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Adhesion of *Pseudomonas aeruginosa* Pilin-Deficient Mutants to Mucin

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Attachment of *Pseudomonas aeruginosa* to epithelial cells or tracheobronchial mucin is mediated by surface adhesins. Pili, composed of monomeric pilin subunits, make up one such class of adhesins. The formation of pili and flagella in *P. aeruginosa* is under the control of the alternative sigma factor *rpoN*. Isogenic mutant strains with insertionally inactivated *rpoN* genes were constructed with strains PAK, 1244, and CF613 and were tested for their ability to adhere to respiratory mucin. All *rpoN* mutants showed significant reduction of adherence to mucin relative to that of their wild-type parents. In contrast, the adherence of pilin structural gene mutants was similar to the adherence of wild types. These results provide suggestive evidence that *P. aeruginosa* also binds to mucin via adhesins that are distinct from pilin and are still under the genetic control of *rpoN*. Unlike the laboratory strain PAK, the clinical strains 1244 and CF613 are capable of agglutinating erythrocytes. The *rpoN* mutation had a minimal effect on the interaction of bacteria with erythrocytes, indicating that the transcription of a gene(s) specifying the agglutination phenomenon does not utilize *rpoN*. These findings collectively indicate the existence of several classes of adhesins on the surface of *P. aeruginosa* that may play an important role in colonization of the human respiratory tract.

Pili and flagella, surface appendages of *Pseudomonas aeruginosa*, are involved in adhesion and motility, two of several attributes which appear to be involved in the virulence of this organism. The *rpoN* locus in *P. aeruginosa* has been demonstrated to control the expression of both of these organelles (4, 12), raising the possibility that *rpoN* is a part of a system which controls locomotion and adhesion in this organism. However, there is preliminary evidence that *P. aeruginosa* may possess a nonpilus adhesin(s) for tracheal cells (9), but it is not known whether this adhesin(s) is also under the control of the *rpoN* gene product nor whether it mediates adhesion to mucins. In addition, *P. aeruginosa* has been shown to possess a surface hemagglutinin (2, 3) about which little is known, e.g., it is not known whether this is the same as the nonpilus adhesin alluded to above or whether this substance is also under the genetic control of the *rpoN* gene. In order to examine these questions, we have constructed nonpiliated and *rpoN*-negative strains of *P. aeruginosa* and examined the effects of these mutations on the adhesion of this organism to mucins and on hemagglutination of human erythrocytes. Our studies with two nonmucoid strains show that *P. aeruginosa* possesses a nonpilus adhesin(s) which has a significant capacity for binding mucins and that this adhesin(s) may also be controlled by the *rpoN* gene product. In contrast, expression of the alginate adhesin is not controlled by this gene. Using these same mutants, we also find that hemagglutination is not controlled by the *rpoN* gene and is an activity separate from that of the main nonpilus adhesin.

MATERIALS AND METHODS

Bacterial strains. Nonmucoid strains PAK and 1244 and mucoid strain CF613 have been utilized in previous studies (8, 12). The constructions of the nonpiliated and *rpoN*-negative mutants of PAK (PAK-NP and PAK-N1, respectively) have been previously described (9, 12), as has the construction of CF613-N1 (12). Nonpiliated and *rpoN*-negative mutants of strain 1244 were also constructed as described below. The strains that were used are shown in Table 1, with the relevant genotype or phenotype and source.

Construction of 1244-NP. Briefly, *P. aeruginosa* 1244 was mated with *Escherichia coli* DH5α carrying an insertionally inactivated pilin gene cloned in pUCm19 and *E. coli* DH5α carrying a helper plasmid, pRK2013. The insertional inactivation of the pilin structural gene with a *tet* cassette has been described previously (9). Tetracycline-resistant, carbenicillin-sensitive transconjugants were identified. The replacement of the wild-type pilin gene with the disrupted gene was confirmed by extracting DNA from bacteria and by Southern blot analysis with a radiolabeled *tet* cassette and the pilin gene as probes. These bacteria also appeared nonpiliated by electron microscopy and were resistant to the pilus-specific phage P04.

