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Genetic Analysis of *Pseudomonas aeruginosa* Adherence: Distinct Genetic Loci Control Attachment to Epithelial Cells and Mucins

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Infection of mucosal tissues by the opportunistic pathogen *Pseudomonas aeruginosa* is initiated by attachment of the bacterium to host tissues. To gain a better understanding of this interaction, we used two methods to isolate mutants of *P. aeruginosa* with altered adherence to cultured A549 cells and to mucins. First, from a population of nonpiliated mutants of *P. aeruginosa* mutagenized with transposon Tn*5*G, we have isolated variants that are defective in binding to both A549 cells and respiratory mucins. Using a cloned transposon plus flanking DNA from one such mutant as a DNA probe, we have isolated plasmids from a cosmid bank, which, upon reintroduction to the original mutants, restored adhesion to both A549 cells and mucin. The second strategy to identify genes involved in adhesion used mutagenesis of *P. aeruginosa* N1G, an *rpoN* mutant which is unable to bind to either A549 cells or mucin, with transposon Tn*5* containing an outward-directed promoter. From this bank of mutagenized *P. aeruginosa* N1G, two classes of adhesion variants were isolated; one class attached to A549 cells and to mucin, and the other class restored binding of the *rpoN* mutant to mucin but not to A549 cells. These findings suggest that *P. aeruginosa* can express at least two adhesins distinct from pili, one recognizing receptors shared by epithelial cells and mucins and the other recognizing mucins alone.

The opportunistic pathogen *Pseudomonas aeruginosa* causes severe respiratory tract infection in cystic fibrosis patients. The infection is characterized by chronic colonization of the airways, culminating in cardiopulmonary failure in the later stages of the disease (14). The initial colonizers resemble environmental isolates, or those associated with other diseases, in their nonmucoid phenotype. As the disease progresses, there is a switch to the mucoid phenotype, which then predominates.

The first step in colonization of the respiratory tract is adherence of the invading bacteria to host tissue. Several *P. aeruginosa* virulence determinants have been implicated in mediating this phase of the bacterium-host interaction. These included pili (25, 26), alginate (17), and, more recently, exoenzyme S (2). *P. aeruginosa* has been shown to recognize receptors on both epithelial cells and mucin. The components thought to make up such receptors include asialogangliosides, lactosyl ceramide (1), sialylated oligosaccharides, and the type 1 and type 2 disaccharide units of mucin (15).

Previous work in our laboratories with genetically engineered strains has shown that pili on the surface of *P. aeruginosa* play a minimal role in attachment of the bacteria to mucin, whereas they contribute significantly in attachment to epithelial cells (4, 16). Moreover, the alternative sigma subunit of RNA polymerase (RpoN), which is required for pilin gene expression, also appears to control expression of the nonpilus adhesin(s) responsible for attachment of *P. aeruginosa* to both cells and mucins, since mutants with mutations in *rpoN* showed a defect in binding to epithelial cells and mucins (4, 16, 18). These results suggest that *P. aeruginosa* may express a common nonpilus adhesin for cells and mucins or, alternatively, that mucins and cells are recognized by different adhesins, both of which are controlled by RpoN.

To further elucidate the nature of *P. aeruginosa* nonpilus adhesins, we have isolated a series of transposon-induced mutants that are altered in binding to both epithelial cells and mucin. Analysis of the phenotypes of these mutants suggests the existence of at least two classes of adhesins. One class of adhesin recognizes receptors on both mucins and epithelial cells. A second class of adhesin, isolated in the *rpoN* background after mutagenesis with a transposon possessing an outward-directed promoter, suggests that an additional adhesin may be expressed by *P. aeruginosa*. This second class specifically recognizes receptors on mucins but not on epithelial cells. This report describes the initial isolation and characterization of these mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains, plasmid vectors, and their derivatives are shown in Table 1. All cultures were routinely grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.), Luria broth (L-broth), or minimal A salts (5) supplemented with 50 mM monosodium glutamate and 1% glycerol. Antibiotic concentrations used for the selection and maintenance of resistance markers (in micrograms per milliliter) were as follows: for *P. aeruginosa*, tetracycline, 100; gentamicin, 25; neomycin, 600; for *Escherichia coli*, ampicillin, 100; kanamycin, 50.

Enzymes and chemicals. All restriction enzymes and T4 DNA ligase were purchased from GIBCO-BRL, Inc., Gaithersburg, Md. All cell culture media and reagents were purchased from GIBCO-BRL.

DNA manipulations and analyses. Plasmid DNA was prepared by the method of Birnboim and Doly (3). Agarose gel electrophoresis, DNA restriction digests, DNA ligations, and Southern blots (under high stringency) were done essen-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli		
DH5α	*hsdR recA lacZYA* ϕ80 *lacZΔM15*	GIBCO-BRL
P. aeruginosa		
PAK	Wild type	D. Bradley
PAK-NP	PAK *pilA*::Tc^r	18
PAK-N1G	PAK *rpoN*::Gm^r	7
B164	Nonadherent mutant	This study
RR10	Nonadherent mutant	This study
RR18	Nonadherent mutant	This study
RR20	Nonadherent mutant	This study
A13	Adherent	This study
RO13	Adherent	This study
RO14	Adherent	This study
MS159	PAK *pilA*::Tc^r *fliC*::Gm^r	This study
NP533	PAK *pilA*::Tc^r *fliA*::Gm^r	This study
Plasmids		
pRK2073	Str^r mobilizing plasmid	11
pVK102	Cosmid vector, Nm^r Tc^r	8
pDS1	8.4-kb *Eco*RI fragment of B164 chromosomal DNA containing Tn*5*G insert in *Eco*RI site of pBR322	This study
pDS20C	Adherence-complementing cosmid	This study
pDS31B	Adherence-complementing cosmid	This study
pDS32A	Adherence-complementing cosmid	This study
pPC110	Gm^r cassette	22
pSUP102::Tn*5*-B50	Suicide plasmid used for mutagenesis of PAK-N1G	19

tially as described previously (12). Chromosomal DNA was isolated from *P. aeruginosa* as described previously (21).

