Biochemical and Serological Properties of Purified Flagella and Flagellins of some *Pseudomonas* spp.

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Sheared polar flagella of the type strains of monotrichate *Pseudomonas aeruginosa* and multitrichate *P. fluorescens* and *P. putida* were purified and the sedimentation coefficients of the corresponding flagellins determined by ultracentrifugation; from these values the approximate molecular weights of the major flagellins were calculated to be between 38 000 and 43 000, and other estimates based on methionine content also suggested that the range was between 38 800 and 43 000. Purified flagellins of these three species, and also *P. syringae* pv. *morsprunorum* and *Pseudomonas* sp. ('*Vibrio percolans*'), were digested with trypsin, and the peptide maps obtained after two-dimensional electrophoresis were compared. Altogether 65 peptide spots were delineated and 15 peptides were found to be common to all five species. The amino acid composition of the purified flagellins of the three type species was found to accord qualitatively and quantitatively with other prokaryote flagellins. Antisera were raised against the three purified flagellins, and tested by the Ouchterlony diffusion plate method against (a) purified homologous and heterologous flagellins, (b) deflagellated cells with sheared flagella and (c) crude sheared flagella of a further 67 test strains and species (mainly *Pseudomonas*). With either flagella or flagellin antigens the serological reactions were virtually identical, but not all strains of a given species reacted with the relevant anti-flagellin antiserum. Rapid species identification using a single anti-flagellin antiserum was thus not possible. The flagellar location of the antigenic site(s) was confirmed by two serological methods, including direct immunofluorescent serology.

INTRODUCTION

Bacterial flagella, pili and fimbriae are now under intensive investigation in structural and genetic terms, because these appendages may be important in various tactic phenomena and adhesion, which in turn may affect the virulence of bacteria towards plants or animals. Relevant examples are discussed by Rowbury *et al.* (1983); they concluded that 'this is a controversial field', an opinion which was reinforced with respect to *Vibrio anguillarum* (Chart, 1983), *Roseburia cecicola* (Stanton & Savage, 1984), *Pseudomonas solanacearum* (Kelman & Hruschka, 1973) and *P. phaseolicola* (Panapoulos & Schroth, 1974). Others found that if the motility of *P. aeruginosa* was impaired genetically or immunologically its virulence was reduced (see Holder *et al.*, 1982, for a review). Furthermore, mice immunized with sheared, purified flagella from virulent strains of *P. aeruginosa* were far less susceptible to challenge doses of this organism than were non-immunized animals. Hence more detailed information concerning *Pseudomonas* flagella is highly desirable.

Non-motile cells of *P. aeruginosa* are very rare (<0.2%; Lányi, 1970), and motile cells virtually always possess one polar flagellum; Jessen (1965) found that more than 300 out of 354 strains were exclusively monotrichate. The closely related saprophytic fluorescent pseudomonads typically possess polar multitrichous flagella; one to five fibrils per tuft is characteristic for *P. putida* and three to twenty for *P. fluorescens* (Rhodes, 1958). The flagella of all three species

are similar in length and wavelength, with approx. two helices with wavelengths of 1.8–2.2 μm . How similar they are in biochemical or serological terms is not known because flagellar serotyping is virtually always restricted to sub-speciation within *P. aeruginosa* (Lányi & Bergan, 1978).

The technical problems involved in obtaining a sufficient yield of polar flagella (yields here of 5–20 mg compared with 50–500 mg from an equivalent wet weight of *Proteus* cells) are considerable, and the elimination of pili, fimbriae and somatic contaminants is consequently more important. Nevertheless, a study of the biochemical and serological characteristics of purified flagella of the type strains of *P. aeruginosa*, *P. putida* and *P. fluorescens*, together with a few other representative organisms for comparative purposes, was undertaken here. Antisera raised against highly purified flagellins were tested using both flagella filaments and purified flagellins of the three type strains, and the serological reactions towards 67 further strains of *Pseudomonas* were determined. Amino acid analyses of the three purified flagellins were also carried out, and their relatedness to other flagellins was calculated using the comparative method of Metzger *et al.* (1968).

METHODS

Organisms and culture conditions. The strains used were *Pseudomonas aeruginosa* ATCC 10145 [*Pseudomonas* Working Party (PWP) No. 1]; *P. fluorescens* ATCC 13525 (PWP 18); *P. putida* NCIB 9887 (PWP 25); *P. syringae* pv. *morsprunorum* D270 from Dr W. J. Dowson, Cambridge University; *Pseudomonas* sp. NCIB 8193 deposited as '*Vibrio percolans*' (PWP 10); *Xanthomonas campestris* NCPPB 13528 (PWP 31), and various other strains listed in Table 5; these were from the Rhodes Collection or those issued to the *Pseudomonas* Working Party (Sneath & Collins, 1974). The two media used had the following compositions (g l⁻¹ deionized water): (1) nutrient broth (NB): Oxoid bacteriological peptone L37, 5; Oxoid Lab Lemco L29, 5; NaCl, 5; adjusted to pH 7.2. (2) Yeast extract broth (YEB): Oxoid bacteriological peptone, 10; Oxoid yeast extract L20, 3; NaCl, 5; adjusted to pH 7.2. For solid media Oxoid agar no. 3 was added to give a final concentration of 1.3% (w/v), and 2.0% (w/v) sucrose YEB enhanced the growth of some phytopathogens. Chemically defined media gave much lower numbers of flagellate cells and so were abandoned.

