Comparison of Fatty Acid, Protein, and Serological Properties Distinguishing Outer Membranes of *Pseudomonas anguilliseptica* Strains from Those of Fish Pathogens and Other Pseudomonads

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Whole-cell fatty acid analyses of 79 *Pseudomonas anguilliseptica* strains demonstrated that all 79 strains were closely related and were distinct from other pseudomonads whose fatty acid profiles were reported previously. Outer membranes were isolated from seven representative strains, and the protein patterns were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Four major outer membrane proteins were found in each of the *P. anguilliseptica* strains but not in the outer membranes of 8 other *Pseudomonas* strains or 10 other fish pathogens. The outer membranes of the seven *P. anguilliseptica* strains studied were immunologically cross-reactive. We suggest that outer membrane protein patterns may be a useful taxonomic tool.

Pseudomonas anguilliseptica was first identified in 1972 as the causative agent of red spot disease in cultured Japanese eels (18). Although this name appears on the Approved Lists of Bacterial Names (17), little taxonomic work other than bacteriological characterization studies (13, 18) has been performed on this organism.

In this paper we describe studies on the whole-cell fatty acids and on the outer membrane protein patterns and immunological cross-reactions of *P. anguilliseptica* strains. Furthermore, we isolated the outer membranes of 9 other *Pseudomonas* strains and 10 nonpseudomonal fish pathogens. Our results suggest that outer membranes may be useful in determining taxonomic similarities.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacteria which we used are described in Table 1. Generally, the medium used was 1% (wt/vol) proteose peptone no. 2 medium (catalog no. 0121-01; Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) NaCl. In control experiments we determined that this medium allowed optimum growth of *P. anguilliseptica* and *Pseudomonas aeruginosa* strains and excellent growth of all of the other bacteria used. The doubling time of *P. anguilliseptica* ET7601 was 4.4 h at 20°C, whereas the maximal attainable values of adsorbance at 600 nm were about 1.7. Occasionally, the medium was supplemented with 0.5 to 1% (wt/vol) RILA marine salts mixture (Rila Products, Teaneck, N.J.). All cells were grown at 20°C with shaking and were harvested during the mid-logarithmic phase of growth (absorbance at 600 nm, 0.6 to 0.8). The solid medium used consisted of 1% (wt/vol) proteose peptone no. 2 medium, 0.5% (wt/vol) NaCl, and 2% (wt/vol) agar (Difco).

Whole-cell fatty acid analysis. Whole-cell fatty acids (both lipopolysaccharide- and lipid-associated fatty acids) were analyzed after transmethylation by gas-liquid chromatography, as previously described by Kropinski et al. (11). Peaks were identified by comparison with a standard bacterial fatty acid mixture obtained from Supelco Ltd., Bellefonte, Pa. Pentadecanoic acid was used as an internal standard to assist quantitation; control experiments were performed to detect pentadecanoic acid in individual cells.

Outer membrane isolation and SDS-polyacrylamide gel electrophoresis. Outer membranes were routinely isolated by the method described by Hancock and Nikaido (7) for the isolation of *P. aeruginosa* outer membranes. From three to six bands were present on each four-step sucrose gradient. Generally, only the uppermost band had properties of inner membranes (i.e., high succinate dehydrogenase levels, relatively low 2-keto-3-deoxyoctulosonic acid levels, and little or none of the major outer membrane protein observed in the denser bands). The two to five lower bands were identified as outer membranes based on their high 2-keto-3-deoxyoctulosonic acid levels (1.2 to 5.3 μg/mg of protein), low succinate dehydrogenase levels (<0.1 μmol of dichloroindolephenol reduced per min per mg of protein), and characteristic protein patterns (7). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as previously described (6); a 14% running gel containing 0.07 M NaCl was used.

Antigenicity of outer membranes. Outer membranes (50 μg of protein) from *P. anguilliseptica* strains ET7601 and ET2 were separately injected subcutaneously into DBA mice (Jackson Laboratories, Bar Harbor, Maine). After 2 and 4 weeks, the mice were given subcutaneous booster injections containing 10

TABLE 1. Bacterial strains used in this study[a]

Species	Strain	Source
P. anguilliseptica	NCMB 1949[T]	Japanese eel
	NCMB 1950	Japanese eel
	ET7601	Japanese eel
	ET2	Japanese eel
	S1	Japanese eel
	SE1	Japanese eel
	NE2	Japanese eel
	ME1	Japanese eel
P. fluorescens	ATCC 13525[T]	Prefilter water works tanks
	ATCC 949	Creamery waste
P. aeruginosa	PAO1	Human isolate (originally designated
	ATCC 8689	P. fluorescens)
	ATCC 9721	(originally designated
		P. fluorescens)
P. putida	ATCC 12633[T]	Soil
	K4359	Soil
P. acidovorans	ATCC 9355	Soil
Pseudomonas sp.	BS-3	Black seabream
Aeromonas salmonicida	NCMB 1102	Salmon
	NCMB 2020	Japanese salmon
Aeromonas hydrophila	ET2	Eel
Edwardsiella tarda	ET79054	Eel
Vibrio anguillarum	ET208	Eel
	PB15	Ayu
	HT7602	Yellowtail
"Vibrio anguillicida"	ET517	Eel
	ATCC 33149	Eel
Vibrio cholerae (non O-I)	PS7910	Ayu

[a] All strains were characterized by using standard biochemical tests (13, 14, 18).