Cell culture. A549 cells were obtained from the American Type Culture Collection. The cells were maintained at 37°C in 5% CO_2 in Ham's F12 medium with glutamine supplemented with 10% fetal calf serum and antibiotics (100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.5 μg of amphotericin B per ml). Cells were removed from the tissue culture dish by trypsin treatment (0.25% trypsin–EDTA) following a brief wash with phosphate-buffered saline (10 mM sodium phosphate, 150 mM sodium chloride [pH 7.4]). Protease activity was stopped by the addition of Ham's F12 medium with glutamine plus 10% fetal calf serum and antibiotics.

Cell adherence assay. All bacteria were grown in L-broth (containing the appropriate antibiotics) overnight at 37°C. Before use in adhesion assays, the bacterial concentration was determined spectrophotometrically. An optical density of 1.0 at 600 nm corresponds to 6.0×10^8 bacteria per ml as determined by plating dilutions of bacterial suspension onto L-agar plates and counting the number of CFU following overnight incubation of the plates at 37°C. Aliquots (1 ml) of each culture were centrifuged at 12,000 × *g* in a tabletop microcentrifuge for 2 min to sediment bacteria. The bacterial pellet was then resuspended in 1 ml of Hanks' balanced salt solution supplemented with 10 mM sodium phosphate buffer (pH 7.4) (HBSS). Bacteria were again sedimented by centrifugation and resuspended in HBSS. They were then diluted in HBSS to give approximately 7.5×10^7 bacteria per ml.

Approximately 3×10^5 A549 cells were inoculated into each well of a 24-well tissue culture plate containing sterile 12-mm round glass coverslips and incubated overnight. The monolayers were washed three times with 2.5 ml of HBSS each time and then overlaid with 0.8 ml of this solution. Aliquots (200 μl) of the diluted bacterial suspension were added to duplicate wells. This gives a ratio of approximately 50 bacteria per A549 cell. The plates were centrifuged at 600 × *g* for 5 min at 23°C and then incubated at 37°C for 2 h in 5% CO_2. Then the unbound bacteria were removed by aspiration, and the monolayers were washed four times with 2.5 ml of HBSS each time. The monolayers were then fixed for 15 min in 100% methanol and stained with 2.0% (vol/vol) Giemsa stain (Harleco; Fisher Chemical Co., Pittsburgh, Pa.) in water for 45 min. The stain was removed by three washes in water, and the plates were air dried overnight. The coverslips were mounted on glass slides by using a mounting reagent and viewed under a light microscope at a magnification of ×400. Five separate fields were chosen at random, and the number of cells and the number of adherent bacteria were counted (approximately 150 A549 cells were counted in such a procedure). Since this procedure does not distinguish internalized from surface-bound bacteria, all data are presented as the total number of bacteria associated with the monolayer. Data are presented as the mean and standard deviation for at least 10 fields in a minimum of two separate experiments.

Mucin adherence assay. To test for adhesion to mucin, we grew bacterial strains overnight in tryptic soy broth containing the appropriate antibiotic. The inoculum was adjusted spectrophotometrically and plated on MacConkey agar to obtain the viable CFU per milliliter. The inocula used were between 2×10^7 and 5×10^7 CFU/ml. The microtiter plate wells were coated with respiratory mucins that were prepared as previously described (27), and the remainder of the adhesion assay was performed as previously described (24). Wells without mucin coating were used as controls for nonspecific binding.

Transposon mutagenesis. A random bank of chromosomal transposon Tn*5*G insertions was generated in *P. aeruginosa* PAK-NP as described previously (13). A bank of Tn*5*-B50 insertions in the *rpoN* mutant of *P. aeruginosa* was constructed by mating *E. coli* S17-1 carrying the suicide plasmid pSUP102::Tn5-B50 (18) with *P. aeruginosa* PAK-N1G. Insertions in the chromosome of the *P. aeruginosa* recipient were obtained after plating the mating mixture on L-agar containing gentamicin and tetracycline. After overnight incubation, approximately 60,000 colonies were obtained, pooled, and saved for further studies.

Isolation of mutants of *P. aeruginosa* PAK-NP defective in mucin binding. The screening adherence assay for nonadhering bacteria was performed as follows. Approximately 10^5 CFU of the PAK-NP bank with Tn*5*G insertions, grown in tryptic soy broth until an optical density at 600 nm of 2 was reached, were passed successively over six mucin-coated wells of microtiter plates. The inoculum was allowed to adhere to each well for 30 min, removed, and passed on to the next well. After six passages, the bacteria remaining in the last well were removed and the well was rinsed to collect all nonadherent bacteria. These nonadherent fractions were then plated in their entirety on McConkey agar plates. Individual colonies were then retested for adhesion as described previously (24) to identify nonadhering organisms from this pool of bacteria that were enriched for nonadherence. Three mutants (RR10, RR18, and RR20) that were defective in adhesion, when compared with PAK-NP, were obtained after screening.

Isolation of mutants of *P. aeruginosa* PAK-NP defective in binding to A549 cells. A bank of *P. aeruginosa* PAK-NP

containing randomly inserted Tn5G was screened as follows. The bank was grown overnight at 37°C in L-broth containing 20 μg of gentamicin per ml. The bacteria were sedimented by centrifugation and then washed once in HBSS. They were resuspended in HBSS to a density of approximately 4×10^4 bacteria per ml. Then 200 μl of this suspension was added to a well of washed A549 cells (in a 24-well plate) and incubated for 1 h at 37°C in 5% CO_2. At the end of the incubation the supernatant containing nonadherent bacteria was removed, added to the next well of washed A549 cells, and incubated for 1 h as before. After the bacteria were passaged over six monolayers consecutively, dilutions of the bacterial suspension were plated on L-agar plus 20 μg of gentamicin per ml. After overnight growth, the bacteria were resuspended in L-broth plus gentamicin and grown for 2 h at 37°C. The bacteria were washed and resuspended as described above, to a density of 4×10^4 bacteria per ml. They were passaged over an additional six monolayers of A549 cells consecutively as described above. The bacterial suspension, obtained following 12 sequential passages over A549 cells, was then plated on L-agar containing gentamicin. Individual colonies were picked and tested in the adherence assay described above. Of 250 colonies tested, 1 isolate (B164) was consistently nonadherent.

