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Comparative Serology of the Marine Fish Pathogen *Vibrio anguillarum*

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The different serotyping systems, based on thermostable O antigens, reported for *Vibrio anguillarum* and *V. ordalii* were compared by quantitative agglutination, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequent silver staining or Western blotting (immunoblotting) of purified lipopolysaccharide (LPS), using polyclonal rabbit antisera. The results demonstrate that 16 different serotypes within *V. anguillarum* (designated O1 to O16) can be distinguished. Each of these serotypes is characterized by a distinct polysaccharide banding pattern, as revealed by silver-stained gels of purified LPS. The comparative analysis allowed a complete alignment of the different serotypes for the first three serovars: O1, O2, and O3. Moreover, immunoblotting showed that strains belonging to each of these serotypes had the same LPS banding pattern independent of the origin of the strain. Serotype O2 contains different subtypes, O2a and O2b. While no differences were apparent between these subgroups in silver-stained gels, they could be separated by quantitative agglutination (titer determination) or immunoblotting. *V. ordalii*, the former biotype II of *V. anguillarum*, strongly reacts with anti-*V. anguillarum* O2a antiserum. Strains of the two species can be separated on the basis of different LPS profiles in the high-molecular-weight region of silver-stained gels of purified LPS. The silver-stained LPS profiles of the different serotypes of *V. anguillarum* that have been established are provided for further comparison in the future.

Vibrio anguillarum is an important fish-pathogenic bacterium, causing septicemia and death in both wild and cultured fish throughout the world (8, 22, 33). *Vibrio ordalii*, the former biotype II of *V. anguillarum*, also causes vibriosis with comparable gross pathological symptoms (33). The two species can be separated by standard identification tests (27, 28). In addition to biochemical identification, serology has been widely used to characterize these pathogens. The serological study, based on serologically distinct, heat-stable somatic O antigens, i.e., lipopolysaccharides (LPS), was initiated by the work of Pacha and Kiehn (24), who described three serotypes for *V. anguillarum*: the Northwest salmonid type, the European Vibrio type (isolated from cod), and the Pacific herring type.

Since then, numerous studies have been conducted to improve the epidemiological database and to develop vaccines. As a result of these studies, 3 serotypes have been found in Norway (17), 2 or 3 have been found in the United States and Canada (4, 5, 11, 14, 31), 9 have been found in Japan (18, 19), 10 have been found in Denmark (30), and 8 have been found in Taiwan (29).

The complexity of the subject was further enhanced by the recognition of two subgroups (a and b) within serovar O2 (3, 25, 26) and the fact that *V. ordalii* and *V. anguillarum* O2 strains share common O-antigenic determinants (4, 11, 14). While the relation between the two subtypes of *V. anguillarum* O2 and *V. ordalii* has recently been studied in great detail by Mutharia et al. (23), consolidation of the different typing systems is urgently needed to allow detailed comparisons between the reported studies and to continue further epidemiological work as suggested by Larsen et al. (21).

In the study described here, representative strains from all reported typing systems have been compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of purified LPS, immunoblotting, and agglutination tests with polyclonal rabbit antisera. The results indicate that at least 16 different serotypes exist within the species *V. anguillarum*. Moreover, three of these serotypes are found worldwide, and two are responsible for almost all infections in fish. The techniques applied in this study enable us to separate these different serotypes, and the silver-stained LPS banding patterns are provided for further comparison in the future.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. The strains, maintained on Long preservation medium (36), were reactivated by being streaked onto brain heart infusion agar (Difco) to which 1% (wt/vol) NaCl had been added (BN). The identity of the strains used was verified by methods described previously (12). All tests and procedures were performed starting from overnight cultures incubated at 26°C on BN. In cooperation with D. Parkhurst, University of Washington, Seattle, we attempted to reculture the strains used by Pacha and Kiehn (24). Since these strains were lyophilized between 1953 and 1967, only some of them were found viable. Unfortunately, none of the "Pacific herring type" strains were recovered.