Detection of motility and flagellation. Motility in liquid cultures was checked by the hanging-drop technique, and the detailed flagellation was determined by examination of silver-plated preparations from NA/water slopes using the staining and light microscope method of Rhodes (1958).

Isolation of flagella. Late exponential or early stationary phase growth of cells incubated for 3–5 d at 14 °C on NA or YEA was harvested at room temperature in PBS (0.9%, w/v, NaCl in 0.02 M phosphate buffer pH 8.1). The organisms were removed gently to minimize breakage of flagellar filaments, suspended in PBS, and warmed to 35–40 °C for 15 min with gentle agitation, prior to centrifugation at 8000 g for 20 min. After two more washes in warm PBS to remove exocellular polysaccharides, the concentrated cell suspensions were deflagellated by shaking for 60–90 min (av. 10 strokes s⁻¹) on a Griffin shaker. The deflagellated cells were removed by centrifugation at 12000 g for 30 min at 3 °C, and the sedimented cells resuspended in PBS and centrifuged twice more to recover flagella trapped in the cell mass; pooled supernatants were then centrifuged at 75000 g for 2 h at 3 °C and the sedimented flagella subsequently allowed to resuspend overnight at 4 °C in 3 ml 0.02 M-borate buffer pH 8.5. This procedure was repeated until the final pellet of flagellin was transparent and contained less than 0.1% (wet weight) viable cells. This was determined using the drop count method of Miles *et al.* (1938) and calculating cell weight by assuming that one cell weighed 10⁻¹² g. The final pellet, with counts less than 2000 organisms ml⁻¹, was suspended in 3 ml membrane-filtered distilled water. Completely bacteria-free preparations of native flagella were never obtained, even after more intensive centrifugation, and so <0.1% wet wt cells per flagella sample was finally regarded as acceptable. Great care was taken to avoid the introduction of microbial cells during the subsequent purification procedures: e.g. all the buffer and ammonium sulphate solutions were membrane-filtered.

Preparation and purification of flagellins. The 3 ml suspensions (above) were diluted to 25 ml with distilled water and the pH was slowly adjusted to 2.0 by dropwise addition of 1.0 M-HCl; acid-induced dissociation of flagella into flagellin was facilitated by incubation at 40–42 °C for 30 min. The 'pH 2.0 insoluble material' was removed by centrifugation at 30000 g for 90 min at 20 °C. The flagellin was then precipitated from the supernatant by adjusting it to pH 8.0 using 0.1 M-NaOH, and then adding a saturated solution of recrystallized ammonium sulphate (the original crystals contained numerous yeast cells) to give various final degrees of saturation values. This acid-dissociation followed by ammonium sulphate precipitation procedure was repeated several times, retaining a narrower range of the ammonium sulphate precipitate each time, i.e. flagellin sequentially precipitated using 0–60%, 10–60%, 45–60%, and finally 48–58% (w/w) ammonium sulphate. This procedure was essential to reduce anthrone-positive material from 25% (w/w) to 0.2–0.4% (w/w), and to eliminate 3–5 bands of minor proteins detected by SDS-PAGE. The final ammonium sulphate precipitate was desalted by dialysis against five

changes of sterile distilled water for 16–20 h until free of ammonia (tested using Nessler's reagent). The final material showed only a single band after electrophoresis on 5% (w/v) polyacrylamide gels containing 1% SDS, after running at 10 mA for 8 h and then staining the gels with Coomassie brilliant blue (Shapiro *et al.*, 1967). Such purified flagellins were freeze-dried.

Further tests for purity. Carbohydrate contamination was tested for using the anthrone Molisch reaction (Snell & Snell, 1953); the method of King (1932) was used to determine phosphate content; the presence of tryptophan, a marker for extraneous protein, was checked using the Hopkins–Cole (Hopkins & Cole, 1901) reaction.

Trypsin hydrolysis. Following the method of Emerson & Simon (1971), lyophilized flagellin samples were treated with performic acid (to eliminate artifacts arising from oxidation of methionine during the drying periods), at a concentration of 2.0% (w/v) in 0.02 M-phosphate buffer pH 8.1, and digested with an equal volume of 0.04% (w/v) trypsin (BDH) plus one drop of toluene. Digestion was complete after 20 h at 25 °C (Enomoto & Iino, 1966). Each sample (300 µg) was then chromatographed two-dimensionally as follows: (a) in the first dimension by low-voltage electrophoresis on Whatman 3MM paper at pH 1.5, using formic acid/glacial acetic acid/water (15:5:80, by vol.) at 15 V cm⁻¹ for 5 h at 0 °C, the electrophoretograms then being air-dried; (b) in the second dimension by descending solvent chromatography with freshly prepared *n*-butanol/glacial acetic acid/water (200:30:75, by vol.) for 15 h at 20 °C. The air-dried chromatograms were sprayed with 0.5% (w/v) ninhydrin and the colour allowed to develop for 20 min at 65–70 °C. Thus a characteristic peptide fingerprint map of each flagellin was obtained.

