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EXPERIMENTAL ARTICLES

Immunological and Biological Relationship among Flagellin of *Pseudomonas aeruginosa, Burkholderia cepacia*, and *Stenotrophomonas maltophilia*[1]

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Abstract—The flagellar protein (flagellin) was isolated and purified from strains of *Pseudomonas aeruginosa, Burkholderia cepacia* and *Stenotrophomonas maltophilia*. A significant difference was observed in the molecular weight of different flagellin preparations obtained from these bacterial isolates. Antiserum prepared against *S. maltophilia* flagellin did not react with flagellin of *P. aeruginosa* or/and *B. cepacia* on Immunoblot or in indirect ELISA. In addition the anti-flagellin did not agglutinate *P. aeruginosa* and *B. cepacia*. No inhibition of motility of *P. aeruginosa* and *B. cepacia* was observed in presence of antiserum; though the latter inhibited the motility of *S. maltophilia*. The results of the present study prove that no specific relationship existed among all the studied flagellar proteins obtained from closely related bacteria.

Keywords: Stenotrophomonas maltophilia, flagellin, antigenicity, specific immune response.

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Stenotrophomonas maltophilia is an aerobic gram negative rod which was earlier classified as *Pseudomonas maltophilia*, renamed as *Xanthomonas maltophilia* after further taxonomic analysis in 1981, and finally reclassified as *Stenotrophomonas maltophilia* in 1993. The organism is widespread in the environment and in recent years it has become an important opportunistic pathogen associated with nosocomial colonization and infection [1]. It causes infection of the central nerves system, bone, blood stream and urinary tract as well as respiratory tract [2]. The organism has been reported to colonize the lungs in higher frequency of cystic fibrosis (CF) and immunocompromised patients [3–5].

Flagella are highly complex bacterial organelles which are unusually well conserved among diverse bacterial species. They play an important role in chemotaxis and motility of the organism [6]. Flagella have also been implicated in the adherence of *S. maltophilia* to mucus [7] and epithelial cells [8]. Different studies have used flagella to find specific antigenic relationship between different closely related species, such as members of family *Enterobacteriaceae* [9]. Other studies have been carried out to find antigenic relationship among different species of *Legionella pneumophila* [10], *Salmonella enteritidis, S. typhimurium* [11] and *Clostridium chauvoei* [12].

The present study was carried out to evaluate the relationship between flagellin of *S. maltophilia* and flagellin of related species such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*, all of which have been implicated as respiratory pathogens in recent years.

MATERIALS AND METHODS

Bacterial isolates: Clinical isolate of *S. maltophilia* (Sm2) (Department of Medical Microbiology PGIMER, Chandigarh, India), standard strain of *Burkholderia cepacia* ATCC25609 (Institute of Microbial Technology, Chandigarh, India) and standard strain of *Pseudomonas aeruginosa* PAO (Dr. Barbara H. Igleski, University of Rochester, New York, United States) were used in this study. Bacteria were preserved by lyophilization and were routinely cultured at 37°C on Luria Bertani agar plates. Subcultures were made every week.

Flagellin preparation. Flagellin was prepared from *S. maltophilia, B. cepacia* and *P. aeruginosa* by following the method of Zgair and Chhibber [13]. Bacteria were grown in LB broth over night at 37°C. A 10 mL volume of the respective culture was added to each of three flasks of 500 mL of Luria broth and incubated with shaking at 37°C overnight and pelleted by centrifugation. Pellets were suspended in potassium phosphate buffer (0.01 M, pH 7.0) and sheared for 1 min in commercial blender. The sheared suspensions were centrifuged for 30 min at 5000*g* and then centrifuged

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for 15 min at 16000*g*. The supernatants were centrifuged at 100000*g* for 3 h. The pellets were collected and kept at –80°C.

Anti-flagellin antiserum preparation. Anti-flagellin antiserum was prepared by immunizing a rabbit with pure flagellin preparation of *S. maltophilia*. The complement in antiserum was inactivated by incubating sera at 56°C for 30 min.