Serotyping. (i) Production of antisera. Antisera were produced against O-antigen suspensions of the strains indicated in italics in Table 1. Bacteria grown overnight on BN were harvested in sterile phosphate-buffered saline (PBS; 50 mM phosphate buffer, 0.15 M NaCl [pH 7.2]) to which formaldehyde was added to a final concentration of 0.3% (vol/vol). The suspensions were heated for 1 h at 100°C in a warm-water bath, and bacterial O antigens were harvested, washed, and subsequently resuspended in sterile PBS to an optical density of 0.7 (A_{600}). Aliquots (1 ml) of the resulting O-antigen suspensions for immunization were frozen at −20°C until use.

Polyclonal antisera to these strains were raised in New Zealand White rabbits by repeated injections of O-antigen suspensions. Animals were given subcutaneously injections of 0.5 ml of O-antigen suspension mixed with an equal volume of Freund's incomplete adjuvant (Difco, Detroit, Mich.). Three weeks later, intravenous injections of 0.25, 0.5, 1, and 1 ml of O-antigen suspension without Freund's incomplete adjuvant were given at daily intervals. One week after the last injection, the animals were exsanguinated by cardiac puncture, the blood was allowed to clot overnight at 5°C, and the sera were stored at −20°C.

Antisera raised against the Japanese serotypes A to C and D to I as described

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TABLE 1. *V. anguillarum* and *V. ordalii* strains used in this study and their serotype designations[a]

Strain	Collection no.[b]	Source[c]	Serotype	
			Stated as	According to present system
2.1	NCIMB 1873	D. Parkhurst	N.W. salmon	O1
4.3	NCIMB 1874	D. Parkhurst	N.W. salmon	O1
8		D. Parkhurst	N.W. salmon	O1
61-1	NCIMB 1875	D. Parkhurst	N.W. salmon	O1
78		D. Parkhurst	N.W. salmon	O1
83	NCIMB 1876	D. Parkhurst	N.W. salmon	O1
NCIMB 6	*NCIMB 6*	*NCIMB*	*European vibrio*[d]	*O2a*
V-2911	NCIMB 829	M. Tolmasky	European vibrio	O2a
775	*NCIMB 2286*	*M. Vigneulle*	*Type 775*[e]	*O1*
1669[f]		M. Vigneulle	Type 1669	O2a
VA 408		M. Vigneulle		O1
NCIMB 571	NCIMB 571	NCIMB	Type HI 10	O1
HI 10	NCIMB 2129	R. Wiik	Type HI 10	O1
HI 410	NCIMB 2130	R. Wiik	O2β	O2b
507		M. Tolmasky	507 group	O1
840613-2:1		I. Dalsgaard	O2α	O2a
820723-2:8		I. Dalsgaard	O2β	O2b
1474/1		I. Dalsgaard	O2β	O2b
1570/1		I. Dalsgaard	O2α	O2a
840814-1:10		I. Dalsgaard	O2β	O2b
6018/1	ATCC 43305	T. Jörgensen	O1	O1
1173/1	ATCC 43306	T. Jörgensen	O2	O2a
6062/A	*ATCC 43307*	*T. Jörgensen*	*O3*	*O3*
1356/1	*ATCC 43308*	*T. Jörgensen*	*O4*	*O4*
1384/1	*ATCC 43309*	*T. Jörgensen*	*O5*	*O5*
1406/1	*ATCC 43310*	*T. Jörgensen*	*O6*	*O6*
6192/3	*ATCC 43311*	*T. Jörgensen*	*O7*	*O7*
1733/2	*ATCC 43312*	*T. Jörgensen*	*O8*	*O8*
1247/1	*ATCC 43313*	*T. Jörgensen*	*O9*	*O9*
1347/1	*ATCC 43314*	*T. Jörgensen*	*O10*	*O10*
PT-7601		K. Muroga	A	O2a
PT-24		K. Muroga	A	O2a
PT-77161		K. Muroga	B	O3
PT-493		K. Muroga	B	O3
PT-77050		K. Muroga	C	O1
HT-77003		K. Muroga	C	O1
PT-77128		*K. Muroga*	*A*	*O2a*
ET-208		*K. Muroga*	*B*	*O3*
PT-213		*K. Muroga*	*C*	*O1*
PT-8004		K. Muroga	G	O14
V-123		Y. Ezura	J-O-2	O3
V-106		Y. Ezura	J-O-3	O1
PB-15		*T. Aoki*	*D*	*O11*
PB-28		*T. Aoki*	*E*	*O12*
ET-1		*T. Aoki*	*F*	*O13*
PT-80-187		*T. Aoki*	*G*	*O14*
PT-80-641		*T. Aoki*	*H*	*O15*
PT-80-644		*T. Aoki*	*I*	*O16*
NCIMB 2167[f]	NCIMB 2167	NCIMB		O2a