μg of outer membrane protein, and 7 days after the third injection the mice were bled through the tail. The sera from these blood samples were diluted and tested by an enzyme-linked immunosorbent assay (1) for antibodies to outer membranes; outer membranes were used as the coating antigen, and alkaline phosphatase-linked rabbit anti-mouse immunoglobulin was used as the second antibody. The results were recorded with a Titertek Multiscan spectrophotometer (Flow Laboratories, Inglewood, Calif.) 30 min after para-nitrophenyl phosphate was added. Serum from uninjected DBA mice was used as a control.

RESULTS

Fatty acid composition of P. anguilliseptica. To determine whether the strains of P. anguilliseptica isolated to date represented a single taxonomic species, we determined the whole-cell fatty acid compositions of 79 strains (Table 2) which previously had demonstrated a large degree of similarity in biochemical tests (13). The utility of whole-cell fatty acid composition for differentiating Pseudomonas strains has been clearly demonstrated by Moss and his colleagues (3, 10, 12). The data in Table 2 strongly suggested that P. anguilliseptica represents a single taxonomic species.

Isolation of the outer membrane of P. anguilliseptica and properties of the outer membrane proteins. Fractionation of P. anguilliseptica cell

envelopes on a sucrose density step gradient produced outer membrane bands having different densities but very similar protein compositions (Fig. 1).

A comparison of the outer membrane protein compositions of seven P. anguilliseptica strains revealed striking similarities in protein composition with respect to both major and minor proteins (Fig. 2 and Table 3). However, the differences were large enough to suggest that these individual isolates were not genetically identical.

The properties of the individual outer membrane proteins were studied by changing the solubilization conditions before SDS-polyacrylamide gel electrophoresis. The apparent molecular weights of two proteins were influenced by the temperature of solubilization before gel electrophoresis. One protein, the 36.5K protein, which was found only in strain ET2, had apparent molecular weights of 34,500 after solubilization at 37°C and 36,500 after solubilization at 88°C. Another protein, the 22K protein, which was found in the other six strains (strains NCMB 1949[T] [type strain], NCMB 1950, ET7601, SE1, ME1, and NE2) but not in strain ET2, was also heat modifiable. This protein co-electrophoresed with the 19K protein (apparent molecular weight, 19,000) after solubilization at 37°C, whereas it had an apparent molecular weight of

22,500 after solubilization at 88°C. Heat modification of the 22K and 36.5K proteins was inhibited by the presence of 0.1 M Mg^{2+} in the solubilization mixture. Under such conditions, after solubilization at 88°C both the heat-modified (higher-molecular-weight) forms and the heat-unmodified (lower-molecular-weight) forms of these proteins were observed together in the gel. Similar behavior has been observed for major outer membrane protein G of *P. aeruginosa* (6) and for protein II* of *Escherichia coli* K-12 (15).

The appearance of the 12K protein in gels was influenced in most strains by the presence of ethylenediaminetetraacetate or Mg^{2+} in the solubilization mixture. Mg^{2+} favored the appearance of this protein, whereas ethylenediaminetetraacetate inhibited its appearance. Similar results have been obtained for a Braun type of outer membrane lipoprotein (protein I) of *P. aeruginosa* and have been shown to result from the influence of ethylenediaminetetraacetate and Mg^{2+} on the migration of lipopolysaccharide (which can mask this lipoprotein) in gels (6, 11).
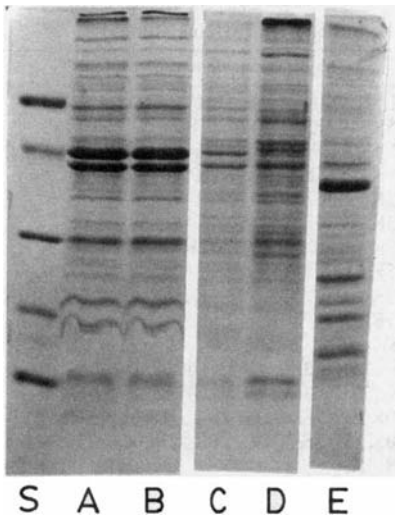

FIG. 1. SDS-polyacrylamide gel electrophoretograms of membrane bands from step sucrose density gradient separation of *P. anguilliseptica* strain ET7601 cell envelopes. Gels A, B, C, and D show the protein compositions of the bands at the interfaces of the original 70 to 64% sucrose step (OM1 band [9]), the 64 to 58% sucrose step (OM2 band), the 58 to 52% sucrose step (M band), and the 52 to 44% sucrose step (IM band), respectively. In gel E the OM2 band of *P. aeruginosa* PAO1 (7) is shown for comparison. The molecular weights of the major outer membrane protein bands were estimated by comparison with a series of molecular weight standards (gel S), including bovine serum albumin (molecular weight, 66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400).