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

Determination of anti-flagellin by indirect ELISA. The 96 microtiter plate (Nunc, Denmark) was coated with flagellin protein of *S. maltophilia, B. cepacia* and *P. aeruginosa* (2 μg of flagellin to each well) and kept overnight at 37°C, the plate was washed four times with 0.1 M PBS containing 0.05% Tween 20 (PBS-Tween) (Himedia, Mumbai, India) at this point, and after each subsequent incubation step. Wells were blocked with 200 μL of PBS containing 3% dried milk and incubated at 37°C for 1 h. One hundred micro liter of rabbit anti-*S. maltophilia* flagellin (different dilutions) suspended in PBS containing 0.1% dried milk was added to each well and plate incubated at 37°C for 1 h. Goat anti-rabbit horseradish peroxidase conjugated antibody ("Sigma") was added to the wells and incubated for 1 h, followed by addition of tetramethylbenzidine substrate (TMB) ("Sigma"). The reaction was stopped with 10% sulfuric acid ("Fluka") and absorbance read at 450 nm (680 microplate absorbance reader ("Biorad", USA) [14]. In Control: a similar procedure adapted for test was followed but instead of immunized rabbit serum, normal rabbit serum was used.

Bacterial agglutination. The method of Montie and Stover (1983) [15] was followed. Briefly, bacterial cells (*S. maltophilia, B. cepacia* and *P. aeruginosa*) were stained with crystal violet and washed three times with PBS (0.01 M, pH 7.2). Bacterial cell suspensions (0.1%) were prepared in PBS. Serial dilutions of rabbit serum (anti-*S. maltophilia* flagellin antiserum) were prepared in U shaped microtiter plates with PBS (1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640). Equal volume (50 μL) of bacterial suspension was added to each well. Plates were incubated at 37°C overnight. Negative control of normal rabbit serum instead of immunized rabbit serum was used. The results were noted the following day.

Motility inhibition test. Expression of swarming of *S. maltophilia, B. cepacia* and *P. aeruginosa* was checked by inoculating bacteria in plate containing 10 mL of swarm-agar (L-broth + 0.3% agar) as described earlier [16] and modified by Chhibber and Zgair (2009) [8]. Inhibition of motility of *S. maltophilia, B. cepacia* and *P. aeruginosa* in presence of flagellin-specific antiserum (anti-*S. maltophilia* flagellin) was also tested at a dilution of 1 : 320. The motility of each strain in presence and absence of anti-serum was checked after 48 hours of incubation.

Statistical analysis. All the experiments were carried out in triplicate and all values have been taken as mean value and standard deviation calculated. The differences between test and controls were analyzed by using Student's *t*-test calculated by employing Origin 8.0 version Software. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Molecular weight of *S. maltophilia, P. aeruginosa* and *B. cepacia* flagellin. Figure 1 shows that all flagellin preparations were pure as only single band was observed in each lane. The molecular weight of flagellin of *P. aeruginosa, B. cepacia* and *S. maltophilia* was 54, 46 and 42 kDa respectively. These results confirm variation in the molecular weight of flagellin of each bacterial species included in this study.

Antigenic cross reaction between flagellin of *S. maltophilia* and flagellin of *P. aeruginosa* and *B. cepacia*. The antiserum against flagellin of *S. maltophilia* was prepared by immunizing rabbit with pure flagellin of *S. maltophilia*. The presence and purity of anti-*S. maltophilia* flagellin was checked by employing Immunoblot assay.

The denaturized and separated protein (flagellin of each bacterial species) in 12% polyacrylamide gel was transferred onto polyvinylidene difluoride (PVDF) membrane. The blots were reacted with antiserum of *S. maltophilia* flagellin to check immunological cross reaction between anti-flagellin of *S. maltophilia* and flagellin of *S. maltophilia, P. aeruginosa* and *B. cepacia*. Figure 2 shows that immune serum reacted with flagellin of *S. maltophilia* (a). No immunological reaction was observed between anti-*S. maltophilia* flagellin and flagellin of *P. aeruginosa* (b) and flagellin of *B. cepacia* (c).

The result of bacterial agglutination test (Fig. 3a) showed that anti-*S. maltophilia* flagellin agglutinated with *S. maltophilia* only and titer of bacterial agglutination was 1/320. While no agglutination reaction was found between anti-*S. maltophilia* flagellin and *P. aeruginosa* and *B. cepacia* (Fig. 3a). These results confirmed that there was no antigenic relationship between flagellin of *S. maltophilia* and flagellin of *P. aeruginosa* and *B. cepacia*.