Identification of transposon insertions. Chromosomal DNA from mutant strains was digested with one of several restriction enzymes and electrophoretically separated on 1.0% agarose gels. For the strains containing the Tn5G transposon, Southern analysis was performed by using a probe consisting of the gentamicin cassette (isolated from pPC110). All probes were labeled with [^{32}P]dCTP by using a random primer labeling kit (GIBCO-BRL). A probe-reactive 8.4-kb *Eco*RI fragment containing the Tn5G transposon plus 4.3 kb of flanking DNA from digests of B164 DNA was isolated and cloned into *Eco*RI-digested pBR322 to form pDS1.

Identification of complementing cosmids. *E. coli* HB101 containing a PAK gene bank in cosmid pVK102 (6) was plated to a density of approximately 300 colonies per plate. Colonies were transferred to nitrocellulose disks and prepared for hybridization by the method of Maniatis et al. (12). The colonies were screened with the ^{32}P-labeled 8.4-kb *Eco*RI fragment, containing the Tn5G with flanking DNA, isolated from pDS1. For complementation of the adhesion defect, the probe-reactive cosmids were introduced into *P. aeruginosa* B164 by triparental conjugation with pRK2073 as the mobilizing plasmid (11).

Isolation of cell-adhesive PAK-N1G mutants. Approximately 7.5×10^7 *P. aeruginosa* N1G cells, mutagenized with Tn5-B50, were added to the A549 cells grown on coverslips in tissue culture plates. The bacteria were centrifuged onto the monolayer (600 × *g* for 6 min), and they were incubated at 37°C for 2 h in 5% CO_2. The monolayer was then washed four times with HBSS, 200 μl of trypsin-EDTA (0.25%, wt/vol) was added, and the mixture was incubated for an additional 5 min at 37°C. Protease activity was quenched by the addition of 1 ml of Ham's F12 medium containing 10% fetal calf serum. The detached A549 cells were resuspended by mixing the medium gently with a pipette. The complete contents of the well were transferred to a microcentrifuge tube, and the A549 cells were collected by centrifugation. The pellet was solubilized by the addition of 20 μl of 1% (vol/vol) Triton X-100 in phosphate-buffered saline for 5 min at 23°C. Then 1 ml of L-broth was added, and the suspension was plated on a series of L-plates containing gentamicin and tetracycline and grown at 37°C. The bacteria from the plates were resuspended in 1 ml of L-broth, and a 50-μl portion of this suspension was added to 2 ml of L-broth plus antibiotics and grown overnight for the next adherence assay. After 12 such rounds of exposure to A549 cells, the adhering bacteria were plated on L-agar plates containing gentamicin and tetracycline, to obtain isolated colonies. Bacteria from individual colonies were tested in the adherence assay, and of 42 tested, 1 highly adhesive mutant (A13) was isolated.

Isolation of mucin-adhesive mutants of *P. aeruginosa* PAK-N1G. Approximately 10^6 CFU of the Tn5-B50-mutagenized bank of *P. aeruginosa* PAK-N1G was screened for mucin binding in the standard adhesion assay, as described above. The adherent bacteria were recovered, plated directly onto McConkey agar, and incubated overnight at 37°C. Individual colonies were regrown and screened for mucin binding. Two isolates, RO13 and RO14, showed levels of adhesion that were reproducibly similar to that of the wild-type *P. aeruginosa* PAK, and these were saved for further studies.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Whole bacterial cells were denatured by boiling in 2% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol–50 mM Tris-HCl (pH 7.5). These samples were separated on 15% polyacrylamide gels (10), and the proteins were electrophoretically transferred to nitrocellulose (23). The filters were treated with 2% nonfat dry milk in Tris-buffered saline, incubated with antisera, washed, and probed with either horseradish peroxidase-labeled anti-mouse immunoglobulin G (IgG) and IgM (Kirkegaard and Perry, Gaithersburg, Md.) or ^{125}I-protein A (Dupont-NEN, Wilmington, Del.). Monoclonal anti-exoenzyme S was kindly provided by D. E. Woods, University of Calgary. Monoclonal antiflagellin was kindly provided by A. Siadak, Oncogen, Seattle, Wash.

RESULTS

Strategies for isolation of *P. aeruginosa* mutants with altered adhesive properties. We have adapted two genetic approaches to identify *P. aeruginosa* genes that are involved in the adhesion of the bacterium to tissues. These are outlined in Fig. 1. First, nonpiliated *P. aeruginosa* PAK-NP cells were mutagenized with transposon Tn5G. Nonadherent bacteria were isolated following sequential passage over either monolayers of A549 cells or mucin immobilized in polystyrene plates. Nonadhesive mutant B164 was obtained after enrichment on A549 cells, while strains RR10, RR18, and RR20 were identified by their inability to bind to mucin.

The second approach relied on isolation of adhesive mutants of the nonadhering *rpoN* mutant of PAK (4) after mutagenesis with the transposon Tn5-B50 containing an outward-reading promoter (P_{out}) and thus capable of overriding the regulatory defect due to the *rpoN* mutation, provided that it inserted adjacent to an RpoN-controlled gene. After a library of random insertions was obtained in strain PAK-N1G, the bacteria were applied to A549 cell monolayers or immobilized mucin and adherent bacteria were isolated. Adhesive mutant A13 was isolated from the A549 cell screen, while RO13 and RO14 were mutants of PAK-N1G that now adhered to mucin.

Adhesion of strains generated by Tn5 mutagenesis of PAK-NP. Individual isolates, obtained after passage of the mutagenized culture over cells or mucin, were tested for binding to both mucin and cells. Figure 2A shows a typical pattern of adherence of PAK-NP, compared with the mutant B164 (Fig. 2B). Quantitative adherence assays for the mutant strains compared with the parental PAK-NP strain are shown in Fig. 3. Mutant B164, which did not adhere to A549

