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Isolation and Characterization of Flagellar Preparations from *Pseudomonas* Species

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been used previously to characterize the flagellin of *Pseudomonas aeruginosa*. Flagellins from several other clinically important species of pseudomonads have been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their molecular weights have been found to vary among species as follows: *P. maltophilia* B69 Fla, 33,000; *P. stutzeri* HEW, 55,000; *P. aeruginosa* M-2, 53,000. The flagellins of *P. cepacia* strains were divided into two groups based on molecular weight. Type I had a molecular weight of 31,000. The molecular weight of type II was in the range of 44,000 to 46,000. Serologically, type I is a homologous group, whereas type II is a heterologous group. The two flagellin types of *P. cepacia* appear to be analogous to the two major flagellin types of *P. aeruginosa*. Characterization of *P. cepacia* strains by flagellin types may serve as a molecular epidemiological tool.

Pseudomonas aeruginosa, in the past 30 years, has been well documented as a major cause of nosocomial infections in compromised hosts (1, 7). *P. aeruginosa* has also been described as a cause of invasive lethal infections in burn patients (18). Evidence has indicated that motility and chemotaxis may play roles in the pathogenesis of *P. aeruginosa* (4, 13, 16). The use of a flagellar antigen vaccine for protection against infection in burned mice has recently been reported (9).

Recently, there has been more precise and definite identification of pseudomonads in hospitals (8, 10). The increased incidences of other *Pseudomonas* species causing numerous nosocomial infections has encouraged a more thorough examination of the procedure for identification and possible means of control (19). As a first approach to evaluating possible control by vaccination, we isolated flagellins from representative clinical isolates to compare their characteristics and relative homogeneity.

(This work was presented in part at the 83rd Annual Meeting of the American Society for Microbiology, New Orleans, La., 6 to 11 March 1983 [T. C. Montie and G. B. Stover, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D24, p. 63].)

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* M-2 was originally isolated from the small intestine of a CF1 mouse (9). Strain M-2 was obtained from I. A. Holder, Shriner's Burns Institute, Cincinnati, Ohio. The other *Pseudomonas* strains were obtained as follows: *P. cepacia* SMH, M. Camblin, St. Mary's Medical Center, Knoxville, Tenn.; *P. cepacia* E4119 Ca, E8980(1) Col, E2973 Ma, E7427 PR, E7893 Ill, D7072 Ind, A1560 Fla, and 3765 Pa and *P. maltophilia* B69 Fla, Centers for Disease Control, Atlanta, Ga.; and *P. stutzeri* HEW, M. Moody, Baltimore Cancer Research Program, University of Maryland, Baltimore.

The original cultures, received from various sources, were suspended in a solution of 25% (vol/vol) glycerol in saline (0.85% [wt/vol] NaCl) and maintained frozen at −70°C. Stock cultures were maintained at 4°C as dilute suspensions in Luria broth (1% NaCl, 1% tryptone, 0.5% yeast extract; pH 7.0).

Laboratory animals. Female New Zealand white rabbits (Clay Graves Rabbitry, Corryton, Tenn.) weighing 3 kg each were used to produce hyperimmune antisera. Before and during immunization, the animals were kept one to a cage with food and water ad libitum.

Preparation of flagellar antigen. Flagellar antigen preparations (FAg) were prepared from *Pseudomonas* strains by a modification of the method of Montie et al. (15). *P. aeruginosa* and *P. cepacia* were grown in 50 ml of mineral salts medium (17) plus glucose overnight at 30°C in a gyratory water bath, and *P. maltophilia* and *P. stutzeri* were grown in Luria broth. *P. maltophilia* was grown at 37°C, whereas *P. stutzeri* was grown at 30°C. A 10-ml volume of the *P. aeruginosa* or *P. cepacia* culture was added to each of four flasks of 490 ml of mineral salts plus 10 ml of glucose solution (final glucose concentration, 0.5%) and then incubated overnight with shaking at 30°C. A 10-ml volume of the *P. maltophilia* or *P. stutzeri* culture was added to each of four flasks of 500 ml of Luria broth and incubated with shaking; *P. stutzeri* was incubated at 30°C, whereas *P. maltophilia* was incubated at 37°C. Flagellar antigens were prepared from cells grown at 30°C since flagellin is optimally synthesized at low tempera-

tures (10); *P. maltophilia* was grown at 37°C because preliminary experiments revealed that FAg yield was greater at this temperature than at 30°C.

