Electrophoretic Separation and Molecular Weight Characterization of *Pseudomonas aeruginosa* H-Antigen Flagellins

JANICE S. ALLISON, MELISSA DAWSON, DAVID DRAKE, AND THOMAS C. MONTIE*

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845

Received 26 February 1985/Accepted 28 May 1985

We found that preparations of *Pseudomonas aeruginosa* flagellar antigens protected against *P. aeruginosa* challenge in a burned-mouse model. To determine the extent of similarity among known flagellar antigen types, we compared flagellins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A majority of our laboratory strains and clinical isolates, including PAO strains and their derivative mutants RM46 and PJ108, as well as virulent strains M-2 and 1244, had flagellins of 53,000 M₅. These flagellins had the same *M₅* as those of type b-standard strains 170001 and 15084. The heterogenous group of a-type H-antigen flagellins were of smaller molecular weights, ranging from 52,000 M₅ for standard strain 5939 (*a₀, a₁, a₂*) to 45,000 M₅ for standard strain 170018 (*a₀, a₃, a₄*). Standard strains 5933 (*a₀, a₁, a₂*) and 5940 (*a₀, a₂*) had intermediate *M₅* of 51,000 and 47,000, respectively. Differences in *M₅* of 1,000 to 2,000 could be resolved by coelectrophoresis. A series of 26 unknown strains were categorized. Correlations among typing by molecular weight, cross-agglutination reactions with O-adsorbed H antisera, and previous results for H-serum typing are reported.

The efficacy of flagellar antigens (FAgs) in protecting mice against infection with *Pseudomonas aeruginosa* in a burned-mouse model has been reported (9). An important aspect of this research is to understand and determine the extent of similarity among existing *P. aeruginosa* FAgs. An initial report identified flagellin as a 53,000-*M₅* species. This determination was in general agreement with a report by Ansorg and Schmitt (2), who identified a 50,000-*M₅* species for all H types.

Lanyi (12) was the first to categorize *P. aeruginosa* FAgs types into two major groups by using slide and tube agglutination assays. A homologous group (type 1) and a heterologous group (type 2) were used to classify 541 clinical isolates. Type 2 contained a subantigen, a, common to all members, with additional subantigens b to f as possible additions. Ansorg (1) confirmed and more precisely and greatly extended Lanyi's classification by using an indirect immunodiffusion technique. Ansorg (1) assigned the letter b for the homologous group and the letter a for the heterologous group, following the initial Lanyi approach. Ansorg assigned numbers instead of letters to the subgroups. According to Ansorg, the FAgs can be divided into two major groups, b types and a types, the latter comprised of a series of subtypes. All the a types have an *a₀* and usually one or more additional subtypes, *a₁, a₂, a₃*, and *a₄*. The common subantigen system is indicative of some cross-reactive capacity as well as some heterogeneity among strains containing the different subtypes (1, 3). We used the Ansorg classification as the basis for this study.

By coincidence we had initially tested only b-type flagellins when determining the molecular weight of flagellin. However, when we extended these studies it was apparent that the a-type flagellins were smaller than the b-type flagellins (T. C. Montie, J. S. Allison, and M. Dawson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B5, p. 18).

To classify both clinical isolates and our laboratory strains, we compared the FAgs electrophoretically. Our two objectives were (i) to establish the *M₅* of the immunologically well-characterized Ansorg-Lanyi strains (standard strains (1, 12) and (ii) to establish unknowns as a- or b-type strains based on their *M₅*. We found that different antigens had different mobilities. This report summarizes the results of this work, which indicate a correlation between antigenic type and molecular weight (relative mobility). Our results with rabbit antisera and cross-agglutination reactions are in general agreement with both previous immunological results (1, 12) and with mobility in gels.