TABLE 2. Fatty acid compositions of *P. anguilliseptica* strains isolated from Japanese eels[a]

Strain(s)	Fatty acids (% of total)[a]									Total fatty acids (nmol/ mg [cell dry wt])
	11:0	3OH 10:0	UK	12:0	2OH 12:0	3OH 12:0	16:1	16:0	18:1	
NCMB 1949[T]	2	6	5	3	T	4	30	12	38	84.2
NCMB 1950	3	5	4	4	T	5	31	11	36	81.3
ET7601	4	6	5	4	T	5	30	11	36	79.5
ET2	1	6	5	4	T	4	31	13	38	76.4
SE1	2	6	4	4	T	5	33	13	32	78.4
NE2	3	5	4	4	2	5	29	9	39	87.4
ME1	2	6	4	3	T	5	32	15	34	97.7
79 strains	3.6 ± 1.7 (T-9)[b]	5.8 ± 0.7 (4-7)	4.4 ± 0.4 (3-6)	3.6 ± 0.6 (3-5)	0.4 (T-5)	4.6 ± 0.8 (3-6)	31.5 ± 2.4 (27-37)	12.5 ± 1.6 (9-15)	34.1 ± 2.8 (29-39)	82.4 ± 18.6 (43.8-109.7)

[a] For the individual fatty acids, the number to the left of the colon refers to the number of carbon atoms, and the number to the right refers to the number of double bonds; 2OH and 3OH refer to hydroxyl groups in positions 2 and 3 on the carbon chain. UK, Unknown which ran between the 3OH 10:0 and 12:0 peaks and had a retention time of 0.41 relative to the internal standard, pentadecanoic acid. T, Trace amount (i.e., <1% of the total).

[b] Mean ± standard deviation for the 79 strains studied. The numbers in parentheses are ranges.
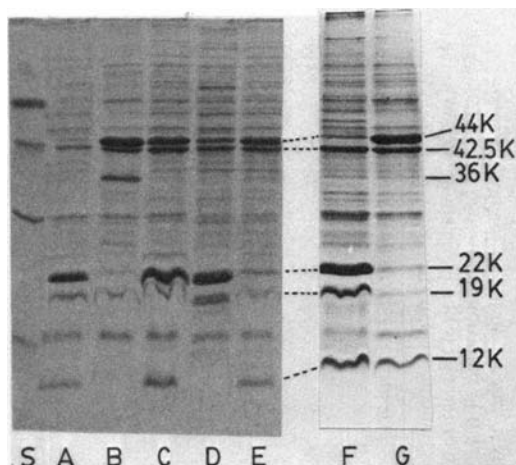
FIG. 2. SDS-polyacrylamide gel electrophoretograms of the outer membranes (OM1 on OM2 bands [see Fig. 1]) of *P. anguilliseptica* strains ET7601 (gel A), ET2 (gel B), SE1 (gel C), NE2 (gel D), ME1 (gel E), NCMB 1949^T (gel F), and NCMB 1950 (gel G). Gel S contained standards (see the legend to Fig. 1).

In addition to altering the solubilization conditions, we also isolated outer membranes from strain ET7601 grown with a variety of salt supplements. This strain (as well as strain NCMB 1949^T) had far lower levels of the 44K protein in its outer membrane (Fig. 2) than other strains when they were grown under our standard conditions (proteose peptone no. 2 medium containing 0.5% NaCl). However, omission of saline from proteose peptone no. 2 medium resulted in production of the 44K protein at levels equal to those present in other *P. anguilliseptica* strains (Fig. 3, gel B). The presence of 0.5% marine salts in the medium prevented the suppression of 44K protein production by 0.5% NaCl (Fig. 3, gels G and C), whereas even 1% marine salts did not suppress the appearance of this protein (Fig. 3, gel I). The concentration of NaCl in the medium apparently influenced the appearance in the gel of at least one other protein. A minor 31,000-dalton protein (31K protein) appeared at its greatest concentration in cells grown on 1% NaCl (Fig. 3, gel D).