Cells were harvested from the overnight cultures by centrifugation for 15 min at 5,000 × *g* and 4°C. Pellets were suspended in potassium phosphate buffer (0.01 M, pH 7.0) and then blended in a Waring commercial blender to shear the flagella. Optimal shearing times for *Pseudomonas* strains, as determined by relative purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were as follows: 30 s for *P. aeruginosa* and *P. maltophilia*, 40 s for *P. stutzeri*, and 15 s for *P. cepacia*. Each suspension was centrifuged for 30 min at 5,000 × *g* and 4°C, and the supernatant was centrifuged again for 15 min at 16,000 × *g* and 4°C. The supernatant fluid was then centrifuged for an additional 3 h at 40,000 × *g* and 4°C in a model L5-75 Beckman centrifuge with a 42.1 rotor. The pellets were carefully removed, suspended in 5 ml of potassium phosphate buffer, dialyzed against distilled water, rapidly frozen using acetone-dry ice, and lyophilized. The dry FAg preparations were stored desiccated at −55°C.

Analytical methods. Molecular weights of FAg preparations were determined by SDS-PAGE, using a Bio-Rad slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.) and by a modification of the procedures of Laemmli (11) and Montie et al. (15). Each FAg preparation was boiled for 1 min in a sample buffer containing 2% SDS before 10 g (dry weight) of FAg was applied to the sample gel. The concentrations of acrylamide in the stacking gel and running gel were 5 and 10%, respectively. Protein was detected by staining with Coomassie blue by the method of Fairbanks et al. (6). The gel was stained with solution 1 (25% isopropanol, 10% acetic acid, 0.05% Coomassie blue R250) for 1 h, with solution 2 (10% isopropanol, 10% acetic acid, 0.005% Coomassie blue R250) for 30 min, and with solution 3 (10% acetic acid, 0.0005% Coomassie blue R250) for 30 min and destained with 10% acetic acid for 1 h.

Preparation of rabbit antisera to FAg. Rabbits were immunized with FAg by a modification of the method described by Lagenaur and Agabian (12). On days 1 and 7, rabbits were given a subcutaneous injection of 250 μg of lyophilized FAg in 0.5 ml of complete Freund adjuvant. A series of four intravenous injections, containing 50, 100, 150, and 250 μg of FAg, respectively, in 0.5 ml of saline, was given at 3-day intervals, starting 20 days after the first subcutaneous injection. Blood was collected from the marginal ear vein 1 week after the last injection. Sera were stored in 0.5-ml portions at −70°C.

Slide agglutination assay. Bacteria were grown for 18 to 24 h at 37°C on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates. A loopful of bacteria was thoroughly mixed with 50 μl of 0.85% (wt/vol) saline on a glass microscope slide. A 20-μl drop of antiserum diluted 10-fold in saline was added to the cell suspension on the slide. The suspension was rocked back and forth and observed continually for 2 min. A reaction was considered positive if agglutination occurred within the period of incubation. Preliminary assays showed that there were no significant differences with cells grown at 37°C, 30°C, and room temperature; therefore, slide agglutinations were performed at the optimal growth temperature of 37°C.