A similar trend was found when ELISA method was employed to detect antigenic relationship between *S. maltophilia* flagellin and flagellin of *P. aeruginosa* and *B. cepacia* (Fig. 3b).

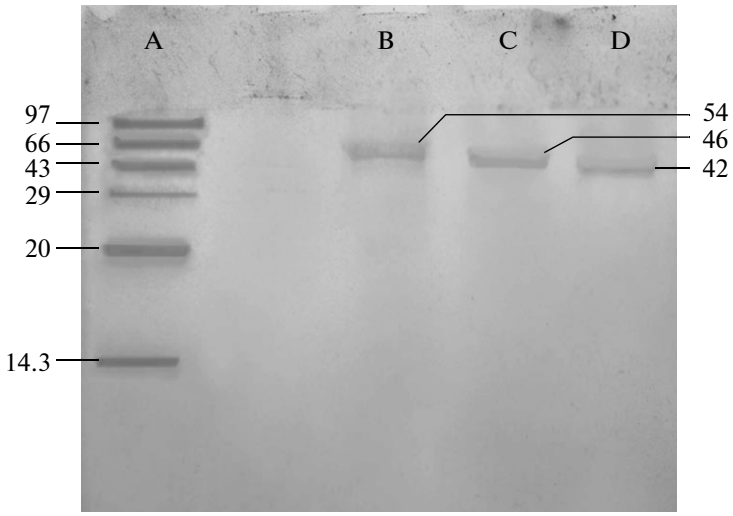

Fig. 1. SDS-PAGE of purified flagellin of *P. aeruginosa, B. cepacia* and *S. maltophilia*. Isolated proteins were denaturized in sample buffer and electrophoresed on 12% separating polyacrylamide gel and stained with Coomassie blue. Lane A: a protein marker. Lane B: pure flagellin protein of *P. aeruginosa* 54 kDa. Lane C: pure flagellin protein of *B. cepacia* 46 kDa. Lane D: pure flagellin protein of *S. maltophilia* (Sm2) 42 kDa.

Motility inhibition test: The effect of anti-flagellin of *S. maltophilia* on the motility of *S. maltophilia, B. cepacia* and *P. aeruginosa* was checked using motility agar, which consisted of LB with 0.3% agar (w/v), with or without antiserum against *S. maltophilia* flagellin. Figure 4B shows the swimming motility of three strains of *S. maltophilia, P. aeruginosa* and *B. cepacia* belonging to closely related genera, on motility agar without anti-flagellin (control). Inhibition of motility was seen only in case of *S. maltophilia* and no effect of anti-serum on *B. cepacia* and *P. aeruginosa* motility was observed (Fig. 4A).

The results in Fig. 5 show that significant difference in the diameter zone of bacterial motility was observed in case of test and control of *S. maltophilia* on growth on either of the media ($P < 0.05$). On the contrary no such difference was seen in case of *P. aeruginosa* or *B. cepacia* ($P > 0.05$).

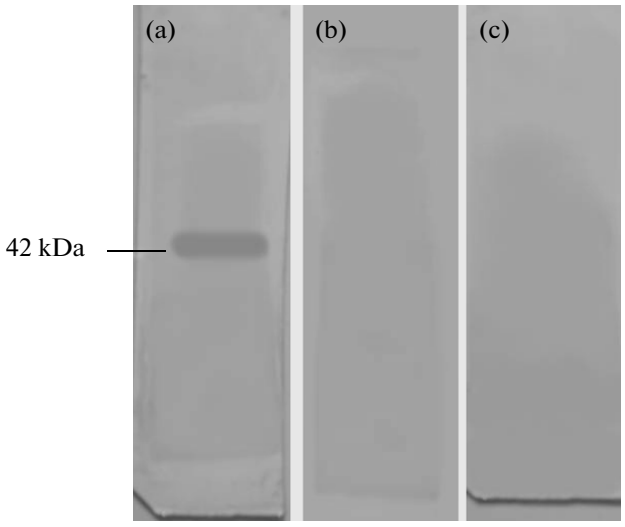

Fig. 2. Immunoblot assay to confirm the antigenic relationship between flagellin of *S. maltophilia* and flagellin of *P. aeruginosa* and *B. cepacia*. The result of Immunoblot assay showed there is no antigenic relationship, (a): blot of flagellin of *S. maltophilia* reacted with anti-flagellin of *S. maltophilia*. (b): Blot of flagellin of *P. aeruginosa* reacted with anti-*S. maltophilia* flagellin. (c): Blot of flagellin of *B. cepacia* reacted with anti-*S. maltophilia* flagellin.