**MATERIALS AND METHODS**

**Organisms and growth media.** Strains used for isolating the different types of flagellin included the following: a series of H-type strains (Table 1) from R. Ansorg (1) (the 5000 series was from the Collection de l’Institut Pasteur, Paris, France, and the 170000 series was obtained from B. Lanyi, National Institute of Hygiene, Budapest, Hungary [3]); SBI (Shriners Burns Institute human isolates) clinical isolates, GNB-1 (general clinical nonburn isolate), 1210, WR-5, and M-2 from I. A. Holder, Shriners Burns Institute, Cincinnati, Ohio; strain 1244 from A. McManus, U.S. Army Institute of Surgical Research, Fort Sam Houston, San Antonio, Tex.; strain 19660 from R. Berk, Wayne State University School of Medicine, Detroit, Mich.; folliculitis strains 2087, 2483, 2492, 3592, 3598, and 3614 from the Tennessee Department of Health, Nashville; and cystic fibrosis sputum strains 86F, 572B, 409G, and 402C from M. J. Thomsen, Case Western School of Medicine, Cleveland, Ohio.

Stock cultures were maintained at 4°C as dilute suspensions in Luria broth. A mineral salts medium adjusted to a pH of 7.0 (15) and with sodium succinate (0.4%) as the carbon source was used as the culture medium for FAgs preparations. Long-term stock cultures were maintained at -70°C as moderately turbid suspensions in 25% glycerol-75% Luria broth.

**Preparation of FAgs.** A modification of the methods of Montie et al. (13) was used to isolate the FAgs. Overnight liquid cultures (50 ml each in 250-ml flasks) in mineral salts medium were used to inoculate two 1-liter flasks containing 500 ml of mineral salts medium; the flasks were then incubated with shaking at 30°C for 15 to 17 h. The cell suspension was centrifuged for 15 min at 5,000 × g at 4°C. The resulting
pellet was suspended in potassium phosphate buffer (0.01 M, pH 7.0) and blended at a low-speed setting in a commercial blender (Waring Industrial Blender) for 30 s to shear the flagella. The 30-s time was experimentally established to achieve maximum flaggella release and minimal amounts of contaminating protein or lipopolysaccharide in the final FAg fraction. This suspension was centrifuged for 15 min at 16,000 × g at 4°C. The preparations were further purified and stored as previously described (9, 13).

Analytical methods. Protein determinations of the lyophilized preparations were made by the Coomassie blue technique of Bradford (5) with crystalline bovine serum albumin as a standard and Bio-Rad reagent (Bio-Rad Laboratories, Richmond, Calif.). The detection of 2-keto-3-deoxyoctulosonic acid was carried out by the method of Karkhanis et al. (10).

Molecular weights were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a Bio-Rad slab gel apparatus (Bio-Rad Laboratories) by a modification of the procedure of Laemmli (11). Use of the large 28-cm gel holder facilitated distinguishing among bands of similar mobilities. The FAg preparation was boiled for 1 min in 2% sodium dodecyl sulfate sample buffer before 1 μg of the preparation was applied to the gel. The stacking gel contained 5% acrylamide, and the separation gel contained 10% acrylamide. Protein was detected by staining with Coomassie blue by the method of Fairbanks et al. (8).

Agglutination reactions. Antisera were raised in female New Zealand rabbits by subcutaneous injection of 250 μg of FAg in 0.85% saline mixed 1:1 with Freund complete adjuvant. Another injection was given after 7 days. Rabbits were bled from the marginal ear vein 10 days after the second injection. Somatic (O) antibodies were adsorbed from the sera with overnight 37°C cultures which were concentrated and heat treated (100°C, 1 h). A concentrated culture sample (400 ml) was added to 15 to 20 ml of antiserum for 1 h at 37°C and then centrifuged at 6,000 rpm in a Sorvall GLC-1 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) for 25 min to remove cells. The adsorption was repeated before O-antibody titers were determined. The slide agglutination reaction was carried out as previously described (6, 14) except that 45 μl of diluted antiserum was added to the cell suspension.

