Adhesion of *Stenotrophomonas maltophilia* to mouse tracheal mucus is mediated through flagella

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Adhesion of *Stenotrophomonas maltophilia*, an opportunistic pathogen, to different surfaces has been reported in the literature. However, its ability to adhere to mucus and the involvement of different bacterial appendages in this process has not been elucidated. In this study, bacterial adhesion to mouse tracheal mucus as well as the role of flagella in the adhesion process were investigated using clinical isolates of *S. maltophilia*. All the clinical isolates adhered to mouse tracheal mucus to varying degrees, showing isolate-to-isolate variation. Isolate Sm2 was selected to study the kinetics of bacterial adhesion to mouse tracheal mucus. The process of bacterial adhesion started after 30 min of incubation, and significant adhesion was detected after 1 h. Bacteria pre-treated with *S. maltophilia* anti-flagellin antibody were used to determine the role of flagellin in bacterial adhesion. The attachment of *S. maltophilia* flagellin preparation to mucus was assessed by enzyme immunoassay. Pre-treatment of the bacteria with anti-flagellin antibody resulted in a significant decrease in adhesion to mucus and this decrease was antibody concentration dependent. A similar observation was made when pure flagellin was allowed to interact with mucus. Pre-treatment of mouse tracheal mucus with flagellin led to a significant decrease in bacterial adhesion at concentrations of 40 and 80 $\mu\text{g ml}^{-1}$ ($P<0.05$). The ability of *S. maltophilia* to adhere to mucus was also reduced when mechanically deflagellated bacteria were checked for this property ($P<0.005$). It was concluded that *S. maltophilia* has the ability to adhere to mouse tracheal mucus and that flagella play an important role in this process. However, further studies using genetically defined mutants lacking flagella are needed to support this observation.

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INTRODUCTION

Bacterial adhesion is an essential step in the pathogenesis of infections of mucosal and prosthetic surfaces (Di Bonaventura *et al.*, 2004). However, the molecular and physical interactions that govern bacterial adhesion to these surfaces require an understanding of both specific and non-specific interactions (Katsikogianni & Missirlis, 2004; Oztuna *et al.*, 2006). *Stenotrophomonas maltophilia* strains of both clinical and environmental origin have been reported to adhere to and form biofilm on abiotic surfaces such as glass, Teflon, polystyrene and stainless steel (Jucker *et al.*, 1996; Baker & Leff, 2005). However, information regarding their ability to adhere to mucus is lacking in the literature. Adhesion of bacteria to mucus has been considered an important step in the pathogenesis of respiratory tract infections (Sajjan *et al.*, 1992). As *S. maltophilia* is an organism frequently associated with infections of the respiratory tract, it is imperative to study its ability to adhere to mucus.

Mucus that coats the surfaces of the gastrointestinal, respiratory and reproductive tracts provides an outermost

barrier against pathogens. It contains glycoproteins that are composed of a peptide backbone linked to carbohydrates, which act as a receptor for bacterial adhesins. This interaction promotes adherence, as has been shown for *Pseudomonas aeruginosa* (Arora *et al.*, 1998).

In the present study, an attempt was made to study the ability of five clinical isolates of *S. maltophilia* to adhere to mouse tracheal mucus and to understand the underlying mechanism of adhesion.

METHODS

Clinical isolates. Five clinical isolates of *S. maltophilia* (Sm1, Sm2, Sm3, Sm6, Sm7) were used in this study. These isolates were obtained from the Department of Medical Microbiology, Post Graduate Institute of Medical Education and Research, Chandigarh, India. The bacteria were preserved by lyophilization, routinely cultured at 37 °C on Luria–Bertani agar plates and subcultured every week.

Flagellin and anti-flagellin preparation. Flagellin was isolated from *S. maltophilia* isolate Sm2 as described previously (Chhibber & Zgairt, 2009). Briefly, *S. maltophilia* Sm2 was grown overnight and pelleted by centrifugation. The pellet was suspended in 0.01 M potassium phosphate buffer (pH 7.0) and sheared for 1 min in a commercial blender. The sheared suspension was centrifuged for 30 min at 5000 g and then for

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15 min at 16 000 g (4 °C). The supernatant was centrifuged at 100 000 g for 3 h (4 °C). The pellet was collected and kept at -80 °C. Anti-flagellin antiserum was prepared by immunizing a rabbit with pure flagellin. Complement was inactivated by incubating the sera for 30 min at 56 °C.

Transmission electron microscopy. The presence of flagella was detected by transmission electron microscopy. Flagella were negatively stained for 2 min with 1% phosphotungstic acid (pH 7.4) on carbon/Formvar copper grids (Electron Microscopy Sciences) as described previously (Girón, 1995).

