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# Recognition of mucin components by *Pseudomonas aeruginosa*

Reuben Ramphal* and Shiwani K. Arora

*Department of Medicine, University of Florida, Gainesville, FL 32610, USA*

***Pseudomonas aeruginosa*** **remains one of the most important bacterial pathogens in lung diseases and especially in Cystic fibrosis. This unusual predilection is best explained by the existence of defects in host defense mechanisms as resulting from the genetic lesion and the presence of a specific colonization niche within the lungs. The niche has been identified as the mucus layer wherein mucin glycoproteins provide a substrate for binding and allows the persistence of this organism in this milieu by a number of possible mechanisms. While this organism is capable of binding to non CF mucins, it is perhaps a combination of factors e.g. increased binding and decreased mucociliary clearance that is responsible for this marked state of colonization in CF. The organism uses chiefly proteins of its flagellar apparatus to initiate this binding and recognizes a variety of oligosaccharides that have been identified in mucins. Among these are both, neutral oligosaccharides and several forms of acidic oligosaccharides derived from the Lewis antigens. There are more than likely a larger repertoire of receptors than those identified and certainly more adhesins present than those currently known. However, the information gathered to date provides an excellent example of the specificity of bacterial interactions with mucins that will certainly be expanded as we study more pulmonary pathogens.**

***Keywords:*** **bacterial adhesion, mucin receptors, *Pseudomonas aeruginosa*, oligosaccharide receptors, Lewis$^x$**

## Introduction

The opportunistic bacterium *Pseudomonas aeruginosa*, has a remarkable propensity to colonize the lungs of humans. Among gram negative bacteria, no other organism has such a predilection. This organism is the most common cause of ventilator associated pneumonia, the most frequent organism found in the CF lung and colonizes some patients with a number of chronic lung diseases. This ability is particularly manifested in hospitalized patients on ventilators and patients with the genetic disorder, cystic fibrosis (CF). There have been a number of explanations for these predispositions, including alterations in the surfaces of cells leading to colonization but these are not discussed in this review. Ventilator associated pneumonia and CF illustrate two means by which this organism may colonize the airways of humans but does not exclude others. In the case of patients on ventilators, injury to airway cells by the endotracheal tube is likely an inciting factor for adhesion to cells [1] followed by growth in mucus that has accumulated in the lungs or covering the endotracheal tube. In CF however, all the evidence points to colonization of the mucus layer as the main event. Colonization of mucus, a location that is normally a defensive site, now becomes a pathogenic event because of the disturbance in mucociliary clearance that occurs in this disease. Another factor that may also play a role is the observed differences in mucins between CF and non CF individuals (discussed in this issue of *Glycobiology*) which may increase mucin binding of this organism. Bacterial colonization at any site requires that specific host receptors be recognized by specific adhesins on the bacterium. In most instances, carbohydrates have been implicated in such interactions but non-specific hydrophobic interactions with lipids cannot be ruled out in a complex milieu such as mucus. In this review, we will examine the evidence that there are specific interactions between components of *P. aeruginosa* and the carbohydrate components of the mucin glycoproteins.

*To whom correspondence should be addressed: Reuben Ramphal, Department of Medicine, University of Florida, Gainesville, FL 32610, USA. Tel.: 352 392 2932; Fax: 352 392 6481; E-mail: ramphr@medmac.ufl.edu

## Mucin binding of *P. aeruginosa*

Early evidence that *P. aeruginosa* was able to recognize mucins came from simple microbiological experiments showing the trapping of this organism by mucus from patients with chronic bronchitis and cystic fibrosis [2]. Some time later direct binding of *P. aeruginosa* to mucus strands was demonstrated *in-vivo* in the mouse and rat trachea [3,4]. This was followed by the demonstration of this organism binding to human respiratory mucins [5], findings that were later corroborated by

studies of human CF materials obtained at post mortem and lung transplantation showing that mucus is the site of airway colonization in CF [6,7]. Initial studies to ascertain the nature of the interaction of *P. aeruginosa* with mucins suggested that sialic acids and N-acetyl glucosamine were involved in adhesion; these were fairly simple inhibition studies but adhesion to mucin was also shown to be neuraminidase sensitive [8] supporting a role for sialic acids. In contrast to these earlier studies with mucins, it should be noted that there is another body of literature that implicated asialo-GM1 on cells as a Pseudomonas receptor [9], implying no role for sialic acids. However the oligosaccharide moiety of asialoGM-1, GalNac beta1-4 Gal, has not been described in mucins. This line of evidence continues to be followed by proponents of the Pseudomonas cellular adhesion hypothesis in CF [10]. More recent findings, that *P. aeruginosa* also binds poorly to cells with the mutant CFTR protein are consistent with the idea that mucus rather than cells is the site of colonization in CF [11] since there has to be a site of colonization in the CF airway.