RESULTS

Initially, three strains were used for the comparison of FAg. These were 170001 (b), 5933 (a0, a1, a2), and 170018 (a0, a3, a4), which are representative of the wide degree of heterogeneity possible in the various H-antigen types and subtypes (1, 3) (Table 1). These strains, along with strains 5940 and 5939, were most carefully and extensively documented by Ansorg (1, 3). The FAg were isolated and compared with that of clinical isolate M-2 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). It was apparent that the flaggellins of different antigenic types migrated differently. The flaggellins of a-type strains 5933 and 170018 migrated faster than those of the unknown M-2 (Fig. 1) or its comparable b-type standard strain 170001 (M, 53,000; Table 1). Strain 5933 was intermediate in its relative mobility between strain 170018 (a type) and strain M-2 (b type). Unidentified strains SBI-S and 1244 (data not shown) had the same mobility as strain M-2, whereas strains 1210 and GNB-1 moved ahead of strain M-2. Strains 1210 and GNB-1 were designated as a types (M, <53,000).

Figure 2 shows plots of the average molecular weights of the five standard antigenic types. Strain 170001 (b type) had an M, of 53,000, strain 5933 (a type) had an M, of 51,000, and strain 170018 (a type) had an M, of 45,000. Strains 5939 and 5940 (a types) had Ms of 52,000 and 47,000, respectively. Thus, the standard a-type strains all had molecular weights below that of the b-type strain.

Certain FAg from unknown clinical isolates were com-

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**TABLE 1.** M, s of flaggellins of standard H-antigen strains and various unknown strains

<table>
<thead>
<tr>
<th>Standard strain*</th>
<th>Antigenic component(s)*</th>
<th>Flaggellin M,</th>
<th>Classified unknown strains*</th>
</tr>
</thead>
<tbody>
<tr>
<td>170001</td>
<td>b</td>
<td>53,000</td>
<td>M-2, 1244, PAO (PAO-1, RM46, PJ108), SBI-I, SBI-H, SBI-S, 409g, 402c, 2483, 2492, 2087, 5142 (a0), 13030 (a0)</td>
</tr>
<tr>
<td>15084</td>
<td>b</td>
<td>53,000</td>
<td></td>
</tr>
<tr>
<td>5939</td>
<td>a0, a1</td>
<td>52,000</td>
<td>CF-LL, 19660</td>
</tr>
<tr>
<td>5933</td>
<td>a0, a1, a2</td>
<td>51,000</td>
<td>1210, 7191</td>
</tr>
<tr>
<td>170012</td>
<td>a type</td>
<td>50,000</td>
<td></td>
</tr>
<tr>
<td>170002</td>
<td>a type</td>
<td>49,000</td>
<td>WR-5, 3592, 3598, 3614, 86F, 572b</td>
</tr>
<tr>
<td>5940</td>
<td>a0, a2</td>
<td>47,000</td>
<td></td>
</tr>
<tr>
<td>170018</td>
<td>a0, a3, a4</td>
<td>45,000</td>
<td></td>
</tr>
</tbody>
</table>

* Standard strains were obtained from Ansorg.

* These antigenic types were differentiated by the indirect fluorescent-antibody and agglutination techniques.

* Based on M,; strains that are printed in italics were also verified by immunological cross-agglutination as either a or b types.

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![FIG. 1. Comparison by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of relative mobilities of standard flaggellins and unknowns. Lanes: A, standard strain 5933 (a0, a1, a2), B, C, and D, strain 1210; E, standard proteins (from the origin down; molecular weights in parentheses): phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400); F, strain M-2; G, strain GNB-1; H, standard strain 170018 (a0, a3, a4); and I, strain SBI-S.](http://iai.asm.org/ Downloaded from)
pared with the standard FAgS. Strain 1210 had a flagellin
molecular weight comparable to that of strain 5933 (Fig. 1).
To confirm this, we mixed the two FAg preparations and
subjected them to electrophoretic analysis. Only a single
band was observed, indicating identity (data not shown).
According to the same method, strain 19660, used in an
infectious mouse eye model (4), had an FAg mobility com-
parable to that of strain 5939. We therefore assigned strain
1210 to the a0, a1, a2 antigen type and strain 19660 to the a0,
a1 antigen type (Table 1).