TABLE 3. Major outer membrane proteins of the bacterial strains studied[a]

Species	Strain	Major outer membrane proteins
P. anguilliseptica	NCMB 1949^T	(44K), 42.5K, 22K, 19K, 12K[b]
	NCMB 1950	44K, 42.5K, (22K), (19K), 12K
	ET7601	(44K), 42.5K, 22K, 19K, 12K[c]
	ET2	44K, 42.5K, 36.5K, 19K, 12K[c]
	SE1	44K, 42.5K, 22K, 19K, 12K
	NE2	44K, 42.5K, 22K, 19K, 12K[c]
	ME1	44K, 42.5K, (22K), (19K), 12K
Pseudomonas sp.	BS-3	44K, 42.5K, 12K
P. fluorescens	ATCC 13525^T	43.5K, 42K, 41K, 33K, 25.5K, 20K, (19.5K), 12K
	ATCC 949	41K, 33.5K, 25K, 20K, 19.5K, 12K
P. aeruginosa	PAO1	(42K), 38K, 25K, 20K, 12K[c]
	ATCC 8689	42K, 38K, 25K, 20K, 18.5K, 12K[d]
	ATCC 9721	42K, 38K, 25K, 20K, 18.5K, 12K[d]
P. putida	ATCC 12633^T	41.5K, 40K, 37.5K, 21K, 20K, (18.5K), 12K
	K4359	41.5K, 40K, 37.5K, 21K, 20K, 18.5K, 12K
P. acidovorans	ATCC 9355	33.5K, 28.5K
Aeromonas salmonicida	NCMB 1102	44K, 40K, (37.5K), 30K, 29K, 12.5K
	NCMB 2020	44K, 40K, (37.5K), 30K, 29K, 12.5K
Aeromonas hydrophila	ET-2	45K, 40K, 37.5K, 28.5K, 24K, 22.5K, 12K
Edwardsiella tarda	ET79054	41K, 38K, 32K, 12.5K
Vibrio anguillarum	ET208	33.5K
	PB15	36K, 27K
	HT7602	45K, 37K, 27K
"*Vibrio anguillicida*"	ET517	44K, 35K, (27.5K)
	ATCC 33149	44K, 42K, 35K, 27.5K
Vibrio cholerae (non 0-I)	PS7910	39.5K, 33.5K, 27.5K

[a] A major protein that comigrated with lysozyme (the 14.4K protein) has been omitted from this table since it may represent contaminating lysozyme which bound to the outer membranes.

[b] The proteins in parentheses are proteins with less dense bands corresponding to the major bands of related bacterial strains.

[c] The 12K protein was observed in SDS-polyacrylamide gels only after solubilization of the outer membranes in the presence of Mg^{2+}.

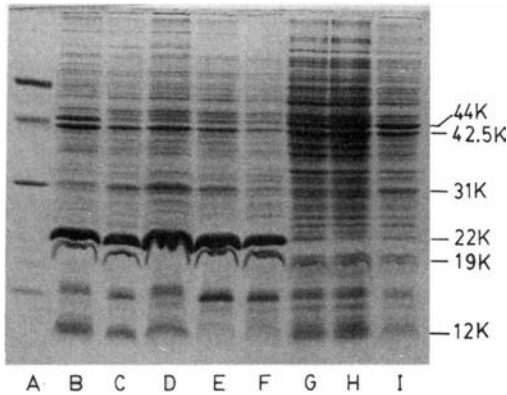[d] The 18.5K protein was probably the heat-unmodified form of the 25K protein (6).

FIG. 3. SDS-polyacrylamide gel electrophoretograms of outer membranes isolated after growth of *P. anguilliseptica* strain ET7601 on proteose peptone no. 2 medium containing the following salt supplements: gel B, no salt supplement; gel C, 0.5% (wt/vol) NaCl; gel D, 1% (wt/vol) NaCl; gel E, 2% (wt/vol) NaCl; gel F, 3% (wt/vol) NaCl; gel G, 0.5% (wt/vol) NaCl and 0.5% (wt/vol) marine salts mixture; gel H, 0.5% (wt/vol) marine salts mixture; gel I, 1% (wt/vol) marine salts mixture. Gel A contained standards (see the legend to Fig. 1).

The inclusion of marine salts in the growth medium had two other effects. First, it resulted in considerably poorer separation of the outer and inner membranes of *P. anguilliseptica* strain ET7601; and second, it resulted in loss of the 22K protein, the major outer membrane polypeptide band of strain ET7601 (Fig. 3, gels G through I).

Comparison with other strains. Outer membranes were isolated from six other species of fish pathogens and five other species of the genus *Pseudomonas*. Examples of separations of these membranes are shown in Fig. 4. In each case excellent separation was achieved, as determined by assays of an outer membrane marker (2-keto-3-deoxyoctulosonic acid) and of an inner membrane marker (succinate dehydrogenase) (data not shown) and by the protein patterns after SDS-polyacrylamide gel electrophoresis (Fig. 4). The outer membrane protein patterns of all of the strains are shown in Fig. 5, and the patterns of major outer membrane proteins are summarized in Table 3. Our results suggest that there are not only large differences in outer membrane protein patterns among species within the same genus but also marked similarities within a species (see below).