Construction of 1244-N3. The disruption of the *rpoN* gene in strain 1244 was accomplished as described previously for PAK (4, 12). Plasmid pKI11, carrying the *tet* cassette in the *rpoN* coding sequence, was introduced into strain 1244. Tetracycline-resistant, carbenicillin-sensitive colonies were identified, and the replacement of the wild-type *rpoN* allele with the insertionally inactivated copy was confirmed by Southern hybridization analysis of DNA. One such isolate, 1244-N3, was further examined and was found to lack pili and flagella and to be resistant to pilus-specific phage P04. For complementation of the *rpoN* mutation, plasmid pPT212 was introduced into recipient *P. aeruginosa* by triparental matings, as described previously (12). When the plasmid carrying the wild-type *rpoN* gene was introduced into strain 1244-N3, the transconjugants simultaneously resumed synthesis of pili and flagella, consistent with complementation of the *rpoN* mutation.

Electron microscopy. Cultures of *P. aeruginosa* were

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TABLE 1. Strains of *P. aeruginosa* used in this study

Strain	Relevant characteristic	Source or reference
PAK-SR	Streptomycin resistant mutant of PAK	4
PAK-NP	Nonpiliated mutant of PAK-SR	9
PAK-N1	*rpoN* mutant of PAK-SR	4
1244	Piliated, nonmucoid strain	8
1244-NP	Nonpiliated mutant of 1244	This study
1244-N3	*rpoN* mutant of 1244	This study
CF613	Mucoid isolate from a CF[a] patient	12
CF613-NG	*rpoN* mutant of CF613	12

[a] CF, Cystic fibrosis.

grown to mid-logarithmic phase of growth in L broth. Bacteria were pelleted and then resuspended in 50 mM sodium phosphate–10 mM $MgCl_2$ and mixed with an equal volume of 2% aqueous phosphotungstic acid. A drop of this suspension was placed onto a Parlodion-coated grid (Mallinckrodt, St. Louis, Mo.), and excess liquid was removed by blotting. The samples were examined with a JEOL 100B transmission electron microscope.

Southern blot analysis. Total DNA was extracted from *P. aeruginosa* as described by Strom and Lory (11) and Totten et al. (12) and was digested for 16 h with restriction enzyme *Bam*HI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Fragments were separated by electrophoresis on a 0.7% agarose gel in Tris-acetic acid-EDTA buffer. The DNA was blotted onto Nitran sheets (Schleicher & Schuell, Keene, N.H.) as described by Southern (10). The blot was probed with DNA fragments labeled with [^{32}P]dCTP (Amersham, Inc., Chicago, Ill.) by nick translation.

Adhesion. Human tracheobronchial mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation, as described previously (14). The bacterial strains were grown in Trypticase soy broth (BBL Microbiology Systems) overnight at 37°C, and the inoculum was adjusted by spectrophotometer to between 2×10^7 and 5×10^7 CFU/ml. Strains containing plasmids which coded for antibiotic resistance were grown in broth containing gentamicin (10 μg/ml). Microtiter plates were coated with mucins at a concentration of 50 μg/ml. The adhesion assay was performed as previously described (13). Each strain was tested a minimum of five times. The results are mean values derived from these experiments.

Adhesion inhibition by pili. In order to rule out the possibility that nonpiliated strain 1244-NP still carried the adhesin for mucin that may have been present on the pili of strain 1244, the following experiment was performed. Mucin-coated microtiter plate wells were incubated with pili from strain 1244 (a gift of J. Sadoff, Walter Reed Army Institute of Research, Washington, D.C.), at concentrations of 20 and 40 μg/ml. The pilus samples were allowed to incubate in the wells for 60 min at 37°C. The wells were then washed to remove excess pili, and adhesion experiments were carried out with strains 1244 and 1244-NP.