The applicability of the coelectrophoresis technique was
evident in a number of additional determinations (Fig. 3 and
Table 1). A presumed b-type isolate (strain 7191) obtained
from R. Ansorg was demonstrated to be an a type by $M_r$
criteria (Fig. 3). Strain 7191 FAg was resolved from standard
strain 15084 and M-2 FAgS (M-2 is a mouse-virulent b-type
isolate comparable to strain 170001). We assigned an $M_r$
for strain 7191 of 51,000 (Table 1), based on its separation
from standard strain 5939 (a0, a2) ($M_r$, 52,000) and its
coelectrophoresis with strain 5933. In the strain 5939 cate-
gory is cystic fibrosis sputum isolate CF-LL. Another un-
known isolate, GNB-1, was separated from standard strain
5940 (a0, a3) ($M_r$, 47,000). Strain GNB-1 flagellin had an $M_r$
of 49,000, comparable to that of standard strain 170002
flagellin. Unknowns of ca. 48,000 $M_r$ are strain WR-5,
foliculitis strains 3592, 3598, and 3614, and cystic fibrosis
sputum strains 86F and 572b (Table 1). These have not as yet
been matched with any specific a type.

The data in Table 1 summarize all of our findings to date.
A series of three human burn isolates (SBI strains) had
flagellins of 53,000 $M_r$ (Fig. 1 and 3) and were placed in the
b-type category. Other b types included various PAO strains
and strain 1244, a human burn isolate, two cystic fibrosis
sputum strains, 409g and 402c, and three foliculitis strains,
2483, 2492, and 2087. A finding of note is that the strains
designated antigenic type $a_0$ by Ansorg (1) were the same
flagellin type as the b types according to our electrophoretic
criterion (Table 1). They included two strains, 5142 ($a_0$) and
13030 ($a_0$), obtained from R. Ansorg.

The standard strains used in this study were extensively
compared by an indirect fluorescence assay (1, 2) and more
recently by a slide coagglutination technique (3). Results
from direct slide agglutination are reported here both for
further confirmation and for examining the immunological
and molecular weight relationships of unknown strains.
Anti-M-2, -5933, and -170018 sera were adsorbed with
heated cells to remove O-antibody titers. The results (Table
2) are in general agreement with the molecular weight
determinations. Standard strain anti-170001 FAg serum (not
absorbed for O antibodies) reacted strongly and specifically
with all of the b types, including the unknown SBI series,
designated as b types by the electrophoresis assay. Anti-M-2
serum reacted with almost all of the b types; however, the
reaction was not as strong as that of strain 170001. Anti-M-2
serum also agglutinated strains 13030 and 5142, formerly
assigned to type a (1) and now assigned to type b (see

FIG. 2. Molecular weight determinations of flagellins from vari-
ous FAg preparations. The data plotted for each strain represent
the averages of six or more values obtained from separate gel runs.

FIG. 3. Comparison by sodium dodecyl sulfate-polyacrylamide
gel electrophoresis of flagellin mobilities. Lanes: A, strain 7191; B,
strain 15084 (upper band) and 7191 (lower band); C, strain 15084;
D, standard proteins (see Fig. 1 legend); E, strain 7191; F, strains
M-2 (upper band) and 7191 (lower band); G, strain M-2; H, standard
proteins (see Fig. 1 legend); and I, strain SBI-1.
above). These results generally were consistent with the electrophoresis data. Both a-type anti-5933 and -170018 sera reacted only with their counterpart standard strains. No reactions were seen among the a-type antisera and other different heterologous a-subtype strains or b-type strains. Recent results with strain 1210 antisera confirmed its cross-reactivity with strain 5933 and 7191 antigens. The assignments of the classical unknown strains (Table 1) into either the type a or the type b category was verified by experiments with two additional standard b-type antisera and anti-1210 serum (italicized strains in Table 1). Further verification of the presence of common a subtypes in unknowns will require testing of a number of different sera at higher sensitivity levels.