Tests for specific amino acids. Small samples of the flagellins were spot tested for the presence of specific amino acids; ethanolic nitroso-2-naphthol was used to test for tyrosine (Gerngross *et al.*, 1933), the diazo reagent of Mann & Leon (1953) for histidine, and the Sakaguchi reaction for arginine (Carver *et al.*, 1953).

Amino acid analysis. The determination of the amino acid compositions of the purified, lyophilized *P. aeruginosa*, *P. fluorescens* and *P. putida* flagellins was carried out by Dr W. A. Weetman, Department of Experimental Pathology, University of Birmingham, UK. The samples were hydrolysed in constant boiling 6 M-HCl under vacuum at 110 °C for 24 h and then analysed on a Locarte (London, UK) amino acid analyser using ninhydrin as the detection reagent.

Determination of sedimentation coefficients by ultracentrifugation. A sample of flagellin (7 mg ml⁻¹) in either 0.2 M-citrate buffer pH 2.2 or in 0.1% (w/v) SDS in 0.2 M-phosphate buffer pH 7.1 was centrifuged at 59780 *g* at 20 °C in a Beckman Model E analytical centrifuge. The bar angle was altered when necessary, and photographs of the positions of the peaks were taken at predetermined time intervals by employing the Schlieren optical system (exposure, 20 s). Measurements from the photographic plates were made with a Gaertner micrometer, and uncorrected sedimentation coefficients calculated using the standard formula. From these values the approximate molecular weights were determined.

Serological studies. Antiflagellin antisera against the three species, *P. aeruginosa*, *P. fluorescens* and *P. putida* were kindly prepared by Dr M. B. McIlmurray of the Wellcome Research Laboratories, Beckenham, Kent, UK. High titres were obtained in mouse ascitic fluid, and each antiserum tested against (a) purified homologous and heterologous flagellins; (b) cells deflagellated by shearing (centrifuged once only); (c) the crude preparation of sheared flagella. Immunodiffusion through Ouchterlony plates containing a 2–4 mm depth of 0.75% (w/v) Oxoid Ionagar No. 2 in 0.9% (w/v) saline in 0.05 M-phosphate buffer pH 8.1 proved to be the most reliable technique for demonstrating the antigen–antibody precipitin reactions. The prepared plates were incubated in sealed bags at 37 °C for 1–3 d. Precipitation was usually optimal after 48 h, and intensified after overnight refrigeration at 0 °C. A positive control was included in every plate, and tryptic digests of the flagellins showed no precipitates when tested with homologous or heterologous antiflagellin antisera. Crude flagella and flagellin preparations from a further 67 strains (listed in Table 5) were similarly screened.

Proof of the flagellar location of the antigenic site. Further proof that the flagellins were the antigens in the immunodiffusion plates was obtained by running the flagellin sample electrophoretically on 7.5% (w/v) polyacrylamide gel in the absence of SDS. Such gel strips were then halved longitudinally; one half was stained with Coomassie blue to locate the flagellin, and the other half placed on a 0.75% (w/v) Ionagar plate and allowed to react against a channel of homologous antiflagellin antiserum. The two halves were then realigned and compared.

Immunofluorescent serology. Air-dried smears of washed cells of *P. putida* were treated with specific anti-flagellin antiserum in a moist atmosphere at 37 °C for 24 h, washed for 8 h in flowing 0.9% (w/v) saline. After re-drying the smear, rabbit anti-mouse immunofluorescent serum was applied and adsorption effected during incubation in a moist atmosphere for 24 h at 37 °C. After re-washing with 0.9% (w/v) saline for 8 h, the specifically stained flagella were located by light-ground UV microscopy following the techniques of Walker *et al.* (1971).

RESULTS

Motility and flagellation. The rapid darting motility of all 67 isolates of pseudomonads used here was constantly confirmed in hanging-drop preparations, and exactly polar flagella resolved in silver-plated preparations. *P. aeruginosa* was virtually always monotrichate (rarely 2 polar

Table 1. *Flagellar wavelengths of polarly flagellate prokaryotes*

Values for air-dried bacteria were determined from light microscope micrographs of silver-plated bacteria and compared with the wavelengths of detached, purified, self-assembled flagella suspended in 50% (w/v) ammonium sulphate. Values are the means of 50–80 measurements.