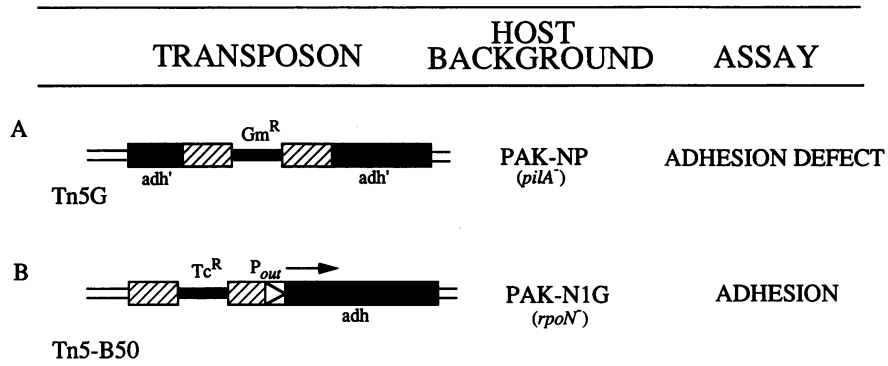

FIG. 1. Strategies for isolation of *P. aeruginosa* mutants with altered adhesive properties. Hatched lines represent transposon sequences. (A) Nonpiliated *P. aeruginosa* PAK-NP, which binds to both mucins and A549 cells, was mutagenized with transposon Tn*5*G. Mutants defective in adhesion should have a Tn*5*G insertion in a gene (*adh*) encoding a structural or regulatory function involved in non-pilus-mediated adherence. (B) An *rpoN* mutant of *P. aeruginosa* PAK-N1G, which does not bind to either mucins or A549 cells, was mutagenized with transposon Tn*5*-B50. This transposon may activate the production of an adhesin if inserted upstream of an adhesin that is not expressed in the *rpoN* background. Thus, mutants were screened for restoration of adherence. Abbreviations: adh, adhesin gene; adh′, adhesin or adhesin regulatory gene; P_{out}, neomycin phosphotransferase promoter from Tn*5*-B50.

cells, also showed reduced adhesion to mucin (Fig. 3A). Similarly, strains RR10, RR18, and RR20, which fail to bind to mucin, do not adhere to epithelial cells to any significant degree. In the mucin-binding assay, the adherence of RR18 and B164 was at least 90% lower than that of the parental PAK-NP, while RR20 showed virtually no adhesion. In contrast, all mutants showed the same low-level adherence to A549 cells of ca. 0.5 bacterium per A549 cell (Fig. 3B). These nonadhesive mutants were not simply a result of insertions of the Tn*5*G into the *rpoN* gene, since the mutants do not possess many of the characteristics of previously characterized *rpoN P. aeruginosa* strains (22).

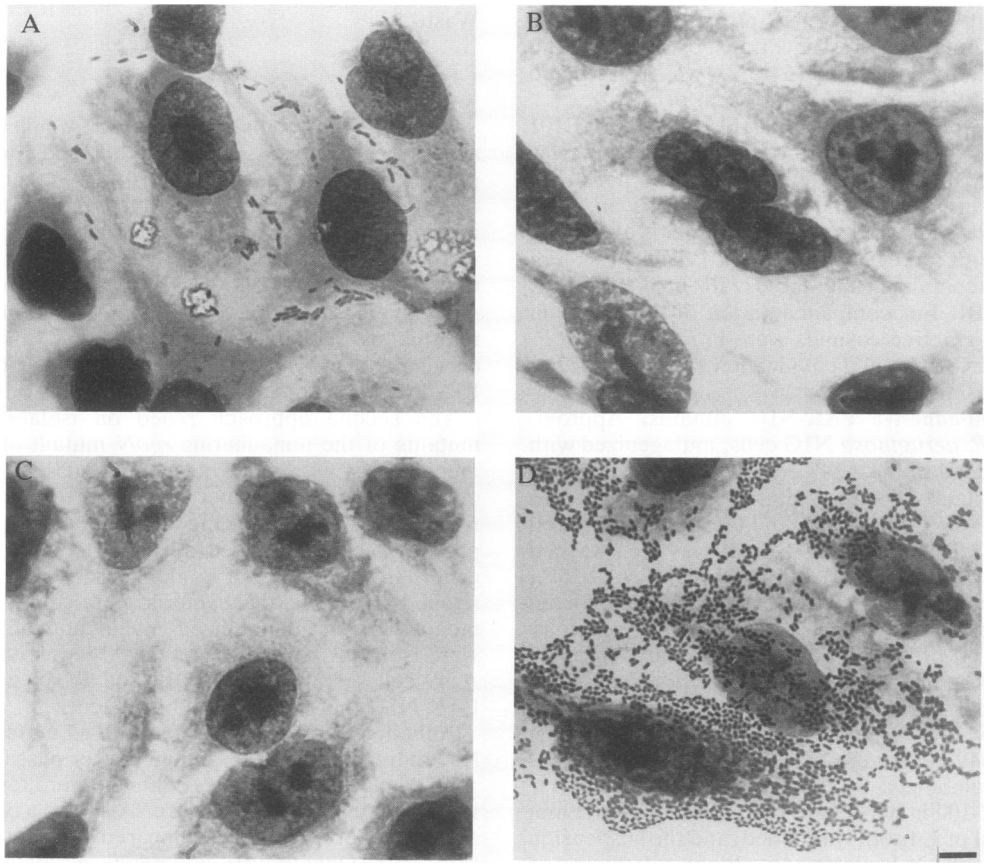

FIG. 2. Adherence of various *P. aeruginosa* mutants to A549 cells. (A) PAK-NP; (B) B164; (C) PAK-N1G; (D) A13. The adherence assay was performed as described in the text. Bar, 5 μm.

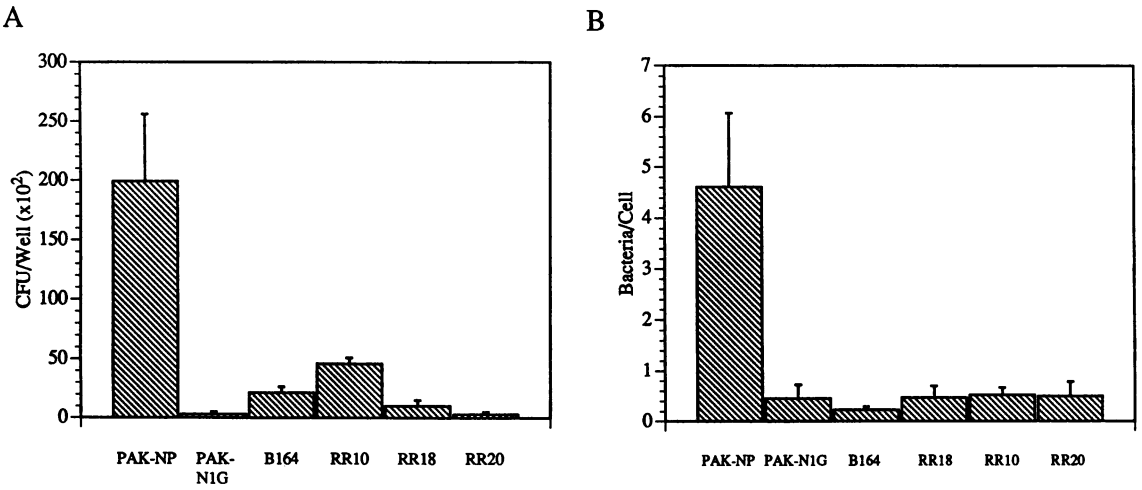

FIG. 3. Quantitation of adherence of PAK-NP Tn5G mutants to A549 cells and respiratory mucins. (A) Adherence of mutants to mucins. Bacteria were examined for their ability to bind to respiratory mucin in a microtiter plate assay as described in Materials and Methods. The results are presented as the number of CFU per well obtained after plating. (B) Adherence of mutants to A549 cells. Bacteria were examined for their adhesion to A549 cells grown on glass coverslips as described in Materials and Methods.

