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EXPERIMENTAL ARTICLES

Stenotrophomonas maltophilia Flagellin Is Involved in Bacterial Adhesion and Biofilm Formation[1]

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Abstract—*Stenotrophomonas maltophilia* is an emerging drug-resistant pathogen and an important opportunistic pathogen. *S. maltophilia* flagellin was purified using serial ultracentrifugation. The purity of flagellin was checked by SDS-PAGE. The antibodies were raised in rabbits. The presence of anti-flagellin and the titer of flagellin were detected by immunoblotting and bacterial agglutination techniques. Two methods (viable bacterial count and spectrophotometric methods) were applied to evaluate bacterial adhesion and biofilm formation. Pretreatment of *S. maltophilia* with dilutions of anti-flagellin (from 1/40 to 1/640) reduced the ability of *S. maltophilia* to adhere and form biofilms on polystyrene ($P < 0.05$). In the present study, the inhibition of bacterial adhesion to polystyrene was dose-dependent. The positive correlation was observed between the antibody dilutions and bacterial adhesion (CFU/mL) ($r > +0.5$, $P < 0.05$), while, the negative correlation ($r < -0.5$, $P < 0.05$) was observed between the percentage of adhesion inhibition and anti-flagellin dilutions. The current study proved the direct role of *S. maltophilia* flagellin in bacterial adhesion to and biofilm formation on polystyrene.

Keywords: bacterial adhesion inhibition, correlation coefficient, flagellin, biofilm formation

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Stenotrophomonas maltophilia is being reported with increasing frequency as an important nosocomial pathogen. It is an opportunistic pathogen colonizing patients in intensive care settings, especially those with underlying debilitating conditions such as immunosuppression, malignancies, and implantation of medical devices [1–5]. Bacterial adherence is the first step in the pathogenesis of infections of prostheses or mucosal surfaces. *S. maltophilia* strains of both clinical and environmental origin have been reported to adhere and form biofilms on abiotic surfaces such as glass, Teflon, polystyrene, and stainless steel [4, 6, 7], as well as on biotic surfaces [8]. The biofilm is a community of bacteria immobilized and embedded in an organic polymer matrix composed of polysaccharides and proteins of bacterial origin [9]. Adhesion to abiotic surfaces is mediated by various appendages. In case of *S. maltophilia*, it has been shown that binding to abiotic surfaces is mediated by flagellalike structures [6]. Flagella are composed of several thousand copies of flagellin subunits, the major component being flagellin C (FliC) [7]. To study this binding, either plate count or spectrophotometry was recommended [10]. Flagella have been involved in adherence to mucus and cells and colonization by *P. aeruginosa* [11, 12], *Vibrio cholera* [13], *Vibrio anguillarum* [14], *Helicobacter pylori* [15], and *Burkholderia pseudomallei* [16]. Furthermore, flagella contribute to the invasiveness of *Campylobacter jejuni* [17], *Salmonella typhi* [18] and *Proteus mirabilis* [19]. All these evidences confirm that motility plays an important role directly or indirectly in bacterial adhesion to biotic surfaces [20].

In the present study, the purified flagellin subunit and the ability of this protein to control bacterial adhesion to polystyrene microtiter plate were evaluated.

MATERIALS AND METHODS

Clinical isolate. A clinical isolate of *S. maltophilia* (Sm2) was used in this study. This isolate was procured from the Department of Medical Microbiology, Post-Graduate Institute of Medical Education and Research, Chandigarh, India. Bacteria were preserved by lyophilization and maintained at 37°C on Luria Bertani (LB) agar plates (Himedia, Mumbai, India) subcultures were made weekly.