[a] Antisera were produced against O-antigen suspensions of the strains in italics.
[b] NCIMB, National Collection of Industrial and Marine Bacteria; ATCC, American Type Culture Collection.
[c] D. Parkhurst, School of Medicine, University of Washington; M. E. Tolmasky, Department of Microbiology and Immunology, Oregon Health Sciences University; I. Dalsgaard, Fish Disease Laboratory, Danish Institute for Fisheries and Marine Research; T. Jörgensen, The Norwegian College of Fisheries Science, University of Tromsö; M. Vigneulle, Laboratoire de Pathologie des Animaux Aquatiques, CNEVA; R. Wiik, Institute of Marine Research, Ministry of Fisheries, Bergen, Norway; Y. Ezura, Faculty of Fisheries, Hokkaido University; K. Muroga, Faculty of Applied Biological Science, Hiroshima University; T. Aoki, Department of Biological Resources, Faculty of Agriculture, Myazaki University.
[d] Also referred to as type NCIMB 6; J-O-1.
[e] Also referred to as 775 group, fast-growing 775 type.
[f] *Vibrio ordalii.*

by Kitao et al. (18, 19) were kindly provided by K. Muroga, Faculty of Applied Biological Science, Hiroshima University, Hiroshima, Japan, and by T. Aoki, Faculty of Agriculture, Miyazaki University, Miyazaki, Japan, respectively.

(ii) Serotyping and titer determination. O-antigen suspensions for agglutination tests were prepared as described by Sörensen and Larsen (30). Serotyping and titer determinations were performed in 96-well microtiter plates by serial twofold dilutions of the antisera in PBS starting from an initial 1:10 dilution. After the serial dilution of the antisera, an equal volume of O-antigen suspension was added to each well (final volume, 100 μl) and the microtiter plates were incubated overnight at 26°C in a moistened chamber. The titer was recorded as the reciprocal of the highest dilution which gave positive agglutination.

LPS purification. LPS was prepared by the proteinase K method described by Hitchcock and Brown (15). Bacteria, harvested and washed in sterile PBS, were resuspended to a transmission of 30% (at 525 nm) in sterile PBS. The cell pellet

obtained from 1.5 ml of this suspension was resuspended in 50 μl of Laemmli sample buffer (20) and heated for 10 min at 100°C. After heating, the residual bacterial debris was removed by centrifugation (8 min at 13,000 × *g*) and the supernatant was transferred into a new Eppendorf tube. An aliquot (10 μl) of proteinase K solution (2.5 μg of proteinase per μl of sample buffer) was added to the sample, and the mixture was incubated for 30 min at 60°C. This proteinase K treatment was performed twice. The resulting LPS samples were stored at −20°C.

SDS-PAGE and Western blotting. SDS-PAGE of the LPS samples for silver staining was carried out by the method of Laemmli (20) in the Bio-Rad Mini Protean II slab cell system. LPS samples (8 μl) were loaded in each well and electrophoresed at 200 V through a 12.5% acrylamide gel. Gel and buffer compositions were as prescribed in the Mini Protean II slab cell instruction manual.

LPS was silver stained by the method of Hitchcock and Brown (15); however, the fixation time was reduced to 20 min and the washing times between the oxidizing step and the silver-staining step were reduced to eight 3-min washes. After staining, gels were preserved in a preservation solution containing 10% acetic acid and 5% glycerol in distilled water. For all steps, samples were shaken (55 rpm) in an orbital shaker.