Attempts were made to delineate the mucin receptor(s) using glycopeptides from human respiratory mucins [12,13]. Neutral and acidic fractions glycopeptide fractions of mucus obtained by pronase digestion, followed by ion exchange chromatography and gel filtration were found to be the best receptors for *P. aeruginosa.* Neuraminidase treatment of some of these fractions also resulted in a reduction of binding [13]. Paradoxically, no binding was observed with the most acidic fractions which contained oligosaccharides that were heavily sulfated [12]. Additionally, binding to salivary mucins, a site not generally colonized in CF, therefore not potentially altered by bacteria, was greater in CF than non CF samples [13], suggesting there may be increased *P. aeruginosa* binding to mucins in this disease.

## Oligosaccharide receptors for *P. aeruginosa*

The availability of oligosaccharides from human milk, that were structurally similar to those found in human mucins allowed further work on this problem since it was difficult to obtain respiratory mucin oligosaccharides in quantity. A series of these milk oligosaccharides were conjugated to phosphatidylethanolamine dipalmitoate or hexadecylaniline providing hydrophobic tails, and were studied for binding of *P. aeruginosa*, by overlaying radiolabeled bacteria on the "neoglycolipids" run on thin layer chromatograms [14,15] and by direct bacterial binding to the neoglycolipids in microtitre plates. Bacterial binding occurred with the type 1 and 2 disaccharide units and their sialylated derivatives (Table 1). Thus these studies supported the idea that sialic acid and *n*-acetyl glucosamine were parts of the receptor(s).

Recently, Scharfman and colleagues have reexamined this question of receptors in mucins using another approach [16]. Neoglycoconjugates were synthesised by linking oligosaccharides to polyacrylamide then examining mixtures of bacteria and glycoproteins by fluorescence analysis. They have shown that sialyl-Lewis$^x$ was the best receptor for a certain strain of *P. aeruginosa* (Table 1). This finding was consistent with earlier

**Table 1.** Carbohydrates moieties implicated in the adhesion of *P. aeruginosa* to mucins

| *Carbohydrate determinant* | *Localization of the bacterial adhesin* | *Detection with* | *Reference* |
|---|---|---|---|
| Gal$\beta$1-3GlcNAc$\beta$1-3Gal$\beta$1-4Glc-<br>Gal$\beta$1-4GlcNAc$\beta$1-3Gal$\beta$1-4Glc- | Piliated bacteria | Neoglycolipid | [14] |
| Gal$\beta$1-3GlcNAc$\beta$1-3Gal$\beta$1-4Glc-<br>Gal$\beta$1-4GlcNAc$\beta$1-3Gal$\beta$1-4Glc- | Non-piliated bacteria | Neoglycolipid | [15] |
| NeuAc$\alpha$2-3Gal$\beta$1-3GlcNAc | Piliated bacteria | Neoglycolipid | [15] |
| Gal$\beta$1-4[Fuc$\alpha$1-3]GlcNAc-<br>(Lewis x) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |
| Flagellin and flagellar protein FliD from strain PAO1 | | | [25] |
| 3-sulfo-Gal$\beta$1-4[Fuc$\alpha$1-3]GlcNAc-<br>(3′sulfo-Lewis x) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |
| NeuAc$\alpha$2-3Gal$\beta$1-4[Fuc$\alpha$2-3]GlcNAc-<br>(3′sialo-Lewis x) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |
| Flagellar protein FliD from strain PAO1 | | | [25] |
| NeuAc$\alpha$2-3Gal$\beta$1-4[6-sulfo][Fuc$\alpha$1-3]<br>GlcNAc-<br>(3'sialo-6-sulfo-Lewis x) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |
| Gal$\beta$1-3[Fuc$\alpha$1-4]GlcNAc-<br>(Lewis a) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |
| Fuc$\alpha$1-2Gal$\beta$1-4[Fuc$\alpha$-3]GlcNAc-<br>(Lewis y) | Non-piliated bacteria | Fluorescent<br>Neoglycoconjugate | [16] |

**Table 2.** Putative mucin adhesins of *P. aeruginosa*

| *Adhesin* | *Reference* |
|---|---|
| Mucoid exopolysaccharide or alginate | [18] |
| Membrane associated proteins—48, 46, 25, 22 kD of strain PAK | [20] |
| Membrane associated proteins—48, 46, 28, 25, 22 kD of strain 1244 | [20] |
| FliD—flagellar cap protein of strain PAK | [22] |
| FliD—Flagellar cap protein and flagellin of strain PAO1 | [25] |
| 16 kD protein of strain 1244 | [24] |

observations on sialic acid. By this method, other oligosaccharide groups such as Lewis$^a$, Lewis$^x$ Lewis$^y$ and sulfo-Lewis$^x$ were also receptors of lesser affinity. (Structures of these groups can be found in this issue, Lamblin et al.) Of note, the sialyllactosamine oligosaccharide found by the neoglycolipid [15] assay was not defined by this method suggesting a caution that exact identities of receptors may be influenced by the methods used.