Flagellin and anti-flagellin preparation. *S. maltophilia* (Sm2) flagellin was prepared according to the procedure described earlier [8]. Briefly, *S. maltophilia* (Sm2) was grown in LB broth overnight and collected by centrifugation. The pellet was suspended in potassium phosphate buffer (0.01 M, pH 7.0) and treated for 1 min in a commercial blender. The homogenized suspension was centrifuged for 30 min at 5000 *g* and

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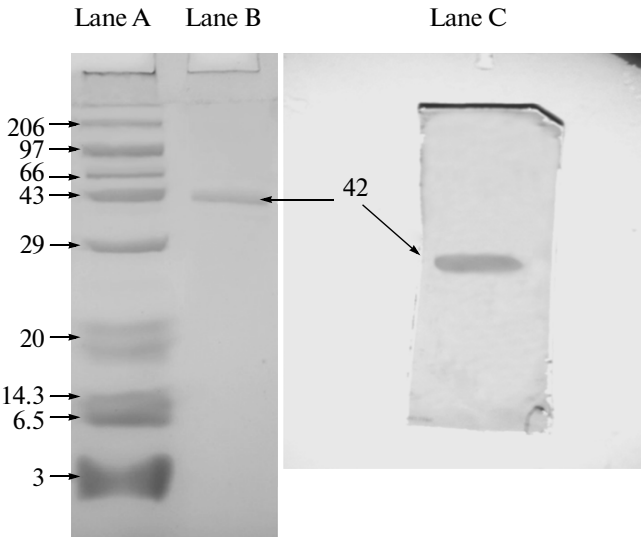

Fig. 1. SDS-PAGE and Immunoblot analysis of *S. maltophilia* (Sm2) purified flagellin. The isolated protein was denatured in the sample buffer, electrophoresed on 12% separating polyacrylamide gel, and stained with Coomassie blue. Lane A, protein marker (206, 97, 66, 43, 29, 20.1, 14.3, 3 kDa). Lane B, pure flagellin protein of *S. maltophilia* (Sm2) 42 kDa. Lane C, immunoblot of flagellin.

then for 15 min at 16000 *g*. The supernatant was centrifuged at 100000 *g* for 3 h. The flagellar filament was converted from polymeric protein to monomeric protein by heating the suspension. The flagellin preparation was stored at −80°C. The anti-flagellin antiserum was prepared by immunizing rabbit with flagellin. The complement was inactivated by incubating the sera at 56°C for 30 min and the presence of anti-flagellin was detected by immunoblotting. Moreover, the titer of anti-flagellin was assayed by using the bacterial agglutination technique.

Western blotting. For western blot, flagellin was denatured, separated in 12% polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Corp, Bedford MA) [21]. The blot was reacted with anti-flagellar antibodies and then the secondary anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Sigma) was added. The reaction was developed with a mixture of diaminobenzidine and 30% hydrogen peroxide (Sigma).

Determination of anti-flagellin by bacterial agglutination. Bacteria were grown for 18 h at 37°C in TSB (Himedia, Mumbai, India). The cells were harvested from the plates and washed three times with PBS (0.01 M, pH 7.2). The cells were stained with crystal violet and washed three timed with PBS (0.1 M, pH 7.2). Bacterial cells suspension was prepared in 0.1% PBS. Serial dilutions (50 μL) of the rabbit serum were prepared in round-bottom microtiter plates with PBS (1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280). The bacterial suspension (50 μL) was added to each well. The plates were incubated overnight at 37°C; the negative control contained PBS instead of the sera was used [22].

Bacterial adhesion and biofilm formation. Overnight cultures of *S. maltophilia* in 3 mL of tryptose soy broth (TSB) (Himedia, Mumbai, India) were washed three times with fresh TSB, and bacterial count was adjusted to 1×10^7 CFU/mL. Bacterial aliquots (200 μL) of the standardized inoculum (10^7 CFU/mL) were added to the wells of sterile flat-bottom polystyrene tissue culture plates (96 wells) and incubated at 37°C either for 4 or 24 h to check the adhesion of *S. maltophilia*. The medium was then discarded, and non-adherent cells were removed by washing three times with sterile PBS (0.1 M, pH 7.2). Quantitation of adhered bacteria was performed both by viable cell enumeration as well as by spectrophotometry as previously described [10], with minor modifications. For plate counts, adherent bacteria (biofilms) were removed from the microtiter wells by scraping, vortexed vigorously, and the counts were determined by plating serial dilutions of the suspension. For the other method, briefly, slime and adhered organisms were fixed by heating at 60°C for 30 min and then stained with Hucker crystal violet (0.4%) for 5 min. After thorough washing with water to remove excess stain, the plates were dried for 30 min at 37°C. The extent of biofilm growth was determined by measuring the absorbance of the stained adherent film upon treatment with acetone : ethanol (30 : 70) at a wavelength of 490 nm.