Immunological cross-reaction of outer membranes. To obtain further evidence for the relatedness of *P. anguilliseptica* strains, we produced antibodies in mice against the whole outer membranes of strains ET7601 and ET2. Sera raised against strain ET7601 outer membranes

reacted strongly with the outer membranes from the other six *P. anguilliseptica* strains and from *Pseudomonas* sp. strain BS-3 (Table 4) and 10- to 40-fold less with *P. aeruginosa* PAO1, *Pseudomonas fluorescens* ATCC 13525^T, and *Pseudomonas putida* ATCC 12633^T outer membranes. All other outer membranes reacted weakly with anti-ET7601 outer membrane serum (Table 4). There was no measurable reaction with normal mouse serum.

The results with antisera directed against strain ET2 outer membranes were somewhat different. The strongest reactions were with the
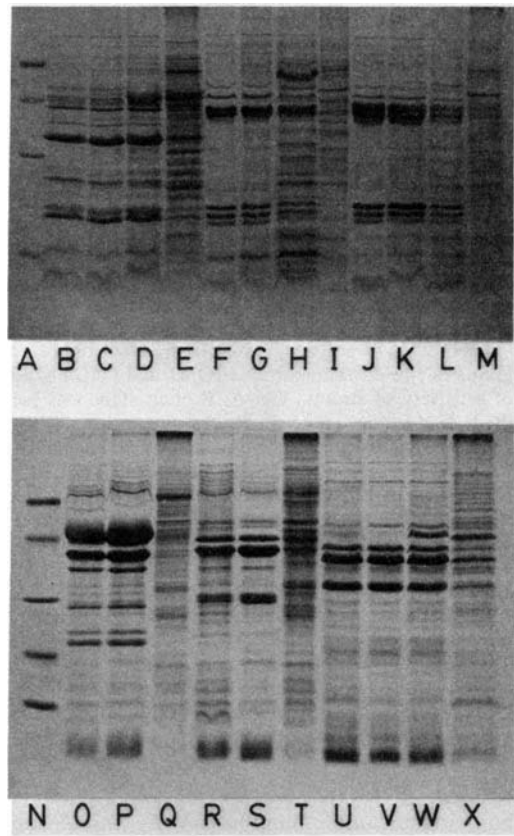

FIG. 4. SDS-polyacrylamide gel electrophoretograms of bands from step sucrose density gradient separation of cell envelopes from *P. fluorescens* ATCC 13525^T (gels B through E), *P. putida* K4359 (gels F through I), *P. putida* ATCC 12633^T (gels J through M), *Aeromonas hydrophila* ET2 (gels O through Q), *Aeromonas salmonicida* NCMB 1102 (gels R through T), and *Edwardsiella tarda* ET79054 (gels U through X). By analogy with previously described separations (9) and those described in the legend to Fig. 1, the gels represent OM1 (gels B, F, J, O, R, and U), OM2 (gels C, G, K, P, S, and V), M (gels D, H, L, and W), and IM (gels E, I, M, Q, T, and X) bands. Gels A and N contained standard proteins, as described in the legend to Fig. 1.
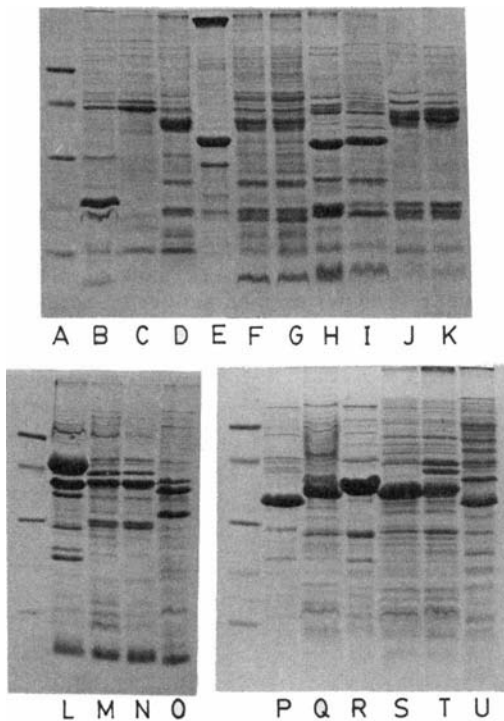
FIG. 5. SDS-polyacrylamide gel electrophoretograms of the outer membrane (OM1 and OM2) bands of a variety of strains. Gel A, Protein standards (see the legend to Fig. 1); gel B, *P. anguilliseptica* ET7601; gel C, *Pseudomonas* sp. BS-3; gel D, *P. aeruginosa* PAO1; gel E, *P. acidovorans* ATCC 9355; gel F, *P. aeruginosa* ATCC 8689; gel G, *P. aeruginosa* ATCC 9721; gel H, *P. fluorescens* ATCC 949; gel I, *P. fluorescens* ATCC 13525^T; gel J, *P. putida* K4359; gel K, *P. putida* ATCC 12633^T; gel L, *Aeromonas hydrophila* ET2; gel M, *Aeromonas salmonicida* NCMB 1102; gel N, *A. salmonicida* NCMB 2020; gel O, *Edwardsiella tarda* ET79054; gel P, *Vibrio anguillarum* ET208; gel Q, *V. anguillarum* PB15; gel R, *V. anguillarum* HT7602; gel S, "*Vibrio anguillicida*" ET517; gel T, "*V. anguillicida*" ATCC 33149; gel U, *Vibrio cholerae* (non O-I) PS7910.