Organism*	Flagellar wavelength (µm)	
	Air-dried cells	Self-assembled filaments
<i>P. aeruginosa</i> ATCC 10145 (PWP 1)	1.8–2.2	0.77
<i>P. fluorescens</i> ATCC 13525 (PWP 18)	1.8–2.0	0.9
<i>P. putida</i> NCIB 9887 (PWP 25)	1.8–2.2	0.88
<i>P. stutzeri</i> ATCC 17592 (PWP 120)	1.8–2.0	0.68
<i>P. syringae</i> pv. <i>morsprunorum</i> D270	1.5–1.8	0.68
<i>Xanthomonas campestris</i> NCP 13528 (PWP 31)	1.6–1.8	Insufficient yield
<i>Pseudomonas</i> sp. (' <i>Vibrio</i> <i>percolans</i> ') NCIB 8193 (PWP 10)	2.7–3.1	Short units failed to aggregate

* PWP, *Pseudomonas* Working Party (Sneath & Collins, 1974)

Table 2. *Comparison of the number of flagella peptides shared by each pair of five polarly flagellate bacteria, revealed by comparing their peptide maps*

	<i>P. fluorescens</i>	<i>P. putida</i>	<i>P. aeruginosa</i>	<i>P. syringae</i> pv. <i>morsprunorum</i>	<i>Pseudomonas</i> sp. (' <i>Vibrio percolans</i> ')
<i>P. fluorescens</i> ATCC 13525	26				
<i>P. putida</i> NCIB 9887	22	38			
<i>P. aeruginosa</i> ATCC 10145	18	19	27		
<i>P. syringae</i> pv. <i>morsprunorum</i> D270	18	15	19	31	
<i>Pseudomonas</i> sp. (' <i>Vibrio percolans</i> ') NCIB 8193	21	21	18	18	30

flagella); *P. putida* possessed 1–5 flagella, although monotrichy was rather common; *P. fluorescens* had 3–20 flagella (3 rather frequently). The wavelengths of some native flagella are given in Table 1, together with the wavelengths of detached ammonium-sulphate-aggregated flagella. Why the values of the latter are approximately only half those of the air-dried cells is not known. The shorter and more loosely coiled flagella of *Pseudomonas* sp. ('*Vibrio percolans*') with a wavelength of 2.7 µm yielded short fibrils which would not aggregate, perhaps due to traces of flagellin polymerization inhibitors recently reported by Ikeda *et al.* (1984) for the short-flagellum mutant of *Salmonella typhimurium*.

Tryptic digests and peptide mapping. Two-dimensional separation of the tryptic hydrolysates of the flagellins yielded a characteristic 'fingerprint' or peptide map of peptides for each flagellin; 5 flagellins were thus carefully compared and a comparative summary of their peptide maps is presented in Table 2. Only spots appearing in the same positions on at least 3 of the 5 replicates obtained for each strain were recorded; a total of 65 peptide spots was thus marked. The number of peptides which consistently appeared for each species was: *P. aeruginosa*, 27; *P. fluorescens*, 26; *P. putida*, 38; *P. syringae* pv. *morsprunorum*, 31; *Pseudomonas* sp. ('*Vibrio percolans*') 30. A

Table 3. Amino acid composition of the flagellins of three type strains of *Pseudomonas*

Amino acid	Amount of amino acid in flagellin of:					
	<i>P. fluorescens</i> ATCC 13525		<i>P. putida</i> NCIB 9887		<i>P. aeruginosa</i> ATCC 10145	
	$\mu\text{mol mg}^{-1}$	mol per 40 kg protein	$\mu\text{mol mg}^{-1}$	mol per 40 kg protein	$\mu\text{mol mg}^{-1}$	mol per 40 kg protein
Aspartic acid	1.6358	42.5	0.8702	41.3	0.9294	46.5
Glutamic acid	1.1348	29.5	0.5588	26.5	0.6395	32.0
Lysine	0.5743	14.9	0.3848	18.3	0.3064	15.3
Arginine	0.4361	11.3	0.2796	13.3	0.2097	14.6
Tyrosine	0.1360	3.5	0.0667	3.2	0.0497	2.5
Methionine	0.1190	3.1	0.0587	2.8	0.0582	2.9
Serine	1.1583	30.1	0.6181	29.4	0.5511	27.6
Threonine	1.4423	37.5	0.6747	32.1	0.4996	25.0
Glycine	1.2802	33.2	0.7297	34.6	0.7291	36.5
Alanine	2.3615	61.3	1.1604	55.1	1.0462	52.4
Valine	0.6568	17.1	0.3512	16.7	0.4519	22.6
Leucine	0.8470	22.0	0.5113	24.3	0.3930	19.7
Isoleucine	0.6450	16.8	0.4790	22.8	0.4130	20.7
Phenylalanine	0.2636	6.8	0.1123	5.3	0.1509	7.6
Histidine	0.0034	0.1	0.0042	0.2	0.0165	0.8
Proline	0.1366	3.6	0.0794	3.8	0.0491	2.5
Cysteine	ND	0	0.0120	0.6	ND	0
Tryptophan	ND	0	ND	0	ND	0
Σ -N-Methyl lysine (Ammonia)	ND	0	ND	0	ND	0
Total no. of residues (minus ammonia)	(2.0199)	(51.3)	(1.3883)	(64.1)	(1.1543)	(57.8)
		333.3		330.3		329.2

ND, Not detected.