Animals. BALB/c mice, 6–8 weeks old, weighing 20–25 g were obtained from the central animal house of Panjab University, Chandigarh, India. The animals were kept in clean polypropylene cages and fed on a standard antibiotic-free diet (JBD Agencies). The study was conducted following approval from the animal ethics committee of Panjab University, Chandigarh, India.

Crude mucus preparations. Twenty microlitres of sterile PBS (0.2 M, pH 7.2) was passed repeatedly through the tracheal lumen of the mice to wash out tracheal mucus. The slightly turbid mucus suspension was pooled, centrifuged at 10 000 g for 10 min and filtered through a 0.2 μ m Millipore filter to remove cells and debris.

Assay of bacterial adherence to tracheal mucus. The bacterial isolates were grown in tryptic soy broth (HiMedia Laboratories) overnight at 37 °C and the bacterial count was adjusted to 10^7 c.f.u. ml^{-1} . Microtitre plates (Nunc) were coated with crude mucus at a concentration of 100 μ g ml^{-1} (Vishwanath & Ramphal, 1984). Bacteria were added to each well and the plates were incubated at 37 °C for 1 h to check the ability of the different clinical isolates to adhere to mucus. To study the kinetics of adhesion, samples were withdrawn at different time intervals (0.5, 1, 2, 4, 8 and 24 h) after washing the wells five times with 0.2 M PBS (pH 7.2). The adherent bacteria were removed with 0.05% Triton X-100 and enumerated by plating different dilutions on Luria–Bertani plates. All experiments were conducted in triplicate.

Adhesion inhibition assay. The bacterial isolate showing maximum adherence (Sm2) was pre-treated for 30 min with different dilutions of anti-flagellin antibody (1:40, 1:80, 1:160, 1:320 and 1:640) and tested for adherence to mouse tracheal mucus as described above. In another experiment, wells coated with mouse tracheal mucus were pre-treated first with pure flagellin using different concentrations (10, 20, 40 and 80 μ g ml^{-1}) for 30 min to block the receptors and the adherence of isolate Sm2 was then evaluated by following the protocol described above.

Adhesion of flagella-sheared bacteria. After overnight growth, the bacteria were centrifuged, washed and deflagellated using a commercial blender for 1 min. The ability of the sheared bacteria to adhere to mouse tracheal mucus was then determined. The results were compared with those obtained using non-sheared bacteria as a control.

Role of *S. maltophilia* flagellin in attachment to mouse tracheal mucus as evaluated by enzyme immunoassay. The method of Cogan *et al.* (2004) was followed with modifications. Briefly, four rows of a microtitre plate were used. Rows 1–3 were coated with mouse tracheal mucus as described above, whereas row 4 was left uncoated. Each row was washed four times with 0.2 M PBS (pH 7.2) containing 0.05% Tween 20. *S. maltophilia* flagellin (100 μ l of 20 μ g ml^{-1}) was added to each well of rows 1 and 2 and incubated for 1 h at 37 °C. No flagellin was added to rows 3 and 4. After washing with PBS, the wells were blocked with 200 μ l PBS containing 3% dried milk and incubated at 37 °C for 1 h. Rabbit *S. maltophilia* anti-flagellin antibody (100 μ l of 1:320 dilution) suspended in PBS containing 0.1% dried milk was added to each well of rows 1, 3 and 4,

whilst normal rabbit serum was added to the wells of row 2 (control) and the plate was incubated at 37 °C for another 1 h. Goat anti-rabbit horseradish peroxidase-conjugated antibody (1:1000; Sigma) was added to the wells and incubated for 1 h, followed by the addition of tetramethylbenzidine substrate (Sigma). The reaction was stopped with 2 M sulphuric acid (Fluka) and the A_{450} was read. Thus, in this experiment, row 1 was the test row, row 2 was control 1 (CN1: mucus + flagellin + normal rabbit serum), row 3 was control 2 (CN2: mucus + anti-flagellin antibody only) and row 4 was control 3 (CN3: anti-flagellin antibody only).

In another experiment, flagellin pre-treated with different dilutions of anti-flagellin antibody was applied to mucus-coated wells instead of flagellin. The control well consisted of mucus, flagellin and anti-flagellin antibody.

Statistical analysis. All values were calculated as the means $\pm$ SEM. Differences between tests and controls were analysed using Student's *t*-test and the correlation coefficient using Origin version 8.0 software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Adhesion of clinical isolates to mouse tracheal mucus

The results presented in Fig. 1 show that all five clinical isolates tested in this study were able to adhere to mouse tracheal mucus. Maximum adherence was observed with *S. maltophilia* isolate Sm2 followed by Sm3, and was least with Sm1.

