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Adhesion of *Pseudomonas aeruginosa* to Respiratory Mucins and Expression of Mucin-Binding Proteins Are Increased by Limiting Iron during Growth

ANDRÉE SCHARFMAN,[1] HÉLÈNE KROCZYNSKI,[1] CHRISTOPHE CARNOY,[1] EDWIGE VAN BRUSSEL,[1] GENEVIÈVE LAMBLIN,[1] REUBEN RAMPHAL,[2] AND PHILIPPE ROUSSEL[1]*

Unité INSERM 377, 59045 Lille Cedex, France,[1] and Department of Medicine, University of Florida, Gainesville, Florida 32610[2]

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The attachment of *Pseudomonas aeruginosa* to human respiratory mucus represents an important step in the development of lung infection, especially in cystic fibrosis. Local factors in the respiratory tract, such as osmolarity or iron concentration, might influence this colonization. In the present work, we have observed that overall levels of adhesion of two nonmucoid, nonpiliated strains of *P. aeruginosa*, 1244-NP and PAK-NP, to human airway mucins were higher when these strains were grown in a minimal medium of low osmolarity (M9) than when they were grown in a rich medium of higher osmolarity (tryptic soy broth [TSB]). However, increasing the NaCl concentration of M9 to increase the osmolarity did not modify the level of binding. In order to find out whether these differences were due to variations in nutrients, the influence of iron concentration was investigated: the levels of binding of the two strains increased after TSB was depleted of iron and decreased after iron was added to M9. Since the outer membranes from the two strains have been shown to contain proteins reacting with human bronchial mucins, we compared the mucin-binding proteins expressed by the two strains grown in different media. When the nonpiliated strains 1244-NP and PAK-NP were grown in the different media, previously observed mucin-binding bands were detected in the 42- to 48-kDa range but additional mucin-binding bands in the 77- to 85-kDa range were detected when these strains were grown in M9 or iron-deprived TSB. These results demonstrate that the adhesion of *P. aeruginosa* and the expression of mucin-binding proteins in the outer membranes of nonpiliated *P. aeruginosa* are affected by the iron content of the medium in which the bacteria are grown and not by the osmolarity.

Bacterial gene expression is influenced by growth conditions (2, 4, 12–14, 32). Therefore, it is intuitive that the conditions in vivo, which are more complex than those in most microbiological media, may have influences which are difficult to detect under standard laboratory conditions. In this respect, the interaction of *Pseudomonas aeruginosa* with the respiratory tracts of cystic fibrosis (CF) patients may involve a variety of conditions or environmental signals within this niche which influence gene expression and hence the ability of this organism to colonize this environment.

Some of these conditions are being elucidated by studies of respiratory tract mucus, but a full characterization has not been done. Among the conditions that need to be examined for influences on gene expression are the osmolarity of the secretions, the ionic composition, and the effects of the macromolecules that are present in mucus. The influence of osmolarity itself on the secretion of exoenzymes of *P. aeruginosa* has been examined, and it was concluded that certain exoenzymes were reduced under hyperosmolar conditions (5). More recently, the ability of the macromolecules of mucus to activate certain genes of *P. aeruginosa* has been described by Lory et al. (19). Thus, the environment of the respiratory tract may play a role in enabling *P. aeruginosa* to be the successful pathogen that it is.

Within the respiratory lumen of CF patients, *P. aeruginosa* is found in the mucus (17, 28). This bacterium has a marked affinity for mucins in vitro (7, 8, 10, 24–27, 29), and several mucin-binding proteins have been observed in the outer membranes of nonpiliated strains of *P. aeruginosa* (7, 8, 23). The respiratory mucus itself is suspended in the periciliary fluid. Recent measurements of the elemental composition of this fluid in healthy and diseased airways have been performed, and it has been shown that the surface fluid collected from healthy airways was hypotonic (220 mosmol) with respect to plasma, whereas the fluid collected from patients with sustained airway irritation, infection, or CF appeared more isotonic with respect to plasma (18). To investigate the possible influence of osmolarity on the adhesion of *P. aeruginosa* to mucins, two nonpiliated strains were compared after growth in tryptic soy broth (TSB), M9 medium, and M9 medium supplemented with 111 mM NaCl.