DISCUSSION

Recent observations that flagellin can evoke nonspecific and specific immunity has generated interest among scientists, to explore its potential in various infectious diseases. In a study from our laboratory we have reported its ability to evoke innate immunity that was found to be protective against challenge with *S. maltophilia* [17]. Working in this direction, the present study was planned to see whether there existed any similarity among flagellin of *S. maltophilia, P. aeruginosa* and *B. cepacia*. These three organisms have been documented as major pathogens of the respiratory tract [18]. Making a vaccine that protects against all these three pathogens would be more meaningful. The flagellar antigen has been documented to be well conserved among different bacterial species, that acts through its binding to TLR5 [19, 20].

The results of the present study showed that there are major differences among the flagellin of *P. aeruginosa, B. cepacia* and *S. maltophilia*. The difference in the molecular weight of flagellin of all the studied organisms is suggestive of differences in their H-antigenic structure of these organisms. It is not surprising that we did not find any similarity in the molecular

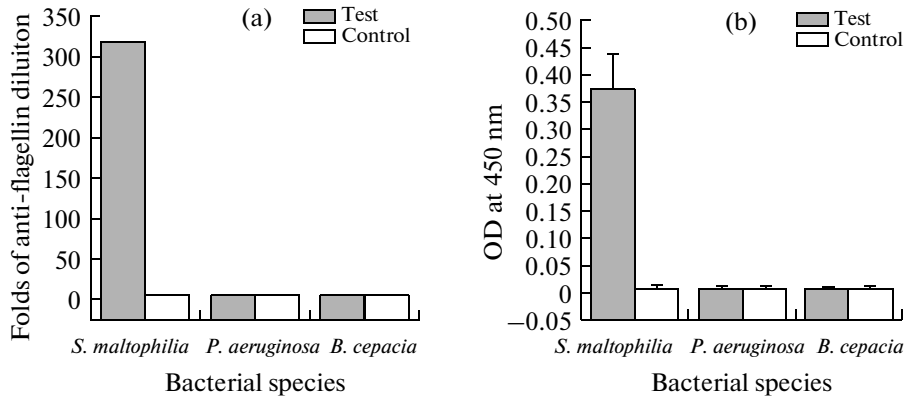

Fig. 3. Cross reaction between anti-flagellin of *S. maltophilia* (Sm2) and flagellin of *S. maltophilia, B. cepacia* and *P. aeruginosa*. Bacterial agglutination method (a) and ELISA method (b). Test: bacteria agglutinated with *S. maltophilia* anti-flagellin.

