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Relationships between Plasmids and Phenotypes of Presumptive Strains of *Vibrio anguillarum* Isolated from Different Fish Species†

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On the basis of plasmid composition as well as serological and biochemical properties, 26 strains identified as *Vibrio anguillarum* isolated from diseased fish could be assigned to two different groups. Except for three reference strains, these strains were isolated from Norwegian fish. The four strains isolated from rainbow trout (*Salmo gairdneri*), the only strain isolated from char (*Salvelinus alpinus*), and three of six strains isolated from Atlantic salmon (*Salmo salar*) harbored a plasmid of 47 megadaltons (MDa). Restriction endonuclease analysis showed that this plasmid and the virulence plasmid pJM1, carried by *V. anguillarum* strain 775, were very similar but not identical. Strains harboring the 47-MDa plasmid had nearly identical biochemical properties and were serotype O1. Strains isolated from reared coastal cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*), free-living saithe (*Pollachius virens*), and partly from reared Atlantic salmon differed from strains harboring the 47-MDa virulence plasmid by not containing this plasmid, by having different biochemical traits, and by being serotype O2. Rainbow trout which were experimentally infected with a strain isolated from cod suffering from vibriosis developed clinical symptoms similar to those in cod but quite different from those usually seen in rainbow trout.

Vibriosis, caused by infection with *Vibrio anguillarum*, affects a number of fish species (6, 16, 27, 29). Pathogenic *V. anguillarum*-like strains have also been isolated from crustaceans and molluscs (4, 5, 19). Although vibriosis has been reported in complete freshwater culture (17), the disease is primarily a marine problem.

Epizootics of vibriosis have occurred in different locations along the Norwegian coast, affecting several anadromous and marine fish species. In the present study, strains of *V. anguillarum* isolated from locally reared rainbow trout, Atlantic salmon, cod, turbot, char, halibut, and free-living saithe suffering from vibriosis have been compared with respect to plasmid composition as well as serological and biochemical properties. The aim of this study was to examine a possible correspondence between groupings of bacterial strains with respect to these characteristics and groupings with respect to the host species from which they were isolated.

MATERIALS AND METHODS

Bacterial isolation and identification. The strains of *Vibrio anguillarum* used in this study are listed in Table 1. Fish with clinical symptoms of vibriosis (12, 14, 15) were selected for bacteriological examination. Samples were taken from the kidney with a sterile swab and inoculated on plates of nutrient agar (Oxoid Ltd., London, U.K.) supplemented with 5% sheep blood and 1% NaCl (final concentration, 1.5%) (NBA). The following characteristics were taken as preliminary identification of *V. anguillarum*: low, convex, semitranslucent, hemolytic colonies consisting of gram-negative, mobile, curved rods which were sensitive to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, 10 μl of a saturated solution per disc) (BDH Chemicals Ltd., London, U.K.), which grew better at 22°C than at 15°C after 48 h of incubation. Spleen imprints fixed and stained with M+D Diff-Quik (Merz+Dade AG, Düdingen, Switzerland) should be positive for curved rods.

Growth conditions. The general growth substrates used throughout this study were tryptone soy broth (Oxoid) supplemented with NaCl to a final concentration of 1.5% (TSB) and NBA. Generally, the *V. anguillarum* cultures (TSB) were incubated aerobically with continuous shaking for 18 to 24 h at 22°C.

Plasmid screening. Two plasmid detection methods were employed; one was described by Kado and Liu (20), and the other was described by Wiik et al. (33). The growth conditions were adjusted to fit those of *V. anguillarum*. Plasmids from *Escherichia coli* V517 ranging from 35.8 to 1.4 megadaltons (MDa) (22) and plasmid pJM1 (47 MDa) from *V. anguillarum* 775 (10, 11, 28) were isolated as size standards. *E. coli* V517 was incubated at 37°C for 15 to 18 h.

Restriction endonuclease cleavage analysis. Plasmid DNA was isolated by the method of Birnboim and Doly (2) with some modifications (33). Restriction endonuclease digestion with *Hin*dIII, *Eco*RI, and *Pvu*II (Amersham International plc, Buckinghamshire, U.K.) was performed in accordance with the specifications of the supplier. Bacteriophage λ DNA (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany [FRG]) digested with *Hin*dIII was used as a standard.

Plasmid amplification. The plasmid amplification experiment was performed according to Wiik et al. (33) with some adjustments. TSB (25 ml) was inoculated with 0.5 ml of an overnight culture of *V. anguillarum* 775 and incubated at 22°C for 5 h. After addition of chloramphenicol, the culture was incubated at 22°C for a further 15 h. As a reference, *V. anguillarum* 775 was cultivated under the same conditions as described above but without chloramphenicol.

Molecular mass. Agarose gel electrophoresis and determination of molecular mass of the plasmids and plasmid fragments were performed according to Wiik et al. (33).

Antisera production. Whole cells of *V. anguillarum*

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† This paper is dedicated to the memory of Emmy Egidius, who died on 3 February 1989.

TABLE 1. Presumptive *V. anguillarum* strains used in this study

Strain	Source[a]	Host	Geographic origin
NCMB 6	NCMB	Cod (*Gadus morhua*)	Denmark
408	LNP	Rainbow trout (*Salmo gairdneri*)	France
775	UO	Coho salmon (*Oncorhynchus kisutch*)	USA
NCMB 2129	NCMB	Rainbow trout (*S. gairdneri*)	Norway
NCMB 2131	NCMB	Rainbow trout (*S. gairdneri*)	Norway
NCMB 2132	NCMB	Rainbow trout (*S. gairdneri*)	Norway
HI 9199	IMR	Rainbow trout (*S. gairdneri*)	Norway
HI 9084	IMR	Atlantic salmon (*Salmo salar*)	Norway
HI 9829	IMR	Atlatnic salmon (*S. salar*)	Norway
HI 10067	IMR	Atlantic salmon (*S. salar*)	Norway
HI 9984	IMR	Char (*Salvelinus alpinus*)	Norway
HI 9588	IMR	Atlantic salmon (*S. salar*)	Norway
HI 7400	IMR	Atlantic salmon (*S. salar*)	Norway
HI 9485	IMR	Atlantic salmon (*S. salar*)	Norway
HI 4791	IMR	Cod (*G. morhua*)	Norway
HI 9260	IMR	Cod (*G. morhua*)	Norway
HI 10116	IMR	Cod (*G. morhua*)	Norway
HI 9552	IMR	Turbot (*Scophthalmus maximus*)	Norway
HI 10101	IMR	Turbot (*S. maximus*)	Norway
NCMB 2130	NCMB	Saithe (*Pollachius virens*)	Norway
NCMB 2133	NCMB	Saithe (*P. virens*)	Norway
HI 9976	IMR	Halibut (*Hippoglossus hippoglossus*)	Norway
HI 9977	IMR	Halibut (*H. hippoglossus*)	Norway