homologous outer membranes, *P. anguilliseptica* SE1 outer membranes, and *P. fluorescens* ATCC 13525^T outer membranes (Table 4). The reactions of all other pseudomonad outer membranes tested except *P. aeruginosa* PAO1 and *Pseudomonas acidovorans* ATCC 9355 outer membranes were 13- to 20-fold weaker. These results suggest that strain ET2 outer membranes contain an immunodominant antigen which is not shared by the other *P. anguilliseptica* strains studied.

DISCUSSION

Our results suggest that outer membrane protein patterns might provide a useful adjunct for indicating genetic similarities and differences within a single species.

A fatty acid analysis of 79 *P. anguilliseptica* strains strongly indicated that these strains were related and could be differentiated from other *Pseudomonas* species, including *P. acidovorans* (12), *P. aeruginosa* (11, 12), *P. alcaligenes* (3), *P. diminuta* (19), *P. maltophilia* (12), *P. pseudoalcaligenes* (3), *P. putida* (12), "*P. putrefaciens*" (3), *P. stutzeri* (19), "*P. syncyanea*" (19), and *P. testosteroni* (12). The most closely related strains on the basis of fatty acid composition were those of *P. aeruginosa*, *P. putida*, and "*P. syncyanea*;" each of these taxa had seven major fatty acid peaks in common with *P. anguilliseptica*, including all of the fatty acids (C12:0, 3OH C10:0, 2OH C12:0, and 3OH C12:0) in *P. aeruginosa* and "*P. syncyanea*" which are found exclusively in the lipopolysaccharide (11, 19). However, fatty acid composition alone is of limited taxonomic use, since, for example, the fatty acid compositions of *P. fluorescens* (2) and *P. aeruginosa* (Nakajima and Hancock, unpublished data) can vary substantially with growth temperature and the appearance of at least one group of fatty acids, the cyclopropanes, is related to the phase of growth during which the bacteria are harvested (2, 5).

We also produced antisera against the outer membranes of two different *P. anguilliseptica* strains. When antiserum against strain ET7601 outer membranes was tested (Table 4), the outer membranes of each of the six *P. anguilliseptica* strains and *Pseudomonas* sp. strain BS-3 reacted as well as strain ET7601 outer membranes, suggesting that these outer membranes shared antigens. However, when antiserum against strain ET2 outer membranes was used, the outer membranes of other *P. anguilliseptica* strains showed enzyme-linked immunosorbent assay titers which were no greater than those of some other *Pseudomonas* species (Table 4). Thus, the usefulness of antigenic relationships in demonstrating the taxonomic relationships among *P. anguilliseptica* strains depends largely upon the antigen used to produce the antiserum. This antigenic specificity is probably due to the lipopolysaccharide, which is generally the immunodominant antigen in gram-negative bacteria and can vary widely within a species. Unfortunately, we were not able to isolate significant quantities of lipopolysaccharide from *P. anguilliseptica* strains by classical techniques, and thus we can shed no further light on the antigenic relationships of the strains within this species or among species in the genus. In contrast, using monoclonal antibody MA1-6, we have recently demonstrated the presence of a common outer membrane protein antigen in *P. aeruginosa* (protein H2), *P. fluorescens*, *P. putida*, *Azotobacter vin-*

TABLE 4. Enzyme-linked immunosorbent assay of outer membranes using antisera raised against the outer membranes of *P. anguilliseptica* strains ET7601 and ET2