Comparative adhesion to respiratory mucins of the nonpiliated strains PAK-NP and 1244-NP grown in TSB, M9, and M9 + NaCl. Human respiratory mucins were prepared as previously described (8) from the sputum of a patient (blood group O) suffering from chronic bronchitis. The two strains (which were kindly provided by S. Lory from the University of Washington), PAK-NP and 1244-NP, were grown in TSB (Difco, Detroit, Mich.) for 20 h and in M9 minimum medium (33 mM Na_2HPO_4, 1.9 mM KH_2HPO_4, 8 mM NaCl, 18 mM NH_4Cl, 1 mM $MgSO_4$, 1 mg of thiamine per liter, and 0.2% [wt/vol] glucose as a carbon source). The osmolarity of the TSB and M9 media was measured with an Osmomat 030 osmometer (Gomotec, Berlin, Germany); it was found to be 300 mosmol/liter for TSB and 210 mosmol/liter for M9. A hyperosmolar medium, M9 + NaCl (321 mosmol/liter), was also prepared by using 111 instead of 8 mM NaCl in the M9 medium. After growth, bacteria were pelleted, washed, and sus-

* Corresponding author. Mailing address: INSERM N° 377, Place de Verdun, F-59045 Lille Cedex, France. Phone: (33) 3 20 29 88 50. Fax: (33) 3 20 53 85 62.

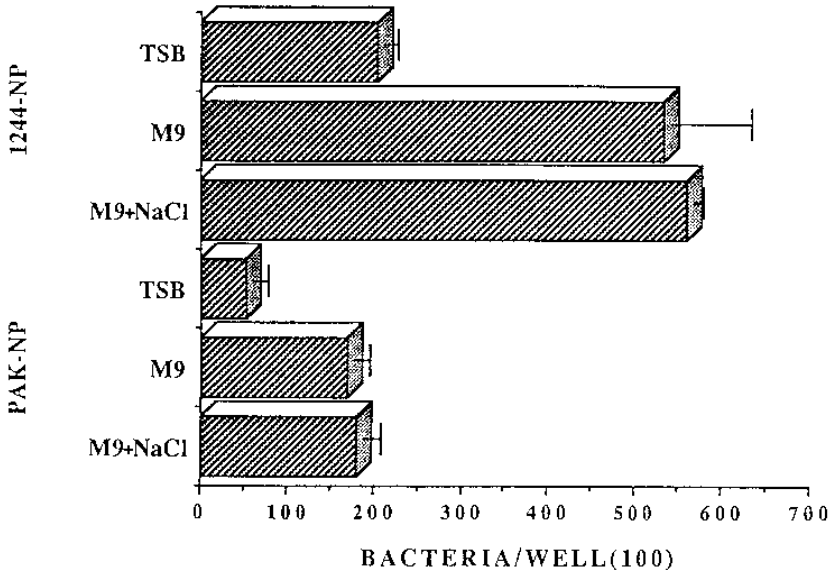

FIG. 1. Adhesion of two nonmucoid, nonpiliated strains of *P. aeruginosa*, 1244-NP and PAK-NP, grown in TSB, M9, and M9 + NaCl. Inocula were standardized to 10^7 CFU/ml. The error bars represent standard deviations.

pended in phosphate-buffered saline. Optical density measurements were used to obtain an inoculum of approximately 1×10^7 to 2×10^7 CFU/ml. The exact inoculum was ascertained by dilution and plating of the suspension. One hundred microliters of the bacterial suspension, containing 1×10^6 to 2×10^6 CFU, was added to each of three wells. The rest of the adhesion assay was done as previously described (31). Since it is not possible to obtain identical inocula from the different cultures, even by using optical density as a guide, the number of organisms per well was adjusted to a standard inoculum of 10^7 CFU/ml before mean values for the different experiments were obtained. When strain 1244-NP was grown in TSB, its attachment to mucins was generally higher than that of strain PAK-NP (7, 25). The growth of the two strains in M9 rather than TSB resulted in significantly increased adhesion to mucin; there was a two- to threefold increase in the number of organisms bound to the wells (Fig. 1). However, the addition of NaCl to M9 did not result in any significant change in adhesion from that seen with M9 alone (Fig. 1).