Table 4. Sedimentation coefficients (Svedberg units) and estimated molecular weights for *Pseudomonas* flagellins

Flagellin concentration was 7.0 mg ml⁻¹ in (a) 0.2 M-citrate buffer pH 2.2, and (b) 0.1% SDS in 0.2 M-phosphate buffer pH 7.1. Approximate molecular weights (which should be regarded as the lower limit of the true values), calculated from S values, are also given.

Source of flagellin		Main component		Minor component	
		S value	Mol. wt	S value	Mol. wt
<i>P. aeruginosa</i> ATCC 10145	(a)	2.35	38 000	11.87	180 000
<i>P. putida</i> NCIB 9887	(a)	2.43	40 000	ND	—
<i>P. fluorescens</i> ATCC 13525	(a)	2.51	41 000	12.39	220 000
	(b)	2.58	41 000	11.08	150 000
<i>P. syringae</i> pv. <i>morsprunorum</i> D270	(a)	2.86	43 000	14.57	250 000

ND, Not detected.

total of 15 peptides were common to all five species, and the number of peptides shared by each pair of species is shown in Table 2.

Amino acid analysis. The amino acid data (supplied by Dr W. A. Weetman) for 3 *Pseudomonas* flagellins are given in Table 3, which shows that all three species had high proportions of aspartic acid, glutamic acid, threonine, alanine and glycine, and small amounts of tyrosine, phenylalanine, methionine, proline and histidine. Tryptophan and Σ -N-methyl lysine (peculiar to some *Salmonella* spp.; Ambler & Rees, 1959) were never detected. A small amount (0.17%, w/w) of cysteine was found in the *P. putida* flagellin.

Determination of sedimentation coefficients by ultracentrifugation. The results of ultracentrifugation of acid-dissociated purified flagellin at pH 2.2 are detailed in Table 4. Exact molecular weights could not be calculated here because no accurate estimations of the partial specific volumes of the proteins, nor of their diffusion constants in the buffers used, were available. The

Table 5. *The reactions of 67 bacterial isolates towards antisera raised against purified flagellins of P. aeruginosa, P. fluorescens and P. putida*

Reactions were determined by the Ouchterlony diffusion technique, with both crude sheared flagella (Fla) after one centrifugation or purified flagellins (Fln) as antigens. Trypsin-digested control flagellins showed no serological reactions.

Species	Reaction of antisera prepared against flagellins of:*					
	<i>P. aeruginosa</i> ATCC 10145 (PWP 1)		<i>P. fluorescens</i> ATCC 13525 (PWP 18)		<i>P. putida</i> NCIB 9887 (PWP 25)	
	Fla	Fln	Fla	Fln	Fla	Fln
<i>P. aeruginosa</i> ATCC 10145 (PWP 1)	+ ^m	+	-	-	-	-
<i>P. aeruginosa</i> ATCC 10145 (PWP 85)	+	+	-	-	-	-
<i>P. aeruginosa</i> NCTC 5940	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 20	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 21	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 24/2	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 282/9	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 282/11	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes 282/14	+	+	-	-	-	-
<i>P. aeruginosa</i> Rhodes strains 24/1, 282/1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13	-	-	-	-	-	-
<i>P. fluorescens</i> ATCC 13525 (PWP 18)	-	-	+	+	-	-
<i>P. fluorescens</i> Rhodes strains 4/3, 8/7, 9, 12; 12/3, 22/1, 22/2, 23/5, 26/3, 45	-	-	-	-	-	-
<i>P. putida</i> NCIB 9887 (PWP 25)	-	-	(+)	-	+ ^m	+
<i>P. putida</i> NCIB 9887 (PWP 86)	-	-	(+)	-	+ ^m	+
<i>P. putida</i> NCIB 9887 (Rhodes 192/1)	-	-	(+)	-	+ ^m	+
<i>P. putida</i> NCIB 9887 (Rhodes 192/2)	-	-	(+)	-	+ ^m	+
<i>P. putida</i> Rhodes 41	-	-	-	-	+	+
<i>P. putida</i> Rhodes T207	-	-	(+)	-	+	+
<i>P. putida</i> Rhodes T202	-	-	(+)	-	+ ^m	+
<i>P. putida</i> Rhodes T206	-	-	-	-	+	+
<i>P. putida</i> Rhodes 43/2	-	-	-	-	sl	+
<i>P. putida</i> Rhodes 43/3	-	-	-	-	sl	+
<i>P. putida</i> Rhodes 43/4	-	-	-	-	+	+
<i>P. putida</i> Rhodes 34	-	-	-	-	+	+
<i>P. putida</i> Rhodes strains 9/2, 9/4, 15/4, 15/5, 25/1, 32, 35/2, 42/3, 43/1, 44, 56/1, KB1, D244, T200	-	-	-	-	-	-
<i>P. syringae</i> pv. <i>morsprunorum</i> D270	-	-	-	-	-	-
<i>P. stutzeri</i> ATCC 17592 (PWP 120)	-	-	-	-	-	-
<i>P. lemoignei</i> NCIB 9947 (PWP 123)	-	-	-	-	-	-
<i>P. stizolobii</i> NCPPB a450	-	-	-	-	-	-
<i>P. cepacia</i> (<i>multivorans</i>) ATCC 17759 (PWP 88)	-	-	-	-	-	-
<i>P. acidovorans</i> NCIB 9681 (PWP 72)	-	-	-	-	-	-
<i>P. pseudoalcaligenes</i> NCIB 9946 (PWP 122)	-	-	-	-	-	-
<i>Pseudomonas</i> sp. (' <i>Vibrio percolans</i> ') NCIB 8193 (PWP 10)	-	-	-	-	-	-
<i>Xanthomonas campestris</i> NCPPB 13528 (PWP 31)	-	-	-	-	-	-
<i>Proteus vulgaris</i> Rhodes 18E	-	-	-	-	-	-