Outer membrane antigens from:		Titer[a]	
Species	Strain	Anti-ET7601 outer membrane	Anti-ET2 outer membrane
P. anguilliseptica	NCMB 1949^T	4.2×10^3	9.5×10^2
	NCMB 1950	4.1×10^3	1.0×10^3
	ET7601	4.5×10^3	4.4×10^2
	ET2	3.7×10^3	8.2×10^3
	SE1	1.2×10^4	1.6×10^3
	NE2	5.5×10^3	6.4×10^2
	ME1	8.5×10^3	6.0×10^2
Pseudomonas sp.	BS-3	3.0×10^3	5.0×10^2
P. aeruginosa	PAO1	3.0×10^2	$\leq 10^1$
	ATCC 8689	8×10^1	4.0×10^2
	ATCC 9721	3×10^1	4.8×10^2
P. fluorescens	ATCC 1325^T	1.6×10^2	3.2×10^3
	ATCC 949	$\leq 10^1$	4.2×10^2
P. putida	ATCC 12633^T	1.0×10^2	6.4×10^2
	K4359	5×10^1	4.4×10^2
P. acidovorans	ATCC 9355	$\leq 10^1$	$\leq 10^1$
Aeromonas hydrophila	ET2	$\leq 10^1$	$\leq 10^1$
Aeromonas salmonicida	NCMB 1102	$\leq 10^1$	$\leq 10^1$
	NCMB 2020	$\leq 10^1$	$\leq 10^1$
Edwardsiella tarda	ET79054	7.5×10^1	$\leq 10^1$
Vibrio anguillarum	ET208	$\leq 10^1$	$\leq 10^1$
	PB15	$\leq 10^1$	$\leq 10^1$
	HT7602	$\leq 10^1$	$\leq 10^1$
"*Vibrio anguillicida*"	ET517	5×10^1	$\leq 10^1$
	ATCC 33149	5×10^1	$\leq 10^1$
Vibrio cholerae (non 0-I)	PS7910	1.1×10^2	$\leq 10^1$

[a] Titers (from an enzyme-linked immunosorbent assay) represent the reciprocal of the dilution of antiserum that resulted in 30% of the value for absorbance at 490 nm obtained for a 10^{-2} dilution of each antiserum (e.g., anti-ET2 outer membrane) when the equivalent outer membrane (e.g., ET2 outer membrane) was used as the antigen.

elandii, and *P. anguilliseptica*, which is not present in the outer membranes of the other strains described here (8).

The data in Table 4 suggest that there are at least two serological groups of *P. anguilliseptica* strains, in agreement with the previous data obtained by using heat- or Formalin-killed cells to produce antisera (14). These data suggest that the basis for differentiating type I (typified by strain ET7601) and type II (typified by strain ET2) *P. anguilliseptica* strains may be more complex than previously suggested (14).

The outer membrane protein patterns further demonstrated that the *P. anguilliseptica* strains were closely related in that five major proteins were observed in most strains under the appropriate growth and solubilization conditions. The major difference was that the 22K protein (a heat-modifiable major outer membrane protein) found in the other six strains studied was not found in strain ET2. Instead, the 36.5K protein (a heat-modifiable outer membrane protein) was uniquely present in this strain. We cannot say whether these heat-modifiable proteins are re-

sponsible for the serological differences discussed above. The 22K protein was present in quite different amounts in the six strains containing this protein, and it could be completely suppressed by the presence of a crude marine salts mixture in the growth medium (Fig. 3). Together with the serological relationship noted above, the presence in *Pseudomonas* sp. strain BS-3 of three major outer membrane protein bands having molecular weights similar or identical to those of *P. anguilliseptica* major outer membrane proteins suggests that this strain should also be classified as a member of *P. anguilliseptica* despite its different origin (Table 1).

Thus, outer membrane protein patterns appear to be useful for showing the similarity among *P. anguilliseptica* strains, and these patterns are clearly different from those of other species, as shown in Table 3 and Fig. 4. One indication of the usefulness of this technique is illustrated by *Pseudomonas* strains ATCC 8689 and ATCC 9721. These strains were preserved in our departmental collection for many years as

strains of *P. fluorescens*. However, the outer membrane protein patterns of these strains were very similar to the outer membrane pattern of our standard laboratory *P. aeruginosa* strain PAO1. Upon investigation, we found that these strains were originally isolated and presented to the American Type Culture Collection as *P. fluorescens* strains and were later reclassified as *P. aeruginosa* strains. We have investigated representative strains from all 17 serological types of *P. aeruginosa* and have found that the major outer membrane protein patterns are very similar to those shown in Fig. 4 and Table 3 (L. M. Mutharia, T. I. Nicas, R. E. W. Hancock, J. Infect. Dis., in press) for *P. aeruginosa* H103. The two *P. putida* strains which we studied are related to one another on the basis of outer membrane protein patterns, as are the two *P. fluorescens*, two *Aeromonas salmonicida*, and two *Vibrio* spp. strains studied (Table 3).