## Mucin adhesins of *P. aeruginosa*

Pili are the most ubiquitous bacterial adhesins described. Indeed pili were shown to mediate adhesion of *P. aeruginosa* to cells [17] and they were examined for a role in adhesion to mucin. Initial studies suggested that pili were involved but inhibition studies showed that pili could not fully inhibit binding of *P. aeruginosa* to mucin [18]. Alginate also seemed to have a weak effect by increasing the binding of organisms to mucin [18]. However, when nonpiliated mutants of *P. aeruginosa* became available, with the use of precise genetic manipulations, pili were not found to be essential for mucin binding. In fact, nonpiliated strains had slightly increased binding over the wild type strains [19]. This suggested that the major mechanism of mucin binding involved a nonpilus adhesin(s) in the strains being examined. In order to find this other adhesin, an approach of using an overlay assay was developed [20]. Outer membranes of *P. aeruginosa* were prepared and run out on SDS-PAGE, transferred to nitrocellulose then blotted with radiolabeled mucins. The blots showed 4 to 5 mucin binding bands, ranging from 48 to 22 kD in mass.

A classic genetic approach was then taken to identify this nonpilus adhesin(s) [21]. Transposon mutagenesis was done on strain PAK to obtain a bank of *P. aeruginosa* with random chromosomal insertions. This bank was then screened by passage over mucin to obtain mutants that were incapable of binding to mucins. Several mutants were obtained and in the course of genetic analysis of the sites of the transposon insertion it was discovered that all the mutants studied were nonmotile [21]. The transposon insertions were in flagellar genes and resulted in nonflagellate bacteria. This suggested that the adhesin in this strain was associated in some way with the flagellum of *P. aeruginosa.* Mutational analysis and complementation of genes involved in flagellar biogenesis led to the identification of *fliD*, as the gene encoding the mucin adhesin, the flagellar cap, that is located at the tip of the flagellum [22]. A mutation in the *fliD* gene resulted in the loss of adhesion as opposed to no effect with a mutation in the gene for flagellin in the strain studied. Furthermore, purified FliD inhibited adhesion (80%) at very low concentrations and other *P. aeruginosa* proteins did not [22]. Thus, in strain PAK, the mucin adhesin appears to be the flagellar cap protein. It is however unlikely that this is the sole adhesin for mucins since strain differences in adhesion are known to exist and these differences are unlikely to be explained by the structure of the flagellar cap protein. In fact there are only two different flagellar cap proteins which are structurally quite dissimilar without immunological cross reactivity [23] but measurements of adhesion of large numbers of *P. aeruginosa* strains over time shows considerable variations in the adhesion of these strains (R Ramphal—unpublished observations). Lastly, another protein of about 16 kD in mass from strain 1244 was found to bind mucins by Reddy, but its identity is unknown [24].

## Recognition of specific mucin receptors by defined mucin adhesins

The flagellar cap protein FliD was now known to be a specific adhesin but the carbohydrate moieties that it recognized were unknown. Using fluorescent neoglycoconjugates as previously done to implicate the sialyl-Lewis derivatives in Pseudomonas mucin binding [16], Scharfman and colleagues recently examined the role of the FliD protein in binding to these derivatives [25]. The FliD protein from one extensively studied strain (PAK) was not involved in binding to the specific receptors that were defined. However, the FliD protein from another extensively studied strain (PAO1) demonstrated a clear association with Lewis$^x$, sialyl-Lewis$^x$ and sulfosialyl-Lewis$^x$. The flagellin protein of this strain also recognized Lewis$^x$. This work demonstrates the complexity of elucidating these interactions. The oligosaccharides are a very small fraction of those that have been identified in mucins [26]. In order to elucidate the full receptor repertoire, a larger battery of neoglycoconjugates are required since the structure(s) recognized by the PAK type of FliD, the most common type of FliD protein of *P. aeruginosa* (80% of CF isolates) has not been elucidated.

## Conclusions

There remain many unanswered questions about mucin binding of *P. aeruginosa* both *in vitro* and *in vivo*. While at least two mucin binding proteins have been described and some receptors have been found, the findings are incomplete. Are there other adhesins on different *P. aeruginosa* strains. Are there adhesins expressed *in vivo* that are induced under the conditions

of the respiratory tract in mucus. Do the other substances found in mucus—lipids, proteins, lactoferrin, DNA, play a role in allowing persistence of *P. aeruginosa* in the respiratory tract. While it is tempting to examine mucin carbohydrates since they provide such a large repertoire of potential binding sites, these other substances also merit consideration. One may also ask what function does the adhesion to mucus and the mucus habitat serve ? Why does this organism prefer this habitat to cellular adhesion in CF ? Some insight may be obtained from a few studies. It has been demonstrated that mucins protect *P. aeruginosa* from opsonophagocytosis [27]. One can speculate that bacteria enmeshed in mucins exhibit a layer of host glycoproteins which does not allow recognition by leucocytes and the host fails to trigger a phagocytic response. Another recent explanation lies in the advantageous location allowing these organisms to switch to anaerobic metabolism and thus synthesize alginate as a protective coat [28]. Whatever the reasons, the specific recognition phenomena displayed by mucins for *P. aeruginosa* probably play a role in both host defense and disease.

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