SDS-PAGE of LPS samples for Western blotting (immunoblotting) were performed on the Phast System (Pharmacia LKB, Uppsala, Sweden) with 12.5% (wt/vol) acrylamide homogeneous precast gels. LPS samples for electrophoresis and subsequent blotting, prepared as discussed above, were diluted 1:10 in sample buffer. Electrotransfer of the LPS to polyvinylidene difluoride membranes (Bio-Rad) was carried out in the PhastTransfer semidry electrophoretic transfer system (Pharmacia LKB). Blots were blocked for 2 h with skim milk in 50 mM Tris buffer (pH 7.6), washed in Tris saline buffer (TS; 10 mM Tris buffer, 150 mM NaCl, 0.1% [vol/vol] Triton X-100 [pH 7.6]), and subsequently incubated overnight with the primary antibody (dilution, 1:2,000 in TS). The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit antibody (Dakopatts, Copenhagen, Denmark), diluted 1:400 in TS, was applied for 1 h, and the blots were developed as described by Blake et al. (2) in a substrate solution containing BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt [Sigma, St. Louis, Mo.]) and nitroblue tetrazolium chloride (Sigma) in an alkaline phosphatase buffer (100 mM Tris buffer, 100 mM NaCl, 5 mM $MgCl_2$ [pH 9.6]).

RESULTS

Antiserum reactions and serotyping. Homologous antiserum titers varied between 1,280 and 10,240 depending on the antiserum. For all antisera, heterologous titers between an antiserum and one of the other strains used for the production of an antiserum were 40 or less. All strains under study were assigned to a certain serotype (Table 1) if their titer with one of the antisera resembled that of the homologous strain, plus or minus one dilution.

Exceptions to this were the results obtained for serotype O2 subgroup b (O2b) strains. While all these strains clearly reacted with the antiserum raised against serotype O2 of *V. anguillarum* (strain NCIMB 6), the titers of the O2b strains were in general lower (e.g., 160 to 640) than the titers of the O2a strains (e.g., 1,280 to 5,120). No differences were found between the titers of O2 and O2a strains. By repeated subculture, a rough-colony variant was selected from strain 1173/1, the type strain for serotype O2 according to the European typing system of Sörensen and Larsen (30). The rough mutant of this strain still showed a titer of 1,280, compared with 2,560 for the smooth-colony variant.

All *V. ordalii* strains tested reacted with the antiserum directed against strain NCIMB 6 (type strain of *V. anguillarum*) similarly to serotype O2a strains of *V. anguillarum*.

Use of the antisera raised against the American-European serotypes (O1 to O3) and the Japanese serotypes (A to C) allowed us to compare these typing systems. All strains that were agglutinated by the antiserum raised against strain 775 (serotype O1) were also agglutinated by the antiserum directed against the Japanese strain PT-213 (serotype C). The same cross-reactivity between antisera was obtained for the antisera against NCIMB 6 (serotype O2) and PT-77128 (serotype A) and for anti-6062/A (serotype O3) and anti-ET-208 (serotype B) antisera. The use of these antisera to type the strains listed in Table 1 resulted in a complete comparison of all previously reported typing systems for the first three serotypes. The results of this comparison are presented in Table 2. Strains 775 and 507, representing serogroups 775 and 507, respectively, of Strout et al. (31), reacted equally strongly with our antiserum raised against strain 775 and not with any of the other antisera. Therefore, we conclude that both these strains belong to the same serotype, e.g., serotype O1. With the exception of the first three serotypes, no cross-reactivity was observed between the different antisera and/or strains used. On the basis of our comparative results, a total of 16 serotypes were established for *V. anguillarum*, corresponding to the 10 serotypes described by Sörensen and Larsen (30) and an additional 6 serotypes (D to I) described by Kitao et al. (18, 19).

TABLE 2. Comparison of serotypes O1, O2, and O3 with serotype designations reported in the literature

Reference	Serotype designation for:		
	O1	O2	O3
Pacha and Kiehn (24)	Pacific Northwest salmonid	European vibrio	
Harrell et al. (14)	Type 775	Type 1669	
Johnsen (17)	Type HI 10	Type NCIMB 6	
Strout et al. (31)	775 and 507 group	569 group	
Gould et al. (11)	Fast-growing 775	Slow-growing 1669	
Ezura et al. (9)	J-O-3	J-O-1	J-O-2
Baudin Laurencin (1)	VA 408		
Kitao et al. (18)	C	A	B
Chart and Trust (4)	I	II	III
Sörensen and Larsen (30)	O1	O2	O3
Goerlich (10)	I	II	
Song et al. (29)	T-O-I	T-O-V	T-O-VI

