclonal antipilus antisera and have demonstrated the production of pilin. Also TEM and pilus specific bacteriophage sensitivity have shown that pili are indeed expressed on the bacteria cell surface on the strains used in this study. We have not used monoclonal antibodies as suggested but agree that if pili specific monoclonal antibodies could be shown to bind at the bacterial cell-mammalian cell adherence interface this would strengthen our interpretations of the results. Unfortunately, at the time this research was conducted, the various monoclonal antibodies we now possess, did not exist.
J.W. Costerton: Figs. 2, 3, 7, 9, 11, 13, and 18 show linear structures amongst the adherent bacterial cells that you appear to interpret as flagella (the legend to Fig. 18 describes a "web of flagella"). Are these structures actually flagella that have been substantially thickened by accretions during processing for SEM? They are morphologically very similar to strands produced by the condensation of exopolysaccharide matrices, during preparation of the piliated and non-piliated cells for SEM, but you describe all of your strains as being alginate-minus "non-mucoid" organisms.
Authors: These linear structures could represent bacterial flagellae or pili which, because of their hydrophobic nature, have formed braids consisting of intertwined pili. It is possible, although unlikely, that these fibers are artifacts of preparation as suggested by Dr. Costerton. Even though our strains were all non-mucoid there is always a small amount of exopolysaccharide on the bacterial surface which could produce these fibers during the fixing process.