Hemagglutination assay. In order to test whether the nonpilus adhesin(s) for mucin is the same as the hemagglutinin, the relevant strains were tested as follows. Human type O erythrocytes were obtained from the blood bank of the University of Florida teaching hospital. They were washed four times with phosphate-buffered saline (PBS) and suspended at 2% (vol/vol) in PBS. The bacteria to be tested were adjusted by spectrophotometer to about 10^9 CFU/ml. A 1/10-ml volume of erythrocytes was mixed with 0.1 ml of the bacterial suspension in wells of glass agglutination slides, which were gently agitated for 2 h at 37°C. Control wells contained erythrocytes alone in PBS. Agglutination was quantitated from 0 to 4+, with 4+ showing complete agglutination. Inhibition of hemagglutination was tested by suspending the bacteria in PBS containing the substance to be tested.

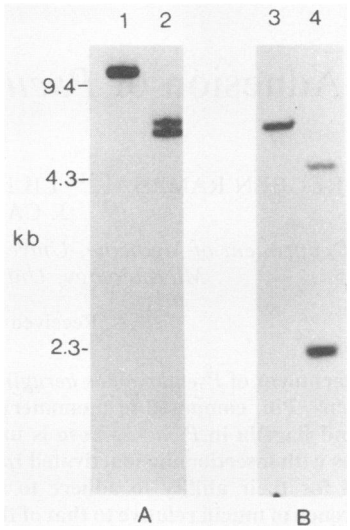

FIG. 1. Southern blot analysis of *rpoN* and pilin gene mutants of *P. aeruginosa* 1244. *Bam*HI digests of DNA from each strain were probed with radiolabeled *rpoN* probe (A) or pilin gene probe (B). DNA digests of each strain are shown as follows: lanes 1 and 3, 1244; lane 2, 1244-N3; lane 4, 1244-NP.

RESULTS

Construction of mutants affected in pilin synthesis in *P. aeruginosa* 1244. We introduced mutations in the *rpoN* and pilin genes of strain 1244, a *P. aeruginosa* clinical isolate, in order to obtain additional isogenic bacterial derivatives for studies of host-cell interaction. Figure 1A shows the Southern blot analysis of DNA from strains 1244 and 1244-N3 digested with *Bam*HI and probed with the radiolabeled *rpoN* gene from plasmid pK110 (4). The probe reacted with a single 10.2-kb fragment in the 1244 digest, while the two fragments of ca. 6.4 and 5 kb reacted in the digest of 1244-N3. These results are consistent with insertion of the 1.4-kb *tet* DNA cassette containing an internal *Bam*HI site into the chromosome of 1244 at the *rpoN* locus. When *Bam*HI-digested DNA of strain 1244 was probed with a pilin gene probe from plasmid pMS27 (6), a single 5.5-kb fragment reacted, while the probe reacted with two fragments (4.6 and 2.3 kb) in a digest of 1244-NP DNA (Fig. 1B). Strain 1244-NP is therefore a pilin structural gene mutant.

The phenotypes of mutants were also confirmed by electron microscopy. Figure 2A is a micrograph of a representative cell of strain 1244 displaying both pili and flagella. Strain 1244-N3 lacks both of these surface organelles (Fig. 2B), which is consistent with the known properties of *rpoN* mutants previously reported (12). Strain 1244-NP lacks only pili, whereas it has a polar flagellum (Fig. 2C). Introduction of the cloned *rpoN* gene on broad-host-range plasmid pPT212 simultaneously restored the abilities of 1244-N3 to form pili and flagella (Fig. 2D).