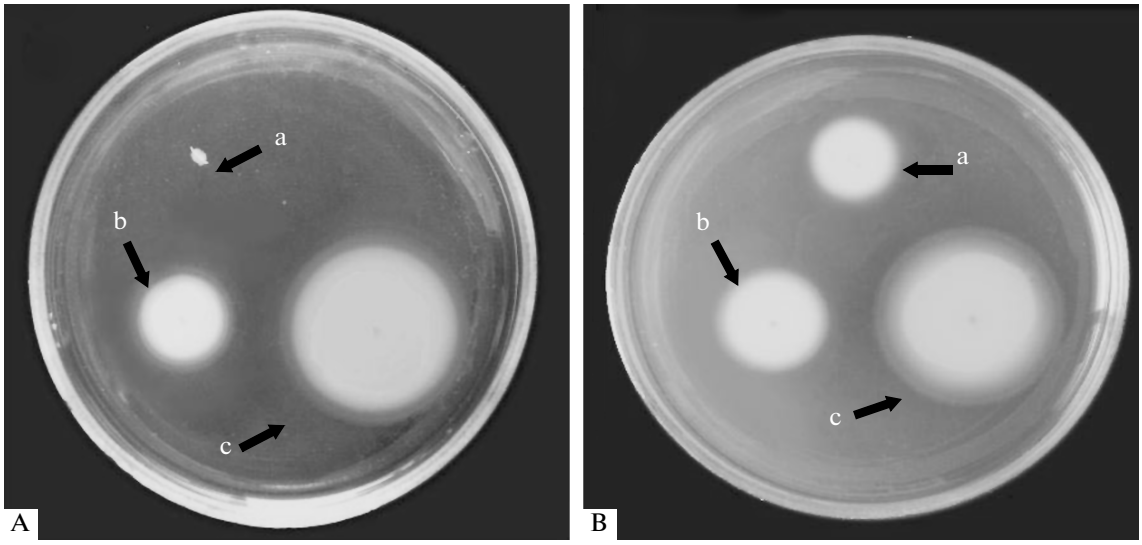

Fig. 4. Motility inhibition assay. A: motility of *S. maltophilia* was inhibited in presence of 1 : 320 flagellin antiserum. No inhibition was seen in case of *B. cepacia* and *P. aeruginosa*. B: control negative which shows all bacteria succeeded to move through the motility agar in the absence of flagellin antiserum, a: *S. maltophilia*, b*: B. cepacia* and c*: P. aeruginosa*.

weight of all these species, as these are taxonomically distant [21]. Further attempts also proved that there existed no serological cross reactivity among the flagellin of all these three organisms.

In 1983, Montie and Stover purified flagella from several pseudomonads, including *P. maltophilia* strain B69 (now referred to as *Stenotrophomonas maltophilia*) [15], and found that B69 produced a flagellin that had a molecular mass of 33 kDa. They did not found antigenic relationship among flagella of *P. aeruginosa* and *P. cepacia* (*B. cepacia*) and *P. maltophilia* (*S. maltophilia*). De Oliveira-Garcia et al. (2002) purified flagella from standard strain of *S. maltophilia*. Its molecular weight was 38 kDa [22]. These workers studied the antigenic and molecular relationship with *P. aeruginosa*. Very weak antigenic relation was detected between flagellin of *S. maltophilia* and *P. aeruginosa*. These results are in line with our findings on antigenic relationship though there were differences in the molecular weight of flagellin from these three organisms.

The findings of this study confirm that the flagellin induced specific immunity may not provide protection against other related organisms as there existed no antigenic cross reactivity among their H-antigen, but its ability to evoke non-specific immunity can be play an important role in the control of respiratory tract infections. Kinnebrew et al. (2010) proved that systemic administration of flagellin to antibiotic-treated mice dramatically reduced vancomycin-resistant enterococcus (VRE) colonization by enhancing mucosal resistance due to development of innate

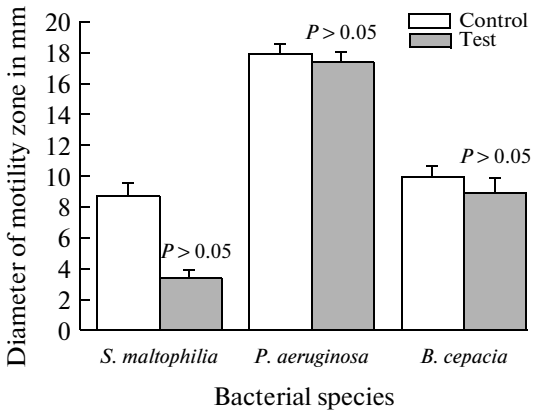

Fig. 5. The diameters of the motility zone on motility media with anti-Sm flagellin (gray bars, test) and without anti-Sm flagellin (white bars, control). The significant decrease in motility diameter was in case *S. maltophilia* only ($P < 0.05$).

immunity against multi-drug-resistant organisms [23]. In another recent study intranasal pretreatment of mice with purified *P. aeruginosa* flagellin led to strong protection against intratracheal *P. aeruginosa*-induced lethality, which was attributable to markedly improved bacterial clearance, reduced dissemination, and decreased alveolar permeability [24]. Muñoz et al. (2010) suggested that flagellin treatment improved *S. pneumoniae* clearance in the lungs that led to increased survival of infected host [25]. The work is in progress in our laboratory to ascertain the protective potential of innate immune response generated following intranasally instillation with flagellin.

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