Silver staining and Western blotting of purified LPS. To confirm the results obtained by serotyping, the 16 different O groups were analyzed by SDS-PAGE and silver staining (Fig. 1). Figure 1 clearly shows that all 16 serotypes of *V. anguillarum*, established by comparative serotyping, are characterized by individually distinct LPS banding patterns. The LPS banding pattern obtained for all the serotypes can be separated into three distinct zones: a high-molecular-weight (HMW) zone near the top of the gel, an intermediate-molecular-weight (IMW) zone around the center of the gel, and a low-molecular-weight (LMW) zone near the bottom of the gel. For most of the serotypes, the banding pattern observed in the different zones is the same; however, the spacing of the individual bands increases from top to bottom. In serotype O5 (Fig. 1A, lane 5), these different zones are not apparent and the LPS profile obtained is rather homogeneous. However, as with the other serotypes, the interband spacing increases near the bottom of the gel. Proteinase K, the enzyme used in the preparation of the LPS, is visible as a dark band near the top of the gel (Fig. 1B, lane PK). All strains assigned to a certain serotype (Table 1) had the same LPS banding pattern as did the strain used for the production of the antiserum with which they were agglutinated (data not shown).

The different titers obtained for the two serotype O2 subgroups of *V. anguillarum* and the strong homology between the titers of *V. anguillarum* O2a and *V. ordalii* were further studied by SDS-PAGE of the purified whole-cell LPS (Fig. 2). In Fig. 2, lane 1, the LPS pattern obtained for the rough-colony variant of strain 1173/1 is presented. The typical LPS banding pattern observed for the other O2 strains (lanes 2 to 5) is not apparent. However, as discussed above, this rough-colony variant still showed a titer of 1,280 compared with 2,560 for the

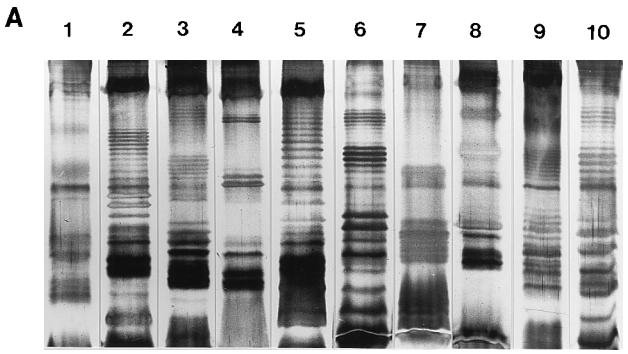

FIG. 1. Silver-stained LPS patterns of purified LPS of serotypes O1 to O16. (A) Serotypes O1 to O10. Lanes: 1, 775 (O1); 2, NCIMB 6 (O2); 3, 6062/A (O3); 4, 1356/1 (O4); 5, 1384/1 (O5); 6, 1406/1 (O6); 7, 6192/3 (O7); 8, 1733/2 (O8); 9, 1247/1 (O9); 10, 1347/1 (O10). (B) Serotypes O11 to O16. Lanes: 1, PB-15 (O11); 2, PB-28 (O12); 3, ET-1 (O13); 4, PT-80-187 (O14); 5, PT-80-641 (O15); 6, PT-80-644 (O16); PK, proteinase K.

smooth-colony variant. In Fig. 2, lanes 2 to 5, the LPS profiles of two O2a strains (PT-7601 and PT-24) and two O2b strains (820723-2/8 and 1474/1), respectively, are presented. No clear difference can be seen between the LPS patterns of the two subgroups of this serotype. The low heterogeneity observed in the staining intensity of different bands between strains PT-7601 and PT-24 (lanes 2 and 3) seems to be caused by a strain-specific dominance of LPS bands with a certain MW. The LPS pattern of *V. ordalii* 1669 is shown in lane 6. The LPS bands in the IMW and LMW zones of this species show a strong homology with the profile of the *V. anguillarum* serotype O2a and O2b strains. In the HMW region, the profile of *V. ordalii* does not show clear and tightly separated bands as do the *V. anguillarum* strains; instead, only weakly stained bands are present over a larger portion of the gel.