Adhesion results. (i) Strain PAK (Table 2). Strain PAK and

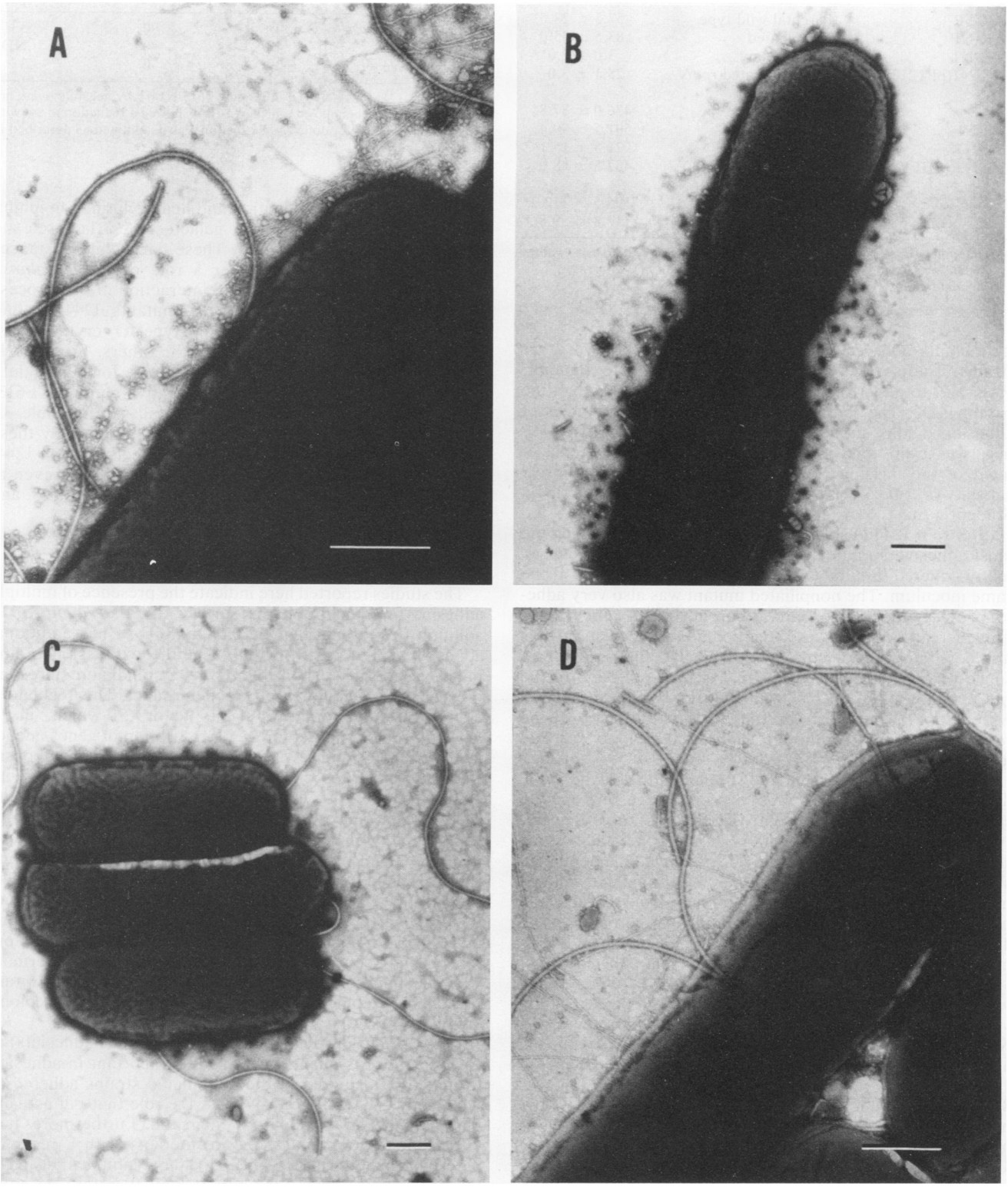

FIG. 2. Electron micrographs of strain 1244 and its isogenic mutants. The following strains are shown: 1244 (A), 1244-N3 (B), 1244-NP (C), and 1244-N3 with plasmid pPT212 (D). Bar, 300 nm.

TABLE 2. Adhesion of *P. aeruginosa* mutants to mucins

Strain	Relevant characteristic	CFU/well (10^2)[a]
PAK-SR	Parental wild type	57.5 ± 23.6*
PAK-NP	Nonpiliated	83.5 ± 35.0
PAK-N1	*rpoN* mutant	3.0 ± 2.0**
PAK-N1(pPT212)	Complemented *rpoN*	28.1 ± 3.0
1244	Parental wild type	476.0 ± 87.8*
1244-NP	Nonpiliated	591.5 ± 99.4
1244-N3	*rpoN* mutant	21.5 ± 15.1**
1244-N3(pPT212)	Complemented *rpoN*	482.5 ± 48.0
CF613[b]	Parental wild type	395.5 ± 112.2*
CF613-N1[b]	*rpoN* mutant	117.8 ± 59.8**