Location of the transposon in the chromosome of the nonadhering mutants. Southern blot analysis of chromosomal DNA digests, with a radiolabeled probe consisting of the gentamicin cassette, was used to compare the insertion site of Tn5G in the various adhesion-defective mutants (Fig. 4). Digestion of DNA from RR10 and RR20 with restriction enzymes that do not have recognition sites in Tn5G (*Bam*HI, *Eco*RI, and *Sal*I) produced unique probe-reactive fragments. The same enzymes yielded identical fragments for B164 and RR18 DNA, which were distinct from those observed for RR10 and RR20. This hybridization pattern suggests that the Tn5G transposons in B164 and RR18, which were isolated by different adhesion assays, may be located close to each other, or identically, whereas RR10 and RR20 carry insertions in different genes. Analysis of DNA digested with *Bgl*II, an enzyme recognizing a single site within the gentamicin cassette region of Tn5G, showed two different probe-reactive patterns for all strains, including RR18 and B164. Therefore, the transposon Tn5G inserted into the same *Eco*RI, *Bam*HI, and *Sal*I fragment in RR18 and B164; however, the insertion is in different locations within these fragments. Insertions in RR10 and RR20 are in different sites with respect to each other, as well as to B164 and RR18. This analysis also suggests that at least three different genetic loci may be involved in the expression of the nonpilus adhesin(s).

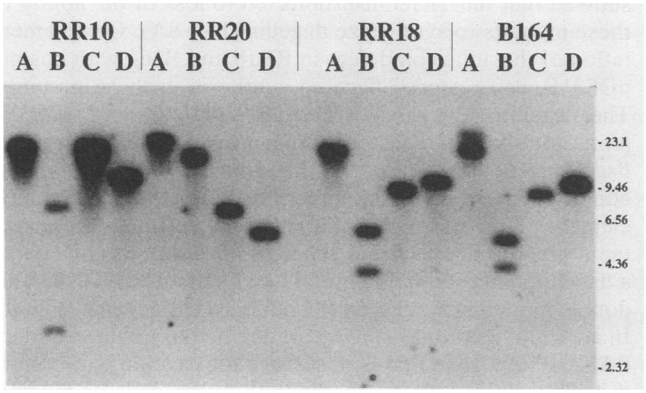

FIG. 4. Identification of Tn5G transposon in nonadhering mutants of PAK-NP. Chromosomal DNA from the designated mutant strains was digested with *Bam*HI (lanes A), *Bgl*II (lanes B), *Eco*RI (lanes C), or *Sal*I (lanes D) and separated on 1.0% agarose gels. Tn5G-containing fragments were identified by Southern analysis by using a probe consisting of the gentamicin cassette. Numbers on the right represent molecular sizes in kilobases.

Isolation of clones complementing the adhesion defect of B164 and RR18. To obtain a DNA probe from the chromosomal region flanking the site of insertion of Tn5G in B164, total chromosomal DNA was digested with *Eco*RI and ligated with *Eco*RI-digested pBR322. Gentamicin-resistant *E. coli* transformants contained an 8.4-kb DNA insert, which corresponds to the size of Tn5G plus ca. 4.3 kb of flanking DNA. This plasmid (pDS1) was used to probe a bank of *P. aeruginosa* PAK DNA in the cosmid vector pVK102. Three probe-reactive clones (pDS20C, pDS31B, and pDS32A) were identified by colony hybridization. Digestion of these cosmids with different restriction enzymes showed common as well as unique fragments, confirming that the clones carry overlapping but distinct regions of the chromosome. The presence of the Tn5G-flanking DNA was confirmed by Southern blot analysis of digests of these clones.

Each of these clones was conjugated into B164 and RR18, and transconjugants were tested for adhesion to A549 cells and mucin. Figure 5 shows that cosmid pDS31B complements the adhesion defect of B164 and RR18 to both A549 cells and mucin. Complementation of the adhesion defect with either pDS20C or pDS32A was not as pronounced as that seen with pDS31B. Complementation of the adhesion defect in both B164 and RR18 with the same cosmid clone confirms that the insertions in these mutants are in linked or identical genes. Partial complementation, noted for both pDS20C and pDS32A, could be due to absence of DNA sequences from the cosmid that contribute to maximal expression of the adhesins.

Adhesion of *rpoN* mutants carrying Tn*5*-B50 insertions. Mutants generated by insertion of the Tn*5*-B50 element into the chromosome of PAK-N1G showed two patterns of adhesion. The level of adhesion of A13 to A549 cells was