It has been shown that the expression of certain outer membrane proteins (OMPs) of *P. aeruginosa* with a variety of different functions may be favored by modifications of the concentrations of different ions, such as Fe^{2+}, PO_4^-, Mg^{2+}, Ca^{2+}, Sr^{2+}, or Mn^{2+}, or of carbon sources (12–14, 32). Since the respiratory tract is considered to be an iron-restricted environment (9), the influence of adding iron to M9 or depriving the TSB medium of iron was tested.

Comparative adhesion to respiratory mucins of the nonpiliated strains PAK-NP and 1244-NP grown in media varying by free iron concentration. The two strains were grown in four different media: M9 (iron [measured by atomic absorption spectrophotometry] = 10 μg/dl), M9 enriched with $FeSO_4$ (M9 + Fe) (iron = 500 μg/dl), TSB (iron = 74 μg/dl), and TSB deprived of iron by addition of a chelating agent, 2,2′-dipyridyl (Sigma, St. Louis, Mo.), at a final concentration of 0.5 mM (1) (TSB − Fe) (free iron = 0). To obtain enough growth in TSB depleted of iron, bacteria were grown for approximately 36 h, since growth was much slower in this medium. The addition of iron to the M9 medium resulted in a decrease in binding to respiratory mucins. Conversely, a three- to fourfold increase in binding to respiratory mucins was obtained by chelation of the iron in TSB (Fig. 2). For strain PAK-NP, the difference in binding between organisms grown in TSB and TSB − Fe was greater than that between organisms grown in M9 and M9 + Fe.

Comparative study of the mucin-binding properties of OMPs from the nonpiliated strain PAK-NP grown in TSB, M9, M9 + Fe, and TSB − Fe. OMPs were prepared as described (8), and those that bound to mucins were detected with [^{125}I]iodide-radiolabeled mucins as previously described (8). The polyacrylamide gel electrophoresis (PAGE) profiles of the OMPs from the nonpiliated strain PAK-NP grown in M9, M9 + Fe, TSB, and TSB − Fe were different (Fig. 3, lanes 1 through 4). Differences were also observed when the corresponding replicas were revealed with radiolabeled mucins. Several bands in the 42- to 48-kDa range were detected on the replicas of the OMPs from the strain grown in the different media, but when the strain was grown in iron-deprived TSB, a new mucin-binding band was visible at 77 kDa (Fig. 3, lane 8). A similar band, although fainter, was observed when the strain was grown in M9 (Fig. 3, lane 5).

Comparative study of the mucin-binding properties of OMPs from the nonpiliated strain 1244-NP grown in TSB, M9, M9 + Fe, and TSB − Fe. The PAGE profiles of the OMPs from the nonpiliated strain 1244-NP grown in M9, M9 + Fe, TSB, and TSB − Fe were also different (Fig. 4, lanes 1 through 4). Differences were also observed when the corresponding replicas were revealed with radiolabeled mucins. Several bands in the 42- to 48-kDa range were detected on the replicas of the OMPs from the strain grown in the different media, but when the strain was grown in M9 or in iron-deprived TSB, two additional bands binding to mucins were detected at 77 and 85 kDa (Fig. 4, lanes 5 and 8).

Thus, the adhesion of the two nonpiliated strains was strongly influenced by the amount of free iron in the medium. In parallel, it appeared that the mucin-binding bands in the 77- to 85-kDa range appeared under iron-restricted growth conditions in M9 or in TSB with the iron chelated but were not visible when the cells were grown in TSB medium or when iron was added to the M9 medium.