Adhesion inhibition assay. The bacterial strain (Sm2) was pretreated with different dilutions of anti-flagellin antibodies (1/40, 1/80, 1/160, 1/320 and 1/640) and tested for adherence to and biofilm formation on polystyrene mictotiter plates. The methods used to investigate adhesion and biofilm formation of *S. maltophilia* to the polystyrene microtiter plate (plate count and spectrophotometry) were used in this section to assay the reduction in bacterial adhesion and percentage of adhesion inhibition to polystyrene.

Statistical analysis. All values are presented as averages and standard error (number ±SE). The correlation coefficient test [Pearson correlation (r)] was used to check the relationship between two groups. The differences were analyzed using Student's t test with the Origin version 8.0 software. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

***S. maltophilia* flagellin purification.** Flagella were separated from the Sm2 isolate by differential centrifugation as described above. The flagellin purity was checked by SDS-PAGE following staining with Coomassie blue. Figure 1 shows the purity of the protein preparation (Lane B). The calculated molecular weight of this protein was 42 kDa.

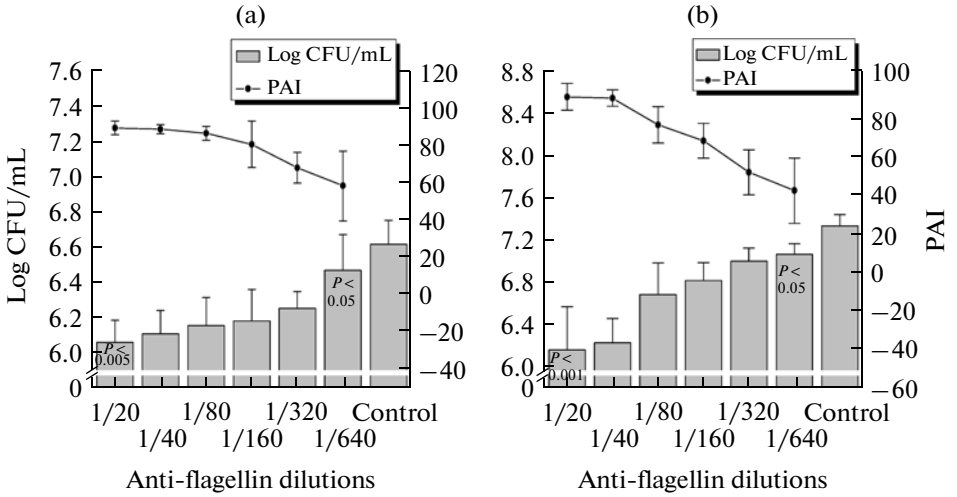

Fig. 2. Adhesion of *S. maltophilia* to polystyrene microtiter plate upon pretreatment with anti-flagellin. (a) Number of adherent bacteria (Log CFU/mL) and PAI at 4 hours, (b) Log CFU/mL and the percentage of adhesion inhibition (PAI) at 24 hours. The bars represent the bacterial adhesion (Log CFU/mL) and line represents the PAI. Each data point represents an average ±SE of 4 independent experiments.

Anti-flagellin preparation in rabbits. The antiserum was prepared by immunizing rabbits with the pure flagellin preparation. The presence and titer of rabbit antibodies to *S. maltophilia* flagellin was determined by the Western blot technique. The lane C in Fig. 1 shows the single band on PVDF. These results confirmed the presence of anti-flagellin in the serum of immunized rabbit and the purity of the flagellin preparation.

Bacterial agglutination. Serial dilutions of the antiserum were incubated with *S. maltophilia* cells, which were stained previously with crystal violet in a round-bottom microtiter plate. The antiserum agglutinated bacterial cells up to the 1/640 dilution, confirming the presence and titer of specific antibodies against *S. maltophilia* flagella.