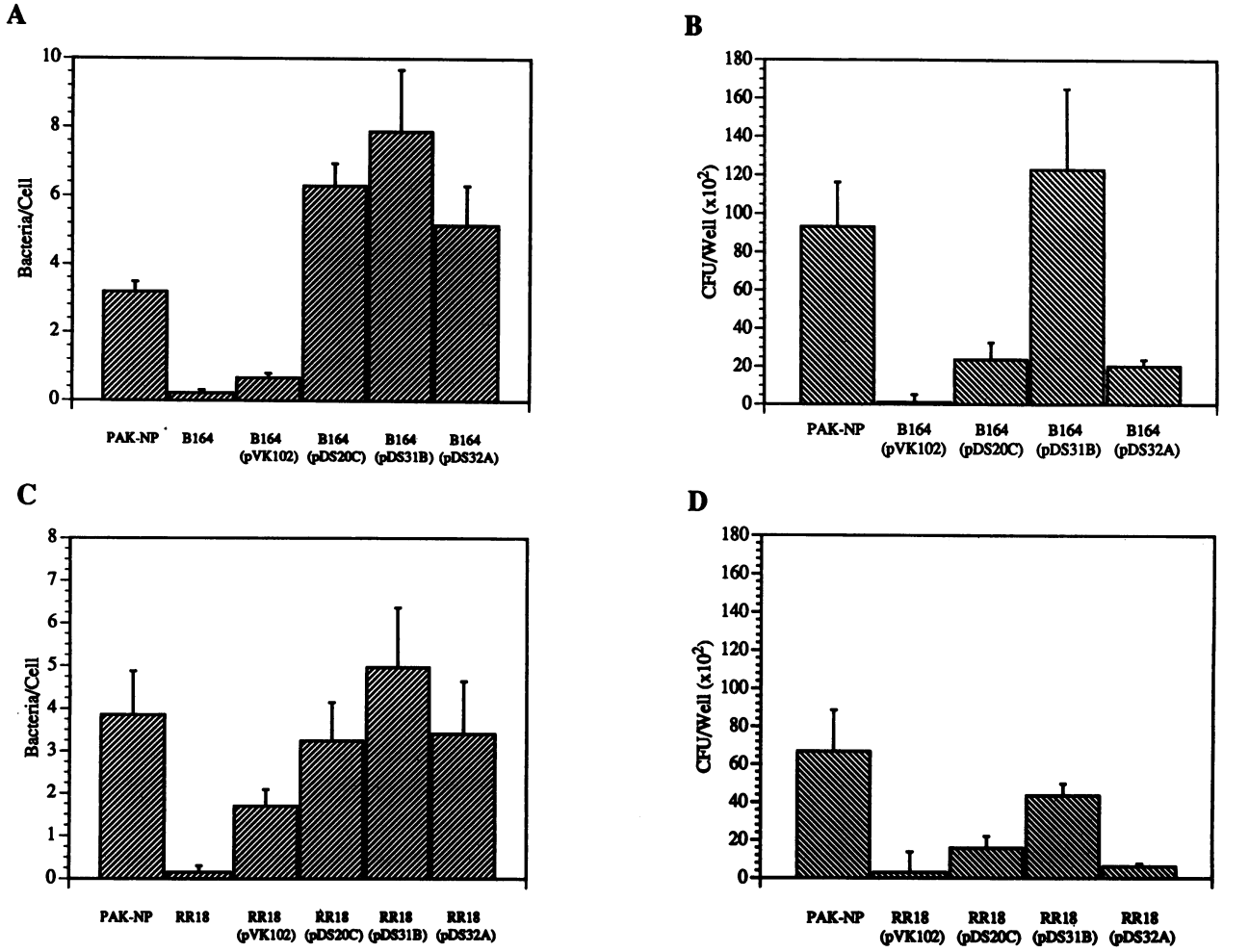

FIG. 5. Restoration of adherence of PAK-NP-derived nonadhering mutants by cosmids. Adherence assays were performed as described in the text. Mutants containing one of the cosmids pDS20C, pDS31B, pDS32A, or pVK102 (control) were examined for their adhesive properties. (A) Cell adherence of complemented B164. (B) Mucin adherence of complemented B164. (C) Cell adherence of complemented RR18. (D) Mucin adherence of complemented RR18.

severalfold higher than seen for the wild-type PAK (Fig. 2C and D and 6A). The same strain also showed a modest increase in adherence to mucin, compared with the *rpoN* parent; however, it was less than that of PAK-NP (Fig. 6B). In contrast, mutants isolated by mucin selection (RO13 and RO14) were nonadhesive for A549 cells. However, they showed strong attachment to mucin, approaching or even exceeding that seen for PAK-NP (Fig. 6B). The three mutants have Tn*5*-B50 insertions in different areas of the chromosome (Fig. 7). Additional enzyme digestions confirmed that the insertion of the Tn*5*-B50 transposon was in a site different from that in any of the nonadhesive Tn*5*G mutants in the PAK-NP background, indicating that the insertion is not due to the activation of a gene(s) that was inactivated in B164, RR18, RR10, or RR20. These results suggest the existence of at least two classes of adhesins, one recognizing receptors on both cells and mucins and the other being mucin specific.

Effect of Tn*5* insertions on expression of other *P. aeruginosa* genes. Because *rpoN* controls the expression of genes for pilin (6), nonpilus adhesin (4), and flagellin (22), we have examined the phenotypes of the nonadhering mutants described in this study for expression of flagellin. Western immunoblot analysis of all adhesion-defective mutants showed that the Tn*5*G insertions led to loss of the ability of these mutants to synthesize flagellin (Fig. 8A). Complementation of the adhesion defect in RR18 and B164 with cosmid pDS31B also restored flagellin synthesis in these mutants. The flagellin gene did not hybridize with cosmid pDS31B (data not shown). These results suggest that the control of flagellin gene expression and the expression of the nonpilus adhesin gene(s) very probably involve at least one common element. To confirm that the adhesion defect in these mutants was not due to the absence of flagellin, we engineered a flagellin mutation in the nonpiliated mutant PAK-NP. This double mutant still adhered to both cells and mucins (Fig. 9). In addition, a mutation engineered in the nonpiliated mutant PAK-NP in a gene that is necessary for flagellin expression, *fliA* (20), did not alter adherence to either cells or mucins. Adhesive mutants isolated by Tn*5*-B50 mutagenesis of the *rpoN* strain PAK-N1G did not express flagellin (Fig. 8A). Furthermore, pilin antigen was not detected in the same bacteria (data not shown), confirming that the adhesive phenotype of these mutants was not due to restoration or hyperproduction of pili but, rather, was the consequence of