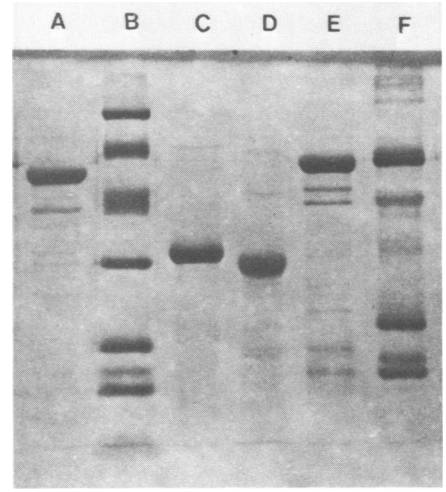

FIG. 1. SDS-PAGE of *Pseudomonas* species FAg preparations. Lane A, *P. aeruginosa* M-2; lane B, Bio-Rad SDS-PAGE premixed standards (molecular weights, 10,000 to 100,000); lane C, *P. maltophilia* B69 Fla; lane D, *P. cepacia* SMH; lane E, *P. stutzeri* HEW; lane F, standard proteins and their molecular weights (from top: bovine serum albumin, 66,000; pepsin, 34,700; trypsinogen, 24,000; and β-lactoglobulin, 18,400).

RESULTS

The gel profiles of flagellin from various *Pseudomonas* species showed large differences among species (Fig. 1). Molecular weights of flagellin were determined by R_f plots, using simple linear regression (Fig. 2, relative mobility versus $\log_{10}$ molecular weight). Flagellin from *P. aeruginosa* M-2 had a molecular weight of 53,000, consistent with previously published reports (15). *P. cepacia* SMH exhibited flagellin of a much lower molecular weight, 31,000. Intermediate in size was the flagellin of *P. maltophilia* B69 Fla, with a molecular weight of 33,000. *P. stutzeri* HEW had a flagellin band with a molecular weight of 55,000.

P. cepacia is becoming a more prevalent organism associated with nosocomial infection, and we have begun to study its virulence in the burned-mouse model (L. L. MacKensie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317; G. B. Stover, D. R. Drake, and T. C. Montie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B158, p. 49). Consequently, we have obtained additional clinical isolates for the above studies and have compared their flagellins. Flagellins from five of these strains were compared with that of *P. cepacia* SMH (Fig. 3). The molecular weights of the flagellins were determined by R_f plots, using simple linear regression (Fig. 4). From these data, we propose that the flagellins from *P.*

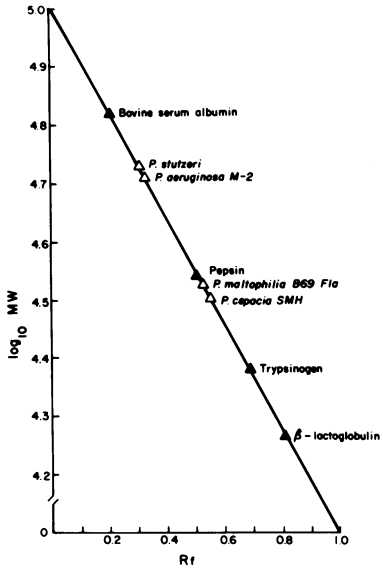

FIG. 2. Molecular weight (MW) determinations by SDS-PAGE. Standard proteins were as described in the legend to Fig. 1.

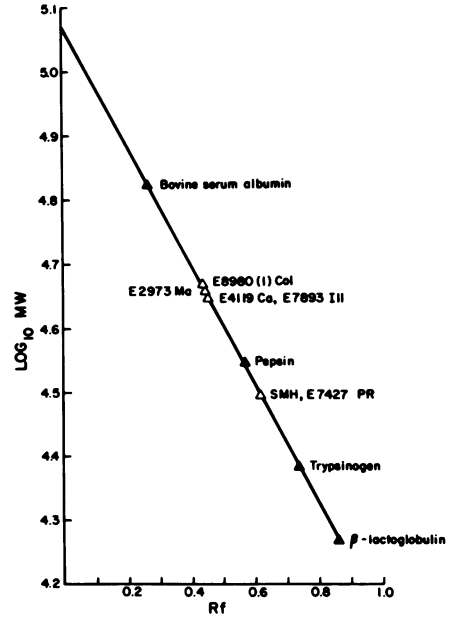

FIG. 4. Molecular weight (MW) determinations by SDS-PAGE. Standard proteins were as described in the legend to Fig. 3.