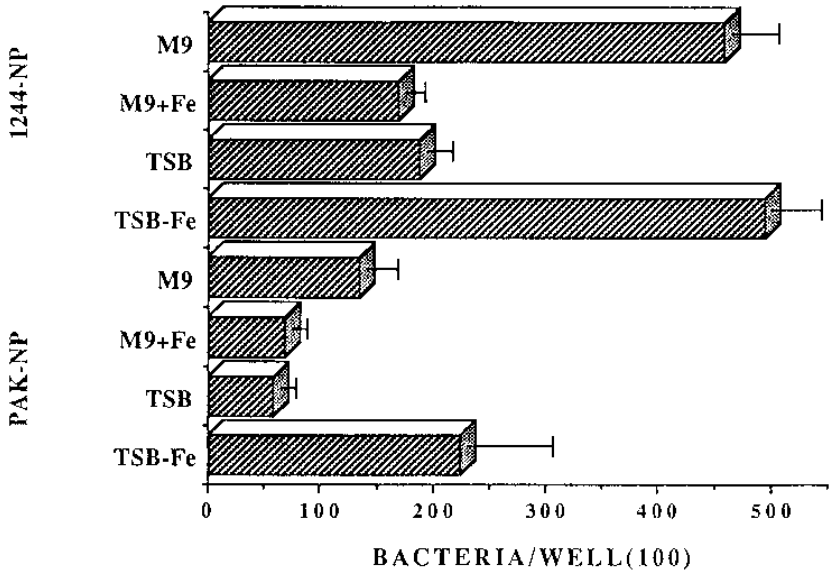

FIG. 2. Adhesion of two nonmucoid, nonpiliated strains of *P. aeruginosa*, 1244-NP and PAK-NP, grown in M9, M9 + Fe, TSB, and TSB − Fe. Inocula were standardized to 10^7 CFU/ml. The error bars represent standard deviations.

Therefore, in addition to what is already known about changes in OMP expression under the influence of different growth media, the present data suggest that the expression of mucin-binding adhesins by *P. aeruginosa* is also affected by the iron content of the growth medium. It has been shown that the adhesion of *Pasteurella multocida* to respiratory tract mucus is increased when this organism is grown under iron-restricted conditions (15), but data concerning the exact free iron concentration in mucus cannot be found in the literature.

The observations of Joris et al. (18), who found differences between the osmolarity of the periciliary fluid of inflamed and normal airways, coupled with reports of the effects of osmolarity on the expression of various *P. aeruginosa* virulence factors (5), suggested that the osmolar conditions of the respiratory tract could have an effect on the adhesion of *P. aeruginosa* to mucins. To mimic the conditions in the respiratory tract more closely, we used osmolar conditions that are close to those found in vivo rather than the nonphysiologic hyperosmolar conditions that are used in other studies (5). Under the conditions we used, osmolarity does not influence adhesion, but other conditions which are found in the lung, i.e., an iron-restricted state, cause significant increases in the adhesion of *P. aeruginosa* to mucins. Normally the lactotransferrin secreted by the mucosa should not allow the presence of free iron in the respiratory secretion. In inflamed states the level of lactotransferrin should increase further, since this protein is then secreted by the mucosa (3) and also by granulocytes (20).

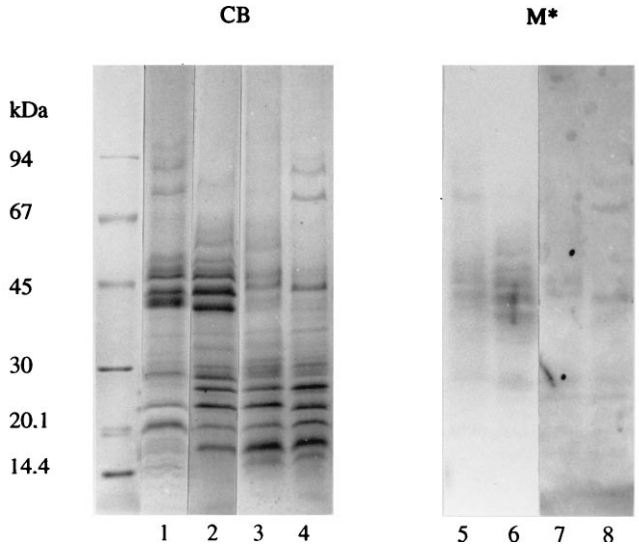

FIG. 3. OMPs from *P. aeruginosa* PAK-NP grown in M9 (lanes 1 and 5), M9 + Fe (lanes 2 and 6), TSB (lanes 3 and 7), and TSB − Fe (lanes 4 and 8) were separated by PAGE and either stained with Coomassie blue (CB) (lanes 1 through 4) or blotted and revealed with ^{125}I-labeled mucins (M*) (lanes 5 through 8). Each lane contained 40 μg of protein.