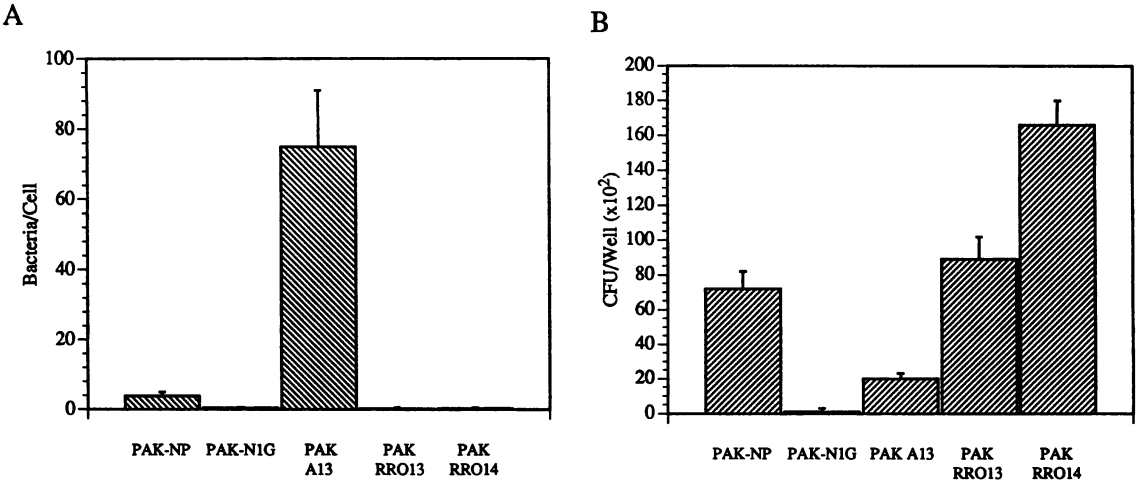

FIG. 6. Adherence of Tn*5*-B50 generated mutants of PAK-N1G to A549 cells and mucins. Adherence assays were performed as described in the text. (A) Adherence of strains to A549 cells. (B) Adherence of strains to respiratory mucins.

constitutive expression of the nonpilus adhesin(s) from the P_{out} promoter of the transposon.

Baker et al. suggested that another bacterial product, exoenzyme S, functions as an adhesin (2). We have examined the nonadhering mutants, as well as adhesive *rpoN* variants, for expression of exoenzyme S. Figure 8B shows that all nonadhering mutants produced exoenzyme S at the level comparable to that in the wild-type parent. Furthermore, the *rpoN* mutation did not cause a block in exoenzyme S synthesis, nor did any of the adhesive mutants in the *rpoN* background produce levels of exoenzyme S differing from that in the parental PAK-N1G. These results suggest that none of the genes affected by insertion of Tn*5*G or Tn*5*-B50 are in loci involved in exoenzyme S expression.

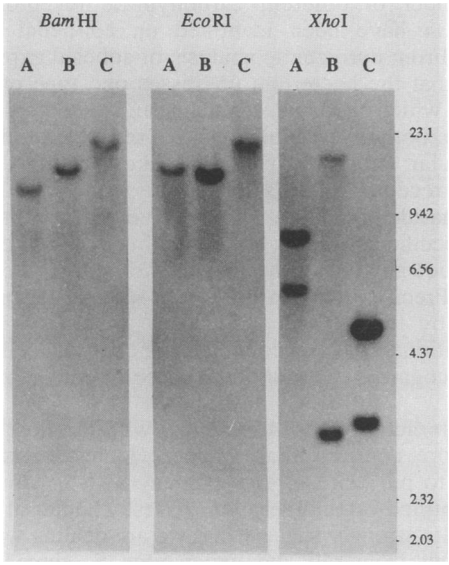

FIG. 7. Identification of Tn*5*-B50 insertions in PAK-N1G-derived adhesive mutants by Southern hybridization. Chromosomal DNA from mutants was digested with either *Bam*HI, *Eco*RI, or *Xho*I, separated on a 1.0% agarose gel, and transferred to nitrocellulose. Transposon Tn*5*B-50-containing fragments were identified by using a ^{32}P-labeled probe consisting of Tn*5* inserted in plasmid ColE1. Lanes: A, RO13; B, RO14; C, A13. Numbers at right represent molecular sizes in kilobases.

DISCUSSION

We have previously reported evidence for the presence of *P. aeruginosa* adhesins distinct from polar pili. This conclusion was based on the observation that genetically engineered mutants in which the pilin structural gene was inactivated retained partial epithelial cell-binding activity and adhered to mucin at parental levels. Moreover, mutations in *rpoN*, coding for a minor sigma subunit of RNA polymerase, resulted in strains that failed to adhere to both cells and mucin. We concluded that RpoN controls transcription of additional adhesin genes in *P. aeruginosa*, and in

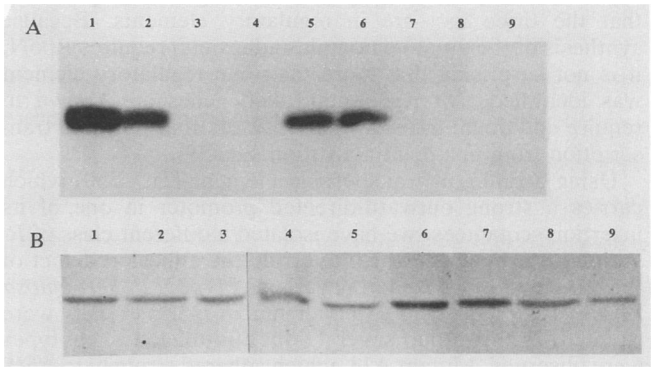

FIG. 8. Immunodetection of *P. aeruginosa* exoenzyme S and flagellin in mutants. Washed whole cells were lysed in 5× SDS-PAGE sample buffer, and the proteins were electrophoresed in discontinuous SDS-PAGE gels (10% polyacrylamide). The proteins were transferred to nitrocellulose and detected with antibody as described in the text. Similar numbers of bacteria were used for each well. (A) Immunodetection of flagellin by using monoclonal anti-flagellin antibody followed by ^{125}I-protein A. Lanes: 1, PAK; 2, PAK-NP; 3, B164; 4, RR18; 5, RR18(pDS31B); 6, B164(pDS31B); 7, PAK-N1G; 8, A13; 9, RO13. (B) Immunodetection of exoenzyme S by using monoclonal anti-exoenzyme S followed by horseradish peroxidase-labeled anti-IgG and anti-IgM. Lanes: 1, PAK; 2, PAK-NP; 3, B164; 4, RR18; 5, RR18(pDS31B); 6, RR18(pVK102); 7, A13; 8, RO13; 9, PAK-N1G.