cepacia strains can be divided into two molecular weight groups. One group had a molecular weight of 31,000, and we are designating this group as type I flagellin. The molecular weight of the second group was in the range of 44,000 to 46,000, and we are designating this group as type II flagellin. Further rationale for categorizing the flagellins into one or the other group was obtained by examination of four additional clinical isolates (Fig. 5). It was found that these four isolates could also be placed in either the type I or the type II group, based on their relative molecular weights. It is interesting to note that the type I flagellin samples exhibited a double-banded pattern on the gels (Fig. 3 and 5). This was observed with all three type I isolates identified.

Although molecular weight patterns gave us evidence to segregate the flagellins of *P. cepacia* strains into two groups, further evidence to

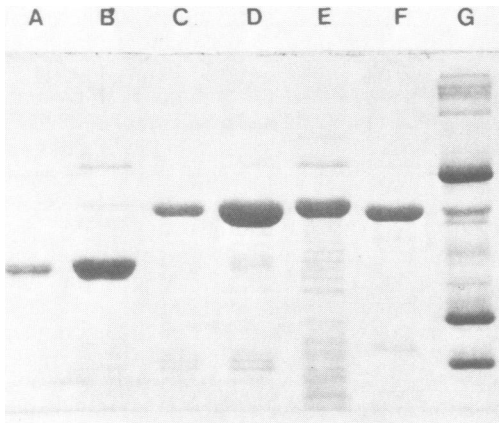

FIG. 3. SDS-PAGE of *P. cepacia* FAg preparations. Lane A, Strain SMH; lane B, strain E7427 PR; lane C, strain E2973 Ma; lane D, strain E7893 Ill; lane E, strain E8980(1) Col; lane F, strain E4119 Ca; lane G, standard proteins and their molecular weights (from top: bovine serum albumin, 66,000; pepsin, 34,700; trypsinogen, 24,000; and β-lactoglobulin, 18,400).

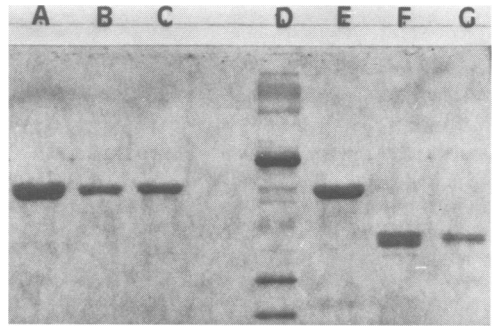

FIG. 5. SDS-PAGE of *P. cepacia* FAg preparations. Lane A, Strain D7072 Ind; lane B, strain A1560 Fla; lane C, strain 3765 Pa; lane D, standard proteins and their molecular weights (from top: bovine serum albumin, 66,000; pepsin, 34,700; trypsinogen, 24,000; and β-lactoglobulin, 18,400); lane E, strain E4119 Ca; lane F, strain F3761 PR; lane G, strain SMH.

TABLE 1. Slide agglutination assays of *Pseudomonas* species with antisera against *P. cepacia* FAg

Species	Agglutination with antiserum against:	
	P. cepacia SMH[a]	*P. cepacia* E8980(1) Col[b]
P. cepacia SMH[a]	+	–
P. cepacia E4199 Ca[b]	–	–
P. cepacia E8980(1) Col[b]	–	+
P. cepacia E7893 Ill[b]	–	–
P. cepacia E2973 Ma[b]	–	–
P. cepacia 3E7427 PR[a]	+	–
P. cepacia D7072 Ind[b]	–	–
P. cepacia A1560 Fla[b]	–	–
P. cepacia 3765 Pa[b]	–	+
P. cepacia F3761 PR[a]	+	–
P. maltophilia B69 Fla	–	–
P. stutzeri HEW	–	–