[a] Differences are statistically significant ($P < 0.001$) for * versus ** values by Student's *t* test.
[b] Mucoid strains.

its mutants adhered poorly compared with the two other strains. There was a tendency for the nonpiliated mutant, PAK-NP, to adhere to mucins in greater numbers than the parent PAK. The same pattern was observed in studies of adhesion of this strain with neoglycolipids (7). The *rpoN*-negative strain was practically nonadhesive, and replacement of the missing gene restored some of the adhesive capacity ($P > 0.01$ compared with that of the parent strain by Student's *t* test).

(ii) Strain 1244 (Table 2). Strain 1244 has been among the most adhesive strains studied by us (8). This strain consistently adhered in numbers 5 to 10 times that of PAK at the same inoculum. The nonpiliated mutant was also very adhesive and again, more so than the parent strain by about 25%. This increase in adhesion of the nonpiliated 1244 strain was also noted in adhesion studies with neoglycolipids showing a single receptor specificity (7). In contrast, adhesion of the *rpoN* mutant of strain 1244 was reduced by 95% compared with that of the parent strain. Replacement of the *rpoN* gene resulted in the restoration of adhesion back to parental levels.

(iii) Strain CF613 (Table 2). Strain CF613 and its *rpoN* derivative CF613-N1 were both mucoid, suggesting that the *rpoN* gene product did not control the expression of alginate. Assuming that the *rpoN* mutant is lacking in pili and that the second adhesin(s) is found on the nonmucoid nonpiliated strains, then adhesion of this mutant should be alginate mediated (8) unless there is still another adhesin. The *rpoN* mutant CF613-N1 adhered at about 30% of parental levels. This suggests that the adhesion of mucoid strains to mucins involves alginate as well as *rpoN*-dependent adhesins.

Effect of pili on adhesion of strain 1244-NP. When mucin was preincubated with purified *P. aeruginosa* pili, the adhesion of piliated strain 1244 to the pilus-treated mucin was reduced by about 54% ($P < 0.001$, Student's *t* test); while the adhesion value (10^2 CFU per well) for the control mucin was 182.2 ± 30.8, the adhesion value for pilus-treated mucin was 83.7 ± 7.6. Doubling the concentration of pilin protein did not reduce this any further. In contrast, pilus treatment of mucin had no effect on the adhesion of nonpiliated strain 1244-NP at any concentration of pilin used; the adhesion value (10^2 CFU per well) for the control mucin was 254.5 ± 19.1, and the value for pilus-treated mucin was 229.5 ± 58.8 (differences not significant).

Hemagglutination studies. Strains PAK, 1244, and CF613, as well as the mutants that were studied for adhesion, were examined for hemagglutination and hemagglutination inhibition by using 2% fucose, 2% galactose, 2% lactose, and 0.025% *p*-nitrophenol (Sigma). These particular substances were chosen to rule out either a role of *P. aeruginosa* internal lectins or a hydrophobic interaction in this process (2, 3). Strain 1244, along with its mutants 1244-NP and 1244-N3, hemagglutinated human type O erythrocytes, strain 1244-N3 minimally less than the others. Strain PAK and its mutants tested completely negative for hemagglutination. Strain CF613 and its *rpoN*-negative mutant, CF613-N1, agglutinated the erythrocytes equally well (Table 3). None of the sugars inhibited the hemagglutination by these strains, but *p*-nitrophenol had minimal effects only on 1244-N3; it did not completely inhibit hemagglutination even at 0.1%. It had no effect on strains 1244, 1244-NP, CF613, and CF613-N1.

TABLE 3. Hemagglutination of *P. aeruginosa* mutants

Strain type	Hemagglutination of strain[a]		
	1244	PAK	CF613
Parental	4+	0	3+
Pilin negative	4+	0	ND
rpoN negative	3+	0	3+

[a] Parental 1244, 1244-NP, CF613, and CF613-N1 (*rpoN* negative) were not affected by *p*-nitrophenol. *rpoN*-negative 1244 showed a reduction to 2+ with *p*-nitrophenol. ND, Not done. Agglutination was quantitated as described in the text.