* +, Definite arc of precipitation; +^m, definite arc of precipitation plus arc representing a minor antigen; (+), faint precipitate probably due to non-flagellar impurities; sl, slight precipitate; PWP, *Pseudomonas* Working Party strain (Sneath & Collins, 1974).

approximate molecular weights suggested by the main peak flagellins were between 38 000 and 43 000, based on the calculation of Weibull (1948) that an S value of 2.4 is approximately equivalent to a molecular weight of 41 000, but that this should be regarded as the lower limit of the true value.

Serological studies. Each of the three anti-flagellin antisera prepared in mice against highly purified flagellins of *P. aeruginosa*, *P. fluorescens* and *P. putida* as antigens, produced a single distinct arc of precipitation when tested against homologous flagellins (3 mg flagellin ml⁻¹ in the test well), or against crude sheared flagella, in the Ouchterlony diffusion plates. Heterologous flagellins or flagella showed no such reactions; sheared homologous cells sometimes showed weak reactions. This was possibly due to residual flagellar stumps on the sheared cells, although in one flagella regeneration experiment with *P. aeruginosa* the majority of the deflagellated cells failed to regenerate flagella; the few which did so produced flagella 0.5 µm long after 30 min, 1.0 µm long after 60 min, and 2.5 µm long after 150 min, compared with flagella 3.5 µm long on the control cells.

The results of similarly testing crude (sheared) flagella preparations and partially purified flagellins derived from 67 additional test isolates are summarized in Table 5. It is evident that each of the three species was not homogeneous; blind testing of duplicates of both *P. putida* and *P. aeruginosa*, however, yielded identical results. With either flagella or flagellin as antigens for a given strain, the serological results were virtually identical.

Proof of the flagellar location of the antigenic site. When purified flagellin preparations were separated electrophoretically on SDS-free polyacrylamide gels, and then the longitudinally halved gels treated with Coomassie brilliant blue on the one half and anti-flagellin antiserum on the other, a single antigen-antibody precipitin line was found to correspond exactly with the position of the band of flagellin protein on the other half of the gel strip.

Immunofluorescent serology. *P. putida* cells stained with specific immunofluorescent anti-flagellin antiserum showed a rather thick single polar flagellum in the UV light microscope. Aggregation of fibrils was suspected.

DISCUSSION

The technique of repeated acid dissociation and ammonium sulphate precipitation eventually yielded purified *Pseudomonas* flagellins which migrated as a single protein on 7.5% (w/v) polyacrylamide gels. Removal of carbohydrate impurities from flagellins was never effected here using DEAE-cellulose fractionation procedures. Possible contamination of our cysteine-containing *P. putida* flagellin caused concern because *P. aeruginosa* pili are known to contain cysteine (Paranchych *et al.*, 1979). However, our serological and ultracentrifugation studies failed to reveal contaminating proteins, and our 0.17% (w/w) cysteine content may be compared with the 0.54–0.75% cysteine-cystine content of *Proteus* and *Bacillus* flagellins 'of highest purity' reported by Kobayaishi *et al.* (1959): cysteine was also found by Montie *et al.* (1982) in the purified flagella preparations of *P. aeruginosa*. Finally, Paranchych *et al.* (1979) convincingly purified the polar pili of *P. aeruginosa* strains PAO and PAK as single polypeptides of molecular weight 18 700 and 18 100, respectively, and no such molecules were detected in our flagellins.

Ultracentrifugation of the *P. aeruginosa* and *P. fluorescens* flagellins for molecular weight determinations resulted in a main peak and a smaller, heavier peak which diffused rapidly. This component had an uncorrected S value approx. fivefold greater than that of the main flagellin, and may represent an aggregate of five protein units, in accord with the observations of Lowry & Hanson (1965) who found two types of fine structures in *P. fluorescens* flagella, one with ten helices, and the other with five longitudinal helical rows of fibrils.

It was not possible to determine the exact molecular weights of the flagellins, but uncorrected sedimentation coefficients suggested values between 38 000 and 43 000 (see Table 4). Assuming a molecular weight of 40 000, the number of lysine and arginine residues in our flagellins was 29–30 for *P. aeruginosa*, 26–27 for *P. fluorescens* and 31–32 for *P. putida*, all in good agreement with the number of peptides produced after tryptic digestion (Table 2). When the methionine content was used to calculate the minimum molecular weight (assuming three residues per mol protein), of flagellins, 41 200 was calculated for *P. aeruginosa* flagellin, 38 800 for *P. fluorescens* and 43 000 for *P. putida*.