Inhibition of bacterial adherence with the antiserum. Inhibition of bacterial adherence to polystyrene was studied by employing anti-flagellin antibodies. The results showed a significant decrease ($P < 0.05$) in bacterial adherence in the presence of different dilutions of anti-flagellin (Fig. 2). The decrease in adhesion was dependent on the antibody amount; a positive relationship was observed between the dilutions of anti-flagellin and bacterial adhesion (r: +0.95, $P < 0.001$ at 4 h; r: +0.807, $p < 0.001$ at 24 h). However, a negative relationship was detected between dilutions of anti-flagellin and the percentage of adhesion inhibition (PAI) in all cases (r: –0.979, $P < 0.001$ at 4 h; r: –0.9318, $P < 0.001$ at 24 h). Similar results were obtained when the inhibition of biofilm formation on polystyrene microtiter plates was studied in the presence of different dilutions of anti-flagellin (Fig. 3). A significant decrease in biofilm formation was detected in the presence of anti-flagellin. Positive relationship between anti-flagellin dilutions and adherent bacteria was observed (r: +0.99, $P < 0.001$). Negative relationship was detected between dilutions of anti-flagellin and PAI (r: –0.99, $P < 0.001$).

DISCUSSION

The treatment of nosocomial infections by *S. maltophilia* is presently difficult, since this pathogen shows high levels of intrinsic or acquired resistance to different antimicrobial agents [23]. Moreover, its ability to

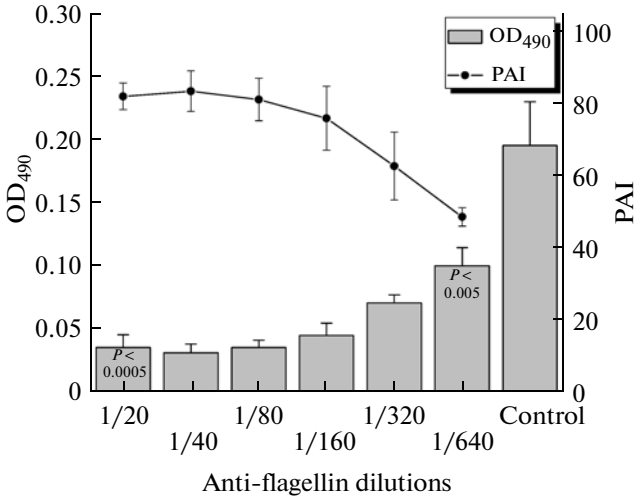

Fig. 3. Biofilm formation of *S. maltophilia* on polystyrene microtiter plate upon pretreatment with anti-flagellin. The bars represent the optical density of crystal violet taken by adherent bacteria on polystyrene and line represents the percentage of adhesion inhibition (PAI). Each data point represents an average ±SE of 4 independent experiments.

adhere to medical devises makes this challenge really serious [24–27]. The major risk factor for *S. maltophilia* infection in hospitalized patients is the implantation of medical devices such as central venous catheters [24], urinary tract catheters [25], prosthetic heart valves [26], and intraocular and contact [27] lenses. Microtiter plate systems for quantifying adherence and biofilm formation have been investigated with many different organisms and stains [28]. Since this technique is simple and inexpensive, it has been used to study the ability of *S. maltophilia* to adhere and form biofilms on abiotic surfaces.

The flagellin preparation in the present study was pure, as was confirmed by a single bind observed on SDS-PAGE. Moreover, the immunoblot test proved the rabbit antiserum was composed of anti-flagellin of *S. maltophilia*. The principle of adhesion inhibition assay is dependent on the ability of anti-flagellin to bind with flagellin located on the bacterial flagella. Thereby, the anti-flagellin blocked the flagellin and prevented attachment of the flagella to polystyrene, meaning that the role of flagella in bacterial adhesion was ruled out. However, other factors like pilin and outer membrane proteins were free (uncoated with antibodies). Analysis of adherence of anti-flagellin-treated bacteria to polystyrene, we found their ability to adhere and form biofilms was significantly reduced as compared with the control (bacteria treated with PBS only). This finding indicates that flagellin plays a central rule in *S. maltophilia* adhesion to polystyrene. However, the decrease in bacterial adhesion to polystyrene was not hundred per cent. Thus the other factors that may play a role in bacterial adhesion to abiotic surfaces should not be ruled out, such as electrical charge, pilin, and outer membrane.

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