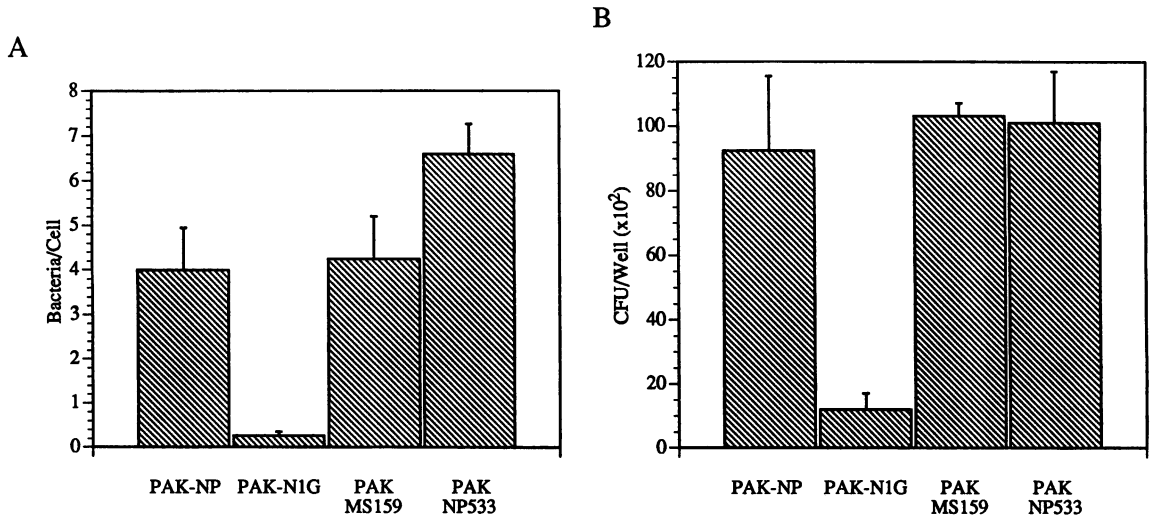

FIG. 9. Adherence of flagellin-negative mutants to A549 cells and respiratory mucins. Adherence assays were performed as described in the text. (NP533, *pilA fliA*; MS159, *pilA fliC*) (A) Adherence of strains to A549 cells. (B) Adherence of strains to mucins.

this study we have undertaken to isolate and characterize mutants with mutations in such genes.

Several classes of mutants were isolated by screening for lack of adherence to mucins and epithelial cells. Mapping of the site of insertion by Southern hybridization revealed that the transposon has inserted into three distinct locations. Mutants B164 and RR18 carry insertions within 1.5 kb from each other, whereas the insertions in RR10 and RR20 appear to be in unlinked loci, within the resolution of the restriction enzyme sites used in the Southern blot analysis. The physical linkage of genes insertionally inactivated in B164 and RR18 was further confirmed by isolation of cosmids, using a DNA probe derived from B164 and demonstrating complementation of the adhesion defect in both B164 and RR18.

Because of the pleiotropic phenotypes of all of the mutants, adversely affecting synthesis of flagellin, we postulate that the three loci are in regulatory elements. Because synthesis of the putative nonpilus adhesin(s) requires RpoN, it is not surprising that more than one regulatory element was identified. All RpoN-controlled genes are known to require additional transcriptional factors that promote transcription from upstream activation sites (9).

Using a different transposable element (Tn*5*-B50) which carries a strong outward-directed promoter in one of its insertion sequences, we have isolated a different class of *P. aeruginosa* mutants, which override the adhesion defect of the *rpoN* mutation. Adhering variants of *rpoN P. aeruginosa* PAK-N1G which bind to either mucin or A549 cells were isolated. In this group several adhesion-related phenotypes were observed. Mutant A13, which adhered strongly to A549 cells, also adhered to mucin. Screening of the PAK-N1G bank on mucin, however, yielded mutants (RO13 and RO14) which bound to mucin but still did not adhere to A549 cells. Southern blot analysis showed that none of these mutants carry insertions in linked loci. Unlike the adhesion-defective mutants isolated by Tn*5*G mutagenesis of *P. aeruginosa* PAK-NP, the Tn*5*-B50 mutants remained nonmotile, presumably because of the original *rpoN* defect (22). It is therefore probable that insertions upstream of the structural genes for several adhesins were isolated. In mutant A13, the promoter of Tn*5*-B50 directs transcription of an adhesin which recognizes a common receptor on cells and mucins. Alternatively, the transposon insertion may direct transcription of either an operon or multiple adhesins that recognize different receptors. Insertions in RO13 and RO14 promote the expression of a mucin-specific adhesin. We cannot exclude the possibility, however, that Tn*5*-B50 controls the expression of regulatory genes. Because the precise level at which *rpoN* is required in the regulatory cascade is not known, adhesion of Tn*5*-B50 mutants may be due to a constitutive expression of *rpoN*-controlled regulatory genes. Our findings nevertheless strongly suggest that there may be additional adhesins which recognize mucin receptors and that these molecules are absent from the membranes of epithelial cells.

A number of different carbohydrate receptors for *P. aeruginosa* have been identified on epithelial cells and mucins. From our genetic analysis of adhesin expression, it is clear that the bacterium possesses one type of common adhesin, which recognizes mucins and receptors on cells; therefore, both of these must have a receptor of identical or very similar structure. The most likely candidate for the common receptor on cells and mucins is the type 1 and type 2 disaccharide units (15). The nature of the receptor for the mucin-specific adhesin is unknown, but possible candidates may include any one of the sialylated carbohydrate chains of mucins. Precise identification of the specific receptors, recognized by the individual bacterial adhesin, will require identification of the component of the bacterial cell envelope which recognizes the individual carbohydrate of mucin and cell.

The interaction of *P. aeruginosa* with the host tissues is much more complex than was previously envisioned. In addition to pili (25, 26) and exoenzyme S (2), our studies have identified yet another two classes of bacterial adhesins. Our results strongly suggest that we are dealing with adhesins distinct from pili and exoenzyme S, since the *rpoN* mutation, which abolishes binding of bacteria to mucins and cells, does not change the level of exoenzyme S expression and none of the nonadhering mutants described in this study have reduced expression of exoenzyme S.

At this time, it is not known which one of these adhesins is expressed under conditions of infection. Moreover, expression of any one of the adhesins may be repressed once

the infection is established or proceeds to the chronic phase such as is seen in cystic fibrosis. Assessment of the role of any one of these bacterial products in infection will require examination of isogenic strains in the appropriate animal models.

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