Table 6. *Comparison of the chemical similarities between different bacterial flagellins*

Data are expressed as the difference index of Metzger *et al.* (1968) calculated from amino acid data obtained from the references indicated.

	<i>P. fluorescens</i> ATCC 13525	<i>P. putida</i> NCIB 9887	<i>P. aeruginosa</i> ATCC 10145	Reference
<i>P. fluorescens</i> ATCC 13525	0			This study
<i>P. putida</i> NCIB 9887	5.1	0		This study
<i>P. aeruginosa</i> ATCC 10145	8.1	7.1	0	This study
(<i>P. aeruginosa</i> PAK pili)	17.7	12.7	18.4	Paranchych <i>et al.</i> (1979)
(<i>P. aeruginosa</i> PAO pili)	15.8	15.0	17.8	Paranchych <i>et al.</i> (1979)
<i>Spirillum serpens</i>	6.8	6.7	6.4	Martinez <i>et al.</i> (1967)
<i>Caulobacter crescentus</i> CB15 (flagellins A plus B)	11.59	12.08	15.68	Sheffery & Newton (1979)
<i>Caulobacter crescentus</i> PCM 103 (polyhooks)	11.09	10.52	11.39	Sheffery & Newton (1979)
<i>Vibrio anguillarum</i> *	14.28	13.7	9.97	Shinoda <i>et al.</i> (1976)
<i>Vibrio parahaemolyticus</i> WP-1*	15.2	15.2	14.4	Shinoda <i>et al.</i> (1976)
<i>Beneckeia neptuna</i> *	14.7	14.9	13.4	Shinoda <i>et al.</i> (1976)
<i>Rhizobium lupini</i> H13-3 (complex flagella)	10.12	9.26	11.53	Maruyama <i>et al.</i> (1978)
<i>Proteus vulgaris</i> P.	13.4	12.2	9.9	Kobayaishi <i>et al.</i> (1959)
<i>Proteus vulgaris</i> IFO 5988	14.6	13.0	9.7	Kurado (1972)
<i>Proteus mirabilis</i>	16.69	14.79	12.67	Schalch & Bode (1975)
<i>Salmonella typhimurium</i> SW 106 1a	10.5	10.1	10.9	Ambler & Rees (1959)
<i>Salmonella typhimurium</i> SW 106 1b	10.4	10.3	10.5	McDonough (1965)
<i>Salmonella adelaide</i> SW 1338	11.2	10.9	10.1	Ada <i>et al.</i> (1964)
<i>Bacillus subtilis</i> SB19	17.3	15.2	14.3	Martinez <i>et al.</i> (1967)
<i>Bacillus subtilis</i> 168 Group 1	18.5	15.8	14.2	Simon <i>et al.</i> (1977)
<i>Bacillus subtilis</i> 10783 Group 2	18.1	16.9	14.4	Simon <i>et al.</i> (1977)
<i>Bacillus subtilis</i> 9799 Group 3	15.7	15.2	16.9	Simon <i>et al.</i> (1977)
<i>Bacillus subtilis</i> 10774 Group 4	17.6	15.7	16.6	Simon <i>et al.</i> (1977)
<i>Bacillus firmus</i> RAB	19.2	20.0	19.5	Guffanti & Eisenstein (1983)

* Purified flagellin of single polar flagella only.

We now appreciate that even highly purified flagellins may have more than one protein component, e.g. *Caulobacter* spp. which possess a 23–25000 and a 28000 mol. wt flagellin (Lagenaur & Agabian, 1977; Sheffery & Newton, 1979; Gill & Agabian, 1982; Johnson *et al.*, 1983). *Rhizobium lupini* H13-3 has a cylindrical core of 43000 mol. wt flagellin surrounded by three bands forming a sheath of a 55000 mol. wt flagellin monomer (Schmitt *et al.*, 1974a; Maruyama *et al.*, 1978), and similar complex flagella with 37000 and 55000 mol. wt proteins were also reported for *Pseudomonas rhodos* (Schmitt *et al.*, 1974b), although this 'unusual *Pseudomonas*' is now reclassified as a *Protomonas*, a new genus of facultatively methylotrophic bacteria (Urakami *et al.*, 1984).

Our literature search for data shown in Table 6 also revealed a range of flagellin molecular weight values of 17–20000 for *Proteus vulgaris* (Erlander *et al.*, 1960), *Serratia marcescens* and *Bacillus subtilis* (Kobayaishi *et al.*, 1959); 33–40000 for 10 strains of *B. subtilis* (Simon *et al.*, 1977); 40000 for *B. firmus* RAB (Guffanti & Eisenstein, 1983); 30–50000 for other *Bacillus* spp. (DeLange *et al.*, 1976); 40000 for unsheathed flagellins of *Vibrio parahaemolyticus*, *V. anguillarum* and *Beneckeia neptunea* (Shinoda *et al.*, 1976); 40000 for *Proteus mirabilis* (Schalch & Bode, 1975); 45000 for flagellar cores of *V. cholerae* (Young *et al.*, 1977); 54000 for *Escherichia coli* (Simon *et al.*, 1978); 55000 for *Salmonella* (Ikeda *et al.*, 1983); 56000 for *Salmonella* SJ25 (Kagawa *et al.*, 1976), and 62000 (and a minor 87000 protein) for the purified flagellins of *Campylobacter jejuni* (Newell *et al.*, 1984). Further literature revealed that flagella hook proteins were more homogeneous, ranging from 43000 to 40000; Homma *et al.* (1984) confirmed a value of 40000 for the hook proteins of several mutants of *Salmonella typhimurium*, but despite the fact