[a] *P. cepacia* FAg type I.
[b] *P. cepacia* FAg type II.

support this approach was obtained by comparing their serological relationships. We have used this approach successfully, together with data of Ansorg (2) and Ansorg (3) et al., in categorizing the flagellins of *P. aeruginosa* strains (T. C. Montie, M. Dawson, and J. Allison, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B5, p. 18). The serological relationships of the two flagellin types of *P. cepacia* were examined. Hyperimmune antisera against flagellar antigens were raised in rabbits. Antisera prepared against b, a_{012}, and a_{034} FAg of *P. aeruginosa* would not react with any of the other pseudomonad strains. Antiserum prepared against *P. cepacia* SMH (type I FAg) would agglutinate only *P. cepacia* strains with type I FAg (Table 1). Antiserum prepared against *P. cepacia* E8980(1) Col (Type II FAg) would agglutinate only *P. cepacia* E8980(1) Col and 3765 Pa (type II FAg); other *P. cepacia* strains with type II FAg were not agglutinated (Table 1).

DISCUSSION

The results show that there are major differences among the flagellin molecular weights of various *Pseudomonas* species. We have recently observed such differences even among strains of *P. aeruginosa*, which have molecular weights of approximately 43,000 to 53,000 (Montie et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B5, p. 18). Initial results have indicated that the differences in *P. aeruginosa* flagellin molecular weights reflect the differences in H-antigenic types, as demonstrated by Ansorg (2) and Ansorg et al. (3). It is not surprising that we find an even larger variation in molecular weights among *Pseudomonas* species, because we are dealing with species that are taxonomically distant (5). Supporting this is the fact that hyperimmune antisera raised against *P. aeruginosa* flagellin types would not agglutinate any other *Pseudomonas* species. By various taxonomic criteria, *P. stutzeri* is close to *P. aeruginosa* (5). This may also be reflected by *P. stutzeri* flagellin having a molecular weight similar to that of the b flagellin of *P. aeruginosa*. *P. cepacia* and *P. maltophilia* are taxonomically distant from *P. aeruginosa* and *P. stutzeri*. This is also reflected by their flagellins having molecular weights dissimilar to those of the flagellins of *P. aeruginosa* and *P. stutzeri*.

A more detailed study of *P. cepacia* isolates showed that flagellins from *P. cepacia* strains could be separated into two distinct groups. Type I exhibited molecular weights of 31,000; type II had molecular weights of 44,000 to 46,000. The molecular weight relationships apparently reflect antigenic relationships since hyperimmune antiserum raised against *P. cepacia* type I flagellin will only agglutinate type I cells; no cross-reactivity with type II strains was observed. However, hyperimmune antiserum raised against *P. cepacia* E8980(1) Col (type II FAg) would agglutinate two of the seven type II strains. This may suggest that there are small differences in type II flagellins analogous to those observed with the various *P. aeruginosa* a flagellar antigen subtypes. These subtypes of *P. aeruginosa* showed some homology, by not only possessing one common subgroup antigen but also having one to four additional subgroups. The b type of *P. aeruginosa* may be analogous to type I of *P. cepacia* because both groups clearly contained, within their own group, flagellins of homologous molecular weights and antigenicity. Further careful examination of additional type II strains is needed to understand the apparently heterologous antigenic relationships among the type II flagellins.

Although type I strains examined to date show homology, we noted a double-banded pattern in these flagellins. This observation suggests either that two monomeric flagellins exist in each flagellum or that two closely related proteins are associated with at least two different flagellar types present in *P. cepacia* type I. *P. cepacia* has been shown to contain three or more flagella (17). More data are needed to determine the exact flagellar composition of type I strains.

Characterization of *P. cepacia* strains into two flagellar antigen molecular weight types may have merit as a possible molecular epidemiological tool. This may serve a role similar to that proposed for other organisms (14). Our data indicate that knowledge of such factors may be a significant aid in strain typing and taxonomic classification.

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