DISCUSSION

The studies reported here indicate the presence of multiple adhesins for mucins on the surface of *P. aeruginosa*. In addition to pili and alginate, *P. aeruginosa* has additional adhesins that differ from pili, and the expression of the putative adhesins responsible for mucin binding requires the presence of a functional *rpoN* gene product. This regulatory element is an alternative sigma factor (5) which, in *P. aeruginosa*, is required for the transcription of genes encoding pili, flagella, and several enzymes of nitrogen metabolism (4, 12). Studies of the interaction of *P. aeruginosa* with cultured respiratory epithelial cells also revealed that the *rpoN* defect causes a much more significant reduction of bacterial attachment than does a mutation in the pilin gene (1). The significance of the various adhesin genes utilizing *rpoN* is not obvious. All *rpoN*-controlled genes are also regulated by additional transcriptional factors that respond to various stimuli and regulate expression of each gene individually or coordinately (5). Identification of such factors for the *rpoN*-controlled adhesins may allow us to determine whether different adhesins expressed by *P. aeruginosa* are regulated by common environmental signals during colonization of the respiratory tract.

These studies also indicate that the nonpilus adhesin(s) of *P. aeruginosa* is quantitatively quite important in adhesion to mucins, since both of the nonpiliated strains adhered as well as their parent strains. Thus, the role that pili actually play in adhesion to mucins is enigmatic. Furthermore, it is clear that this nonpilus adhesin interacts with a receptor different from pili, since whole pili did not inhibit adhesion of the nonpiliated strain 1244-NP. It may therefore be envisioned that the adhesion of nonmucoid *P. aeruginosa* strains may be a two-phase process involving pili initially and then a surface-bound nonpilus adhesin(s) in the final stage. However, even with such a scenario, it is not apparent why pili

should block the adhesion of the piliated strain, since this strain had an intact nonpilus adhesin(s). Conceivably, the binding of *P. aeruginosa* pili to mucins could be a long-range nonspecific hydrophobic interaction which facilitates a closer approximation of the organism with mucins in order to effect a more specific interaction via receptors for the nonpilus adhesin. In this regard, it is noteworthy that a specific receptor for *P. aeruginosa* pili has not been described to date.

The surface hemagglutinating activity of *P. aeruginosa* also requires some discussion. Unlike for other bacteria, this activity does not appear to be pilus mediated (3). Furthermore, it is not an activity shown by all strains. It is also clear that it is not mediated by the nonpilus adhesin. Strain PAK-NP did not show this activity but still adhered to mucin, and the *rpoN* mutations in strains 1244-N3 and CF613-N1 did not result in the complete loss of hemagglutination, whereas the strains showed significant losses in adhesive ability, ascribed to loss of the nonpilus adhesin(s). The surface hemagglutinating activity of *P. aeruginosa* has been reported to be a hydrophobic interaction (2, 3), but we find only a small hydrophobic contribution to hemagglutination by strain 1244-N3 and none by the other strains. Thus, it seems that the hemagglutinin(s) is not necessarily the same as the nonpilus adhesin, since it is not present on strain PAK-NP. It is also quite possible that the residual mucin adhesion shown by the *rpoN*-negative mutants 1244-N3 and CF613-N1 could be mediated by the hemagglutinin, making the system even more complicated.

Thus, the adhesion of *P. aeruginosa* to mucins appears to be a much more complex process than previously envisioned (8), consisting of the interactions of multiple adhesins. In order to dissect this process and to study the receptor structures, it will be necessary, at the very least, to examine pili, alginate, a nonpilus adhesin, and a hemagglutinin. This will be possible with the availability of mutants containing only one adhesin or by the use of the purified adhesins. Additionally, the fact that the genotypically nonpiliated strains possess good adhesive ability suggests that antipilus vaccines to prevent adhesion to mucins may be ineffective.

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