Confirmation of the presence of flagella

Transmission electron microscopic examination of the bacteria confirmed the expression of flagella. Some of the bacteria expressed only one polar flagellum, whilst others expressed more than one (Fig. 2a). Pure flagellar preparation contained numerous such flagella without any bacterial contamination (Fig. 2b).

Fig. 1. Ability of different clinical isolates of *S. maltophilia* to adhere to mouse tracheal mucus. Maximum adherence was observed with isolate Sm2 and minimum with Sm1.

Fig. 2. Transmission electron micrographs showing the polar arrangement of flagella on *S. maltophilia* cells (a) and the purity of the flagellar preparation (b). The cells and polar flagellar preparation were negatively stained. Bars, 0.5 μm (a) and 0.25 μm (b).

Kinetics of *S. maltophilia* adhesion to mouse tracheal mucus

The Sm2 isolate of *S. maltophilia* was used to study the kinetics of adhesion, as maximum adhesion to mouse tracheal mucus was seen with this isolate. The results presented in Fig. 3 showed that bacterial adhesion started after 30 min of incubation, but significant adhesion to mouse tracheal mucus was detected after 1 h ($P < 0.05$). The increase in bacterial numbers continued thereafter and the maximum count was obtained at 24 h.

Effect of *S. maltophilia* flagellin on bacterial adhesion to mouse tracheal mucus

To study the involvement of *S. maltophilia* flagellin as a bacterial adhesin to mouse tracheal mucus, *S. maltophilia* Sm2 was pre-treated with anti-flagellin antibody for

30 min before the binding assay. As shown in Fig. 4, pre-treatment of the bacteria with antibody resulted in decreased adherence of *S. maltophilia* to mouse tracheal mucus. A significant reduction in adhesion was seen compared with the control at all concentrations tested ($P < 0.05$). A negative relationship was detected between anti-flagellin antibody concentration and bacterial adhesion to mouse tracheal mucus, measured in c.f.u. ml^{-1} (correlation coefficient -0.97).

A decrease in adhesion of *S. maltophilia* Sm2 to mouse tracheal mucus was observed after pre-treatment of the mucus for 30 min with pure flagellin compared with untreated mucus ($P < 0.05$) (Fig. 5). This effect was

Fig. 3. Kinetics of adhesion of *S. maltophilia* isolate Sm2 to mouse tracheal mucus. Significant adhesion of bacteria to the mucus surface was detected after 1 h of incubation. Asterisks indicate a significant difference compared with adhesion at 0 h ($P < 0.05$).

Fig. 4. Number of *S. maltophilia* ($\log_{10}$ c.f.u. ml^{-1}) adhering to mouse tracheal mucus following interaction of pre-treatment of the bacteria with different concentrations of anti-flagellin antibody. Interaction with the antibody significantly reduced the bacterial adherence to mouse tracheal mucus ($P < 0.05$). A negative relationship was detected between the anti-flagellin antibody concentration and bacterial adherence (correlation coefficient -0.97). Asterisks indicate a significant difference compared with the control (no antibody) ($P < 0.05$).

Fig. 5. Number of *S. maltophilia* ($\log_{10}$ c.f.u. ml⁻¹) adhering to mouse tracheal mucus following pre-treatment of the mucus with different concentrations of pure flagellin. Pre-treatment of mouse tracheal mucus with flagellin significantly reduced the bacterial adherence at concentrations of 40 and 80 $\mu\text{g ml}^{-1}$ ($P < 0.05$). A negative relationship was detected between flagellin concentration and bacterial adherence (correlation coefficient -0.85). Asterisks indicate a significant difference compared with the control (no flagellin) ($P < 0.05$).

significant at higher concentrations of flagellin (40 and 80 $\mu\text{g ml}^{-1}$) but not at lower concentrations (10 and 20 $\mu\text{g ml}^{-1}$). A negative relationship was found between flagellin concentration and *S. maltophilia* adhesion to mouse tracheal mucus measured in c.f.u. ml⁻¹ (correlation coefficient -0.85). On the basis of these results, we concluded that *S. maltophilia* flagellin is an adhesin for mouse tracheal mucus and that receptors for *S. maltophilia* flagellin are present on mouse tracheal mucus.

Adhesion of flagella-sheared bacteria

The role of flagella in the adhesion of *S. maltophilia* to mouse tracheal mucus was further checked by removing the bacterial flagella in a commercial blender. The results showed a significant decrease in bacterial adhesion of sheared bacteria compared with the adhesion seen with non-sheared bacteria ($P < 0.005$). The $\log_{10}$ value of the mechanically deflagellated bacteria was 4.28 ± 0.27 compared with 5.07 ± 0.14 c.f.u. ml⁻¹ obtained with flagellated bacteria (control) on interaction with mucus.