It seems likely that major outer membrane proteins are strongly conserved within a species. However, limitations on the use of major outer membrane protein patterns in taxonomy should be noted. For example, of the three strains identified as *Vibrio anguillarum* on the basis of biochemical tests, strain ET208 was clearly different from the other two (Table 3). In addition, extensive studies on *E. coli* outer membrane proteins have shown small but significant differences in the major outer membrane proteins of some strains, with up to 36 different patterns (15). However, two proteins having slightly different molecular weights from two strains of *E. coli* frequently cross-react immunologically (9).

It has been suggested recently that on the basis of ribosomal ribonucleic acid homologies (4) and deoxyribonucleic acid hybridization (16), the pseudomonads should be reclassified into five groups. As noted above, *P. anguilliseptica* shows strong similarities in whole-cell fatty acid patterns to *P. aeruginosa* and *P. putida* (12) (Table 2), whereas the outer membranes of these species cross-react immunologically with the outer membranes of certain *P. aeruginosa, P. fluorescens*, and *P. putida* strains. Thus, we suggest that despite its apparent lack of pigment formation (18), *P. anguilliseptica* may well fit into group I of De Vos, which he calls the authentic pseudomonads.

LITERATURE CITED

1. **Buchanan, T. M., and J. F. Hildebrand.** 1981. Antigen-specific serotyping of *Neisseria gonorrhoeae*: characterization based upon principal outer membrane protein. Infect. Immun. **32**:985–994.
2. **Cullen, J., M. C. Phillips, and G. G. Shipley.** 1971. The effects of temperature on the composition and physical properties of lipids of *P. fluorescens*. Biochem. J. **125**:733–742.
3. **Dees, S. B., and C. W. Moss.** 1975. Cellular fatty acids of *Alcaligenes* and *Pseudomonas* species isolated from clinical specimens. J. Clin. Microbiol. **1**:414–419.
4. **De Vos, P.** 1980. Intrageneric and intergeneric similarities of ribosomal RNA cistrons of the genus *Pseudomonas* and the implications for taxonomy. Antonie van Leeuwenhoek J. Microbiol. Serol. **46**:96.
5. **Hancock, I. C., and P. M. Meadow.** 1969. The extractable lipids of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **187**:366–378.
6. **Hancock, R. E. W., and A. M. Carey.** 1979. The outer membrane of *Pseudomonas aeruginosa*: heat- and β-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
7. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* and use in reconstitution and definition of the outer membrane permeability barrier. J. Bacteriol. **136**:381–390.
8. **Hancock, R. E. W., A. A. Wieczorek, L. M. Mutharia, and K. Poole.** 1982. Monoclonal antibodies against *Pseudomonas aeruginosa* outer membrane antigens. Isolation and characterization. Infect. Immun. **37**:166–171.
9. **Hofstra, H., and J. Dankert.** 1980. Major outer membrane proteins: common antigens in *Enterobacteriaceae* species. J. Gen. Microbiol. **119**:123–131.
10. **Kaltenbach, C. M., C. W. Moss, and R. E. Weaver.** 1975. Cultural and biochemical characteristics and fatty acid composition of *Pseudomonas diminuta* and *Pseudomonas vesiculare*. J. Clin. Microbiol. **1**:339–344.
11. **Kropinski, A. M., J. Kuzio, B. L. Angus, and R. E. W. Hancock.** 1982. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **21**:310–319.
12. **Moss, C. W., S. B. Samuels, and R. E. Weaver.** 1972. Cellular fatty acid composition of selected *Pseudomonas* species. Appl. Microbiol. **24**:596–598.
13. **Muroga, K.** 1978. Red spot disease of eels. Fish Pathol. **13**:35–39.
14. **Nakai, T., K. Muroga, and H. Wakabayashi.** 1980. Serological properties of *Pseudomonas anguilliseptica* in agglutination. Bull. Jpn. Soc. Sci. Fish. **47**:699–703.
15. **Overbeeke, N., and B. Lugtenberg.** 1980. Major outer membrane proteins of *Escherichia coli* strains of human origin. J. Gen. Microbiol. **121**:373–380.
16. **Palleroni, N. J., R. Kunisawa, R. Contopoulou, and M. Doudoroff.** 1973. Nucleic acid homologies in the genus *Pseudomonas*. Int. J. Syst. Bacteriol. **23**:333–339.
17. **Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.).** 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. **30**:225–420.
18. **Wakabayashi, H., and S. Egusa.** 1972. Characteristics of a *Pseudomonas* sp. from an epizootic of pond-cultured eels (*Anguilla japonica*). Bull. Jpn. Soc. Sci. Fish. **38**:577–587.
19. **Wilkinson, S. G., L. Galbraith, and G. A. Lightfoot.** 1973. Cell walls, lipids and lipopolysaccharides of *Pseudomonas* species. Eur. J. Biochem. **33**:158–174.