### DISCUSSION

An $M_s$ of 53,000 was previously assigned for the flagellin of *P. aeruginosa* FAg (13). It is now apparent not only that there is variety in the molecular weights of flagellins, but also that the molecular weights are specifically associated with the H-antigen type of the strain. All of the b-type flagellins tested had an $M_s$ of 53,000. The strains used in initial protection studies were, by coincidence, mainly b-type strains (9, 13). In fact, the largest percentage of isolates we maintain are of the b type. Of interest for possible vaccine application are the results of surveys showing that a significant proportion (>59%) of clinical isolates have b-type flagella (1, 12). All of the a-type antigens, except for the $a_0$ type, had flagellins of lower $M_s$ (45,000 to 52,000) (Table 1 and Fig. 1) than did the b-type antigens. This characteristic difference in weight may reflect a peptide region or sequence that differentiates the two major groupings. Recently, amino acid analyses of all of the major H groups with highly purified FAg's showed a close correspondence between molecular weights determined by electrophoresis and those computed from total amino acid composition. Furthermore, immunodiffusion and immunoelectrophoresis results are consistent with molecular weight results and slide agglutination results with standard and unknown purified FAg's (M. Mitterer, T. C. Montie, and F. Dorner, manuscript in preparation). These data are consistent with those of early protection experiments showing that partially purified M-2 antigen protected against b-type strains (SBI strains) and not an a-type strain (GNB-1) (9). Passive protection experiments with O-adsorbed M-2 antisera showed specificity for protection against b-type challenge, but not a-type challenge (strains GNB-1 and 1210) (D. Drake and T. C. Montie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B143, p. 42).

Almost all of our molecular weight results correlate completely with the original H serotyping results reported by Lanyi (12) and Ansorg (1, 3). However, a few discrepancies remain. First, the $a_0$-type strains (1) have both an $M_s$ of 53,000 and immunological typing common to the b-type antigen. Therefore, these typings are probably in error (R. Ansorg, personal communication). Second, strain 7191 has been assigned to the b type, but we have determined that the flagellin has an $M_s$ of 51,000, putting it in the a-type classification, similar to strain 5933. Recent agglutination experiments support the classification of strains 7191 and 1210 as $a_0$, $a_1$, $a_2$ types.

In almost all FAg preparations the amount of protein impurities was <1%, as estimated in the gel profiles of the total protein. An exception was the appearance of one or two low-molecular-weight proteins (15,000 to 20,000) appearing as prominent bands in gel profiles of FAg's of the $a_0$, $a_1$, $a_2$ type, such as those of strain 5933. These small polypeptides appear to be dissociated flagella (possibly protease-nicked flagella) or, less likely, pilus material ($M_s$, 15,000 to 18,000) (16). With strain 5933 or 1210 FAg's we observed increased amounts of some of these bands and decreased amounts of native flagellin that correlated with the length of time in storage. A reduction in the apparent degradation was accomplished by a shift from storage after lyophilization in phosphate buffer to storage in Tris-chloride buffer.

The results obtained are of interest taxonomically because it has been reported that the flagellin of *Pseudomonas stutzeri*, which is grouped with *P. aeruginosa* (7), has an $M_s$ (55,000) similar to that of b-type *P. aeruginosa* (53,000) (14). The flagellins of more distant relatives, *Pseudomonas maltophila* and *Pseudomonas cepacia*, have much lower $M_s$, 31,000 to 45,000, generally below those of all *P. aeruginosa* flagellins (14).

### ACKNOWLEDGMENTS

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We are grateful to Greg Shaw for his demonstrated expertise in the antiserum typing experiments.

### LITERATURE CITED


### TABLE 2. Cross-agglutination reactions with FAg antisera

<table>
<thead>
<tr>
<th>H antisera</th>
<th>M-2 (b)</th>
<th>SBI-H (b)</th>
<th>SBI-1 (b)</th>
<th>SBI-S (b)</th>
<th>170001 (b)</th>
<th>13030 (a0)</th>
<th>5142 (a1)</th>
<th>GNB-1 (a type)</th>
<th>WR-5 (a type)</th>
<th>5933 (a0, a1, a2)</th>
<th>170018 (a0, a1, a2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-2 (b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5933 (a0, a1, a2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>170018 (a0, a1, a2)</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

* FAg components are in parentheses. All sera except 170001 sera were adsorbed for O antibodies.

* Agglutinations were graded as follows: +++, strong; +, moderate to weak; -, none.

* ND. Not done.