Attachment of *S. maltophilia* flagellin to mouse tracheal mucus

An enzyme immunoassay was carried out to confirm the ability of *S. maltophilia* flagellin to bind to mucus. The results showed that flagellin was able to adhere to mucus directly (Fig. 6a). However, no adherence was observed in the controls that lacked either flagellin or anti-flagellin or both. The results confirmed that receptors for flagellin are present on mouse tracheal mucus. The results in Fig. 6(b) showed that attachment of *S. maltophilia* flagellin declined following pre-treatment with different dilutions of anti-flagellin antibody. These results confirmed the presence of adhesins on *S. maltophilia* flagellin that were blocked following treatment with the antibody.

DISCUSSION

The results of this study clearly depict the ability of clinical isolates of *S. maltophilia* to adhere to mucus present on mouse respiratory epithelium. Mucus secreted by specialized epithelial cells in the respiratory tract forms a protective covering, reducing the ability of bacteria to adhere to epithelial cells and thus checking the initiation of

Fig. 6. (a) Ability of flagellin to bind to tracheal mucus as detected by enzyme immunoassay. CN1, Control well coated with mucus, flagellin and normal rabbit serum; CN2, control well coated with mucus and anti-flagellin antibody; CN3, control well with anti-flagellin antibody only. (b) Ability of anti-flagellin antibody to block flagellin attachment to mouse tracheal mucus. The inhibition was antibody concentration dependent. CP, Control well coated with mucus, flagellin and anti-flagellin antibody.

infection (Thorton *et al.*, 2008). The bacteria are eventually cleared through mucociliary clearance (Bals *et al.*, 1999). This may explain the limited pathogenicity of *S. maltophilia* in a healthy host, despite its ability to adhere to airway epithelial cells, which is equivalent to that of *P. aeruginosa* (Wu *et al.*, 2005). As a consequence, *S. maltophilia* is able to cause disease in those individuals who are severely debilitated or immunosuppressed (Graff & Burns, 2002; Miyairi *et al.*, 2005). Exactly what causes these bacteria to establish infection in an immunocompromised host needs to be investigated. It is likely that in this situation the aggregated bacteria may form biofilms by binding to mucus. Yang *et al.* (2008), working with *P. aeruginosa*, demonstrated that biofilms may not necessarily be attached to a surface within the host but may be attached to mucus or suspended in fluid. In another study, evidence was found for *P. aeruginosa* forming aggregates in a matrix in oxygen-depleted mucus from cystic fibrosis patients (Worlitzsch *et al.*, 2002). As biofilm infections are an example of the interplay between the pathogen and the host's genetic makeup, it is likely that *S. maltophilia* in biofilm mode may act as a pathogen when the host's immune status is lowered. This may be responsible for the change of status from colonization to infection. Although it is interesting to speculate that such a mechanism may be responsible for infection of a compromised host, the exact role of such bacteria (in biofilm mode) needs to be investigated further in an animal model.

The motility of an organism is considered an important mechanism in the pathogenesis of pneumonia (Feldman *et al.*, 1998). However, loss of motility due to decreased expression of flagella has been shown to help *P. aeruginosa* and *S. maltophilia* to establish in cystic fibrosis airways (Feldman *et al.*, 1998; Matsumoto *et al.*, 1999). As binding to mucus in this study was found to be through the flagella, binding may hinder the movement of the organisms in the respiratory tract and thus indirectly influence the establishment of these organisms. It is likely that biofilm aggregates attached to mucus by flagella may also protect the organism from the host's immune response. In an earlier study, a similar suggestion was made (Hall-Stoodley & Stoodley, 2009). It has been postulated that bacteria in biofilm mode form a protective mechanism that imparts a beneficial effect to the organism.

In the present study, an attempt was made to delineate the role of flagellin as an adhesin for mouse tracheal mucus. The interaction of flagellin or anti-flagellin antibody with mouse tracheal mucus or bacteria in different experiments suggested the direct involvement of flagella in binding to mucus. This observation was further corroborated using bacteria that had been mechanically deflagellated in a commercial blender. The extent of adhesion of deflagellated bacteria was significantly less than that of their flagellated counterpart. The interaction of flagellin with anti-flagellin antibody before applying to mucus further confirmed its role as an adhesin for mouse tracheal mucus. However, we suggest that the use of genetically defined

non-flagellated mutants in future studies will help further in elucidating the exact role of flagella in this process.

In conclusion, our results suggest that the attachment of *S. maltophilia* to mucus may result in either colonization or infection depending on the immune status of the host. Further studies employing animal models will help in understanding the exact mechanism of virulence of this organism, especially in an immunocompromised host.

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