that the values of approx. 40000 for our *Pseudomonas* flagellins fall within that commonly reported for hook proteins, we confirmed a definite filament location for our purified flagellins by direct immunofluorescent serology.

The gross amino acid composition of our three *Pseudomonas* flagellins (Table 3) is typical of prokaryotic flagella. The similarities in amino acid composition between the flagellins of the three type strains of *Pseudomonas* and other prokaryotes (including some data for purified pili and hooks) are shown in Table 6 in terms of 'difference index' (DI) values, as advocated by Metzger *et al.* (1968). The mol fraction per 40000 mol. wt protein is used as a standard for comparing differences in the fractional content of each amino acid in the flagellin. The sum of the differences (irrespective of sign) is multiplied by 50. On this scale, two proteins with no amino acids in common have a DI value of 100 and identical proteins have a zero value. Table 6 shows that the three *Pseudomonas* spp. and *Spirillum serpens* flagellins form a distinct, closely-related group. However, our calculations suggest that the DI values cannot be interpreted too finely because two *P. aeruginosa* pilins have different values, although they were virtually identical both chemically and physically (X-ray fibre diffraction patterns) according to Paranchych *et al.* (1979). Different DI values for five strains of *Bacillus subtilis* or the two strains of *Proteus vulgaris* (Table 6), confirm this conclusion.

Flagellin cores of polarly monotrichate *Vibrio* and *Caulobacter* are unexpectedly more similar to the flagellins of peritrichate Gram-negative rods (*Rhizobium*, *Proteus* and *Salmonella*) than they are to polarly flagellate *Pseudomonas* and *Spirillum* (Table 6). There is however a marked difference (see Table 6) between the Gram-positive *Bacillus* flagellins and all the other organisms analysed here. Thus the Gram-associated difference in the flagella seems greater than the positional polar-peritrichate difference; this was confirmed in a few test calculations which again revealed higher DI values between peritrichate *Bacillus* and either *Proteus* or *Salmonella* flagellins, compared with DI values for Gram-negative polar flagellins matched against peritrichate flagellins, as shown in Table 6.

Differences in DI values are unlikely to correlate with antigenic specificity [as indeed was found by Kurado (1972) for *Salmonella* and *Escherichia* flagellins] because Yamaguchi & Iino (1969) found that even a single amino acid replacement may markedly change the entire antigenic pattern of the flagellum, whereas other single replacements resulted in only very small changes in antigenicity. The relationships between antigenic specificity and flagellin peptide patterns are fully discussed by Emerson & Simon (1971) and Simon *et al.* (1977) for *Bacillus subtilis*.

Antisera produced against the three *Pseudomonas* flagellins prepared here showed no cross-reactivity over a wide range of other *Pseudomonas* spp. (Table 5) and thus appeared to be species-specific. Each of the flagellin antisera however, did not react with either the sheared flagella or the flagellins of all strains of that species; this was disappointing for diagnostic purposes. Thus the flagella show sub-specific differences, a situation which is also reflected in the many attempts to differentiate *P. aeruginosa* serologically on the basis of thermolabile flagellar antigens (see Ansorg, 1978; Lányi & Bergan, 1978). Pitt (1981*a, b*) describes his eventually successful flagellar (H) serotyping of all except 52 out of 823 clinical strains of *P. aeruginosa* into 22 H-typing patterns, involving 6 H factors either singly or in various combinations. Thus his complex results confirm the genetic data now in the literature, and the findings reported here, which show that even the single polar flagellum of *P. aeruginosa*, or its purified flagellin, is a far from homogeneous entity, although our *P. aeruginosa* flagellin antiserum did not cross-react with any other *Pseudomonas* species tested here. Our results neither support nor negate the 'stabilized mutant' hypothesis to account for monotrichate *P. aeruginosa* and multitrichate *P. fluorescens* in phylogenetic terms, despite the laboratory induction of polarly multiflagellate mutants of *P. aeruginosa* as reported by Suzuki & Iino (1980). This apparently involved a single mutation, the mutation site(s) being linked to a *fla* cluster: for details of the genetic analysis of the flagellar genes in *P. aeruginosa* Tsuda & Iino (1983*a, b*) may be consulted. No biochemical or serological properties of the parent or mutant flagella of *P. aeruginosa* were described by Suzuki & Iino (1980); this would be of interest, as would also the converse experiment, i.e. the induction of stable monotrichate cells of *P. fluorescens* or *P. putida*.

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