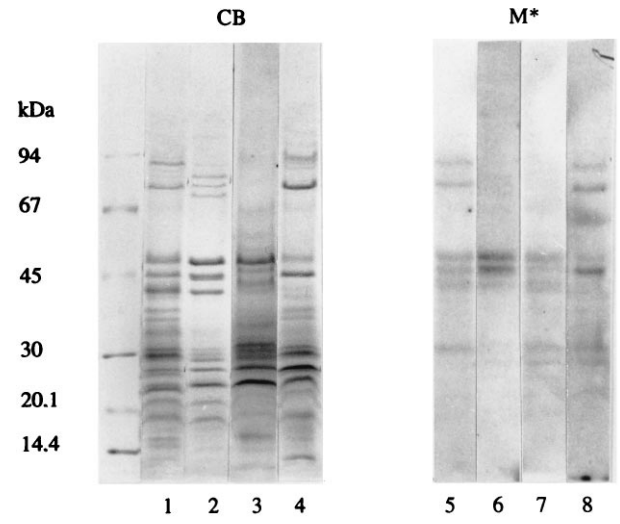

FIG. 4. OMPs from *P. aeruginosa* 1244-NP grown in M9 (lanes 1 and 5), M9 + Fe (lanes 2 and 6), TSB (lanes 3 and 7), and TSB − Fe (lanes 4 and 8) were separated by PAGE and either stained with Coomassie blue (CB) (lanes 1 through 4) or blotted and revealed with ^{125}I-labeled mucins (M*) (lanes 5 through 8). Each lane contained 40 μg of protein.

In CF patients, lung inflammation is usually early, sustained, and severe (6), possibly leading to an increased level of lactotransferrin in the airway (30). Additionally, during infection, transuded transferrin from plasma may also contribute to the trapping of free iron. Therefore, in pathological situations such as CF, the conditions in the bronchi should be similar to those in an iron-restricted medium.

It is not clear whether more of the same adhesins that are found under iron-sufficient conditions are induced or whether entirely new adhesins are also made. The identities of the proteins induced in the 77-kDa range are not known at that time, but they are in the size range of the pyochelin receptor (14), which would be induced under iron-restricted conditions. Whether these induced mucin-binding proteins are themselves involved in the adhesion of the whole bacteria is a question that will be answered after identification and mutagenesis of the genes that code for them. It should also be mentioned that mucin-binding proteins in the 42- to 48-kDa range are quite visible in the presence of iron. It is possible that some of these proteins are buried in the membrane of the living bacteria and may not play a role in the adhesion of the living bacteria to mucins.

The expression of bacterial genes encoding two siderophores, pyochelin and pyoverdin, as well as exotoxin A, a virulence determinant which may increase iron availability by damaging host cells, is coordinated and influenced by iron availability (22). Exotoxin A and pyoverdin are secreted in the sputum of CF patients infected by *P. aeruginosa* (11, 16), suggesting iron limitation. It is possible that increased binding of *P. aeruginosa* to the mucus gel represents another mechanism for facilitating iron extraction from iron-binding molecules entrapped in this gel. In contrast to the secretion of exotoxin A, the expression of pyochelin and pyoverdin genes is directly regulated by a *P. aeruginosa Fur* (ferric uptake regulator) repressor (21), and it will be interesting to find out whether mucin adhesion is also regulated by *fur* or other iron regulatory molecules.

In conclusion, the adhesion of *P. aeruginosa* to human respiratory mucins is more susceptible to modifications in iron concentration than to electrolyte modifications at the surface of the respiratory mucosa. In the future, it will be necessary to verify if the free iron concentration in the bronchi of infected patients with CF is low. This might be of special importance in the development or persistence of *P. aeruginosa* in the lungs of these patients.

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