The apparent homology among the LPS patterns of the strains presented in Fig. 2 was further studied by Western blotting (Fig. 3). The samples used for this blotting came from the same actual purification as did the ones used for silver staining; however, as discussed in Materials and Methods, samples for blotting were diluted 1:10 in sample buffer. Although the rough-colony variant of strain 1173/1 did not show any specific LPS banding in the silver-stained gel (Fig. 2, lane 1), a pronounced banding became apparent in the Western blot (Fig. 3, lane 1), demonstrating the specificity of the antiserum. This result explains the relatively high titer obtained for the rough-colony variant in the serotyping. The differences in titer

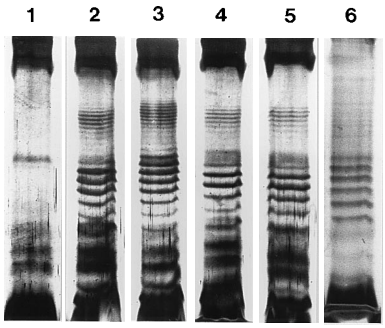

FIG. 2. Silver-stained LPS patterns of serotype O2 (a and b) strains of *V. anguillarum* and *V. ordalii*. Lanes: 1, 1173/1 rough-colony variant (O2a); 2, PT-7601 (O2a); 3, PT-24 (O2a); 4, 820723-2:8 (O2b); 5, 1474/1 (O2b); 6, *V. ordalii* 1669 (O2a).

obtained for the two subgroups of *V. anguillarum* O2 are further emphasized by the blotting results. The two O2a strains (lanes 2 and 3) show a strong reaction with the antiserum raised against strain NCIMB 6, while the two O2b strains (lanes 4 and 5) were only very faintly stained. However, the position and spacing of the bands of the O2b strains are not different from those obtained for the O2a strains.

The heterogeneity in the relative abundance of LPS bands with a certain MW observed between strains PT-7601 and PT-24 in the silver-stained gels is also apparent in the blotting. Figure 3 (lanes 2 and 3) clearly shows that for strain PT-24 (lane 3), the LMW LPS bands are less intensively stained than are the bands with the same apparent MW in strain PT-7601 (lane 2). *V. ordalii* (lane 6) shows the same LPS banding pattern, after Western blotting, as the *V. anguillarum* O2a strains do (lanes 2 and 3).

Only the LPS bands in the IMW and LMW zones of the blot are recognized by the antiserum. No reaction was obtained with the LMW bands observed in the silver-stained gels. The antiserum raised against serotype O2 did not recognize any bands in the blots of strains belonging to another serotype (results not shown).

The Western blot results for strains 6018/1 (serotype O1 [30]), HT-77003 (serotype J-O-3 [9]), PT-213 (serotype C [18]), 775 (serotype 775 [14]), 1173/1 (smooth variant) (serotype O2 [30]), and 6062/A (serotype O3 [30]) are given in Fig. 4, lanes 1 to 6, respectively. The results further substantiate the comparison made between the serotyping systems (Table 2). All strains belonging to serotype O1 (lanes 1 to 4) reacted strongly with the antiserum directed against strain 775. No reaction was

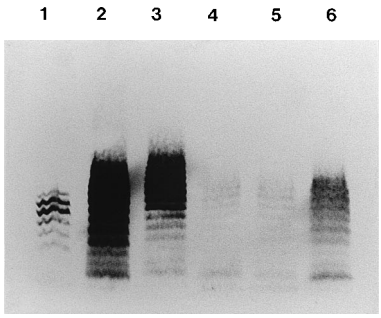

FIG. 3. Western blot of purified LPS from serotype O2 (a and b) strains of *V. anguillarum* and *V. ordalii* with polyclonal rabbit antiserum directed against strain NCIMB 6. Lanes: 1, 1173/1 rough-colony variant (O2a); 2, PT-7601 (O2a); 3, PT-24 (O2a); 4, 820723-2:8 (O2b); 5, 1474/1 (O2b); 6, *V. ordalii* 1669 (O2a).

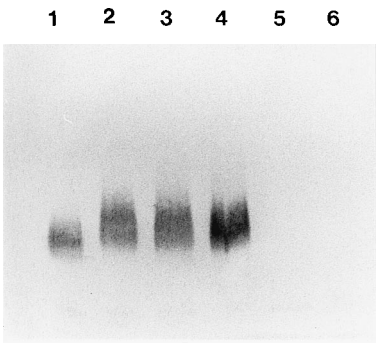

FIG. 4. Western blot analysis of LPS antigens with rabbit immune serum against *V. anguillarum* 775 (O1). Lanes: 1, strain 6018/1 (O1); 2, HT-77003 (O1); 3, PT-213 (O1); 4, 775 (O1); 5, 1173/1 smooth-colony variant (O2a); 6, 6062/A (O3).

obtained with one of the other serotypes (lanes 5 and 6). The LPS of strain 6018/1 (lane 1) has a lower apparent MW than do the LPSs of the other strains used.

The LPS patterns after Western blotting for three representative strains of serotype O3 (6062/A, PT-77161, and PT-493 [Fig. 5, lanes 1 to 3, respectively]) are highly similar. Unlike the results obtained for serotypes O1 and O2, serotype O3 reacts with the antiserum directed against strain 6062/A with a minor zone in the HMW region of the gel. No cross-reactivity was observed between the O3 antiserum and strains belonging to other serotypes.

DISCUSSION

In this study, the previously reported different serotyping systems based on heat-stable somatic O antigens (LPSs) were compared by the use of 19 polyclonal rabbit antisera and representative strains from the different typing systems (Table 1). The serotypes reported from Taiwan (29) were not included (with the exception of T-O-I, T-O-V, and T-O-VI), because they could not be obtained. The agglutination tests performed resulted in the establishment of 16 different serotypes within the species *V. anguillarum*. The first 10 of these serotypes (O1 to O10) represent the groups delineated by Sörensen and Larsen (30), and serotypes O11 to O16 were described as types D to I by Kitao et al. (18, 19). For the designation of these serotypes, we adopted the terminology applied by Sörensen and Larsen (30), i.e., the prefix O followed by a number.

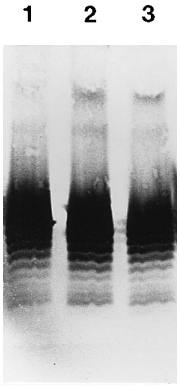

FIG. 5. Western blot analysis of LPS antigens of serotype O3 of *V. anguillarum* with antiserum directed against strain 6062/A. Lanes: 1, 6062/A (O3); 2, PT-77161 (O3); 3, PT-493 (O3).

Although the serotypes of Kitao et al. (18, 19) were described before those of Sörensen and Larsen (30), the typing system of the latter authors was retained to prevent confusion.

The analysis of all strains (listed in Table 1) against all antisera by titer determination made it possible to align all existing typing systems for the first three serotypes (Table 2). While serotypes O1 and O2 occur worldwide and are mainly responsible for diseases in fish (22), serotype O3 is only occasionally isolated from diseased fish and has, to our knowledge, been isolated only in Europe (3, 30) and in Asia (9, 18, 29, 32). To compare the third serotype described by Pacha and Kiehn (24) (the Pacific herring type) with the other serotypes described in the literature, we tried, in cooperation with D. Parkhurst, to reculture the original lyophilized cultures of Ordal and Kiehn. Since none of the strains belonging to this serotype were found viable and since no other strains belonging to this serotype have been described (5), the Pacific herring type had to be excluded from further comparative studies. Both the Northwest salmon type and the European type, described by Pacha and Kiehn (24), are represented in culture collections. The third serotype, described by Strout et al. (31), reacted as strongly with our anti-O1 antiserum as did the homologous strain (strain 775). A high degree of homology between group 507 and group 775 was also found by these authors. However, group 507 was placed separately because of a higher titer obtained with the antiserum directed against strain 507. Johnsen (17) described a third serotype of *V. anguillarum* based on the observation that strain NCIMB 1336 did not react with the antiserum produced against strain NCIMB 6 (O2) or HI 10 (O1) (NCIMB 2129). Strain NCIMB 1336 (ATCC 19105), originally isolated by Tubiash et al. from moribund larvae of hard clams (35), has latterly been assigned to the species *V. tubiashii* (13) and is therefore excluded from this *V. anguillarum* comparison. On the basis of these arguments and our results, the number of different serotypes reported by Ezura et al. (9) and later by Tajima et al. (32) and Horne (16) can be reduced from five to three. The comparison of serotypes given by Sörensen and Larsen (30) overlaps with our results. The homology observed between the different typing systems for the first three serotypes (Table 2) was further substantiated by electroblotting of purified LPS. As in the agglutination tests, the antisera reacted strongly with the strains assigned to the serotype to which they were targeted but not with strains of other serotypes.

SDS-PAGE and silver staining of LPS purified from the strains further confirmed the 16 different serotypes established by the agglutination tests. As shown in Fig. 1, all serotypes are characterized by a distinct LPS banding pattern. Moreover, strains isolated in different parts of the world but belonging to the same serotype showed the same LPS banding patterns. A small variability was observed in the relative abundance among the strains of polysaccharide bands with a particular MW. Most probably, this variability is caused by differences in the number of O-antigenic side chain units (7). However, this phenomenon did not interfere with the recognition of certain serotypes on the basis of the silver-stained LPS pattern.

The silver-stained LPS patterns of serotypes O1, O2, and O3 reported previously (4, 23) resemble to a high degree the results shown in Fig. 1. Therefore, we conclude that SDS-PAGE and silver staining of purified LPS provide a useful tool for a preliminary grouping of strains into serotypes. However, confirmation of the serotypes of these groups must be obtained either by agglutination or by Western blotting with specific antisera.

The sensitivity of silver staining compared with electroblotting is indicated by the staining obtained for a rough-colony

variant of strain 1173/1 (Fig. 2 and 3, lanes 1). No typical LPS banding pattern was obtained for this colony variant in the silver-stained gel; however, typical LPS bands were apparent in the (1:10-diluted) sample after electroblotting and immunostaining. This result is in agreement with the observations of Darveau and Hancock (7), who indicated that an O banding pattern could be obtained (by silver staining) from rough-colony variants if an overdose of an LPS preparation was applied to the gel.

Serotype O2 is without doubt the most complex serogroup of *V. anguillarum*. Not only does *V. ordalii*, the former biotype II of *V. anguillarum*, strongly cross-react with antisera raised against O2 strains of *V. anguillarum* (4; also see above), but also two different subgroups have been described within this serotype: O2a and O2b (25, 26) and O2α and β (3). *V. ordalii* has long been characterized as *V. anguillarum* biotype II (27, 28). In addition, a good correlation existed between biotype and serotype for strains isolated in North America (biotype I strains belonged to serotype O1, and biotype II strains belonged to O2) (11, 14). The relation between serotype and biotype was further stressed by the fact that serotype O2 strains are apparently less common in North America than in Europe and Japan (illustrated by the designation "European Vibrio type" by Pacha and Kiehn [24] and by the fact that true serotype O2 strains of *V. anguillarum* have been described only by Strout et al. [31] and by Mutharia et al. [23]). Our results further substantiate the antigenic homology between serotype O2 strains of *V. anguillarum* and *V. ordalii*. A comparable reaction was observed for both species by titer determination and by electroblotting. Silver staining of purified LPS revealed a difference in banding pattern between *V. anguillarum* O2 and *V. ordalii* in the HMW zone of the gel. However, the antisera produced against the heat-stable O antigens did not recognize these HMW bands and reacted solely with the polysaccharide bands in the IMW and LMW regions.

In a recent study, Mutharia et al. (23) studied serotype O2 in great detail by using polyclonal antisera produced in rabbits and rainbow trout against *V. anguillarum* O2 and *V. ordalii*. As in our study, unabsorbed rabbit sera did not distinguish between the species. However, as demonstrated by Mutharia et al. (23), antisera absorbed with the heterologous strain did. In the same study, the authors suggested that *V. anguillarum* O2 and O2a are identical and also that subgroup O2b strains should not be placed within the O2 serovar.

Our results are in accordance with the first statement; however, some objections can be made regarding the second. Although serotype O2b strains reacted weakly in electroblotting with an anti-O2a antiserum, a high titer was obtained in the agglutination tests. In most studies, serotyping is performed by direct slide agglutination as suggested by Conroy and Withnell (6) and Toranzo et al. (34). The titer obtained for the O2b strains with the anti-O2a antiserum is still sufficiently high to give a clear positive agglutination reaction in the direct slide agglutination test, which could give rise to incorrect results if both subgroups are placed in different serovars. Moreover, as reported by Larsen et al. (21), some O2 strains react only with unabsorbed O2 antiserum. On the basis of these considerations and the fact that the two subgroups are indistinguishable by SDS-PAGE and silver staining of purified LPS, we suggest that the present division of O2a and O2b be maintained. Serotype O2 strains can be assigned to these subgroups by titer determination, electroblotting of purified LPS, or the use of absorbed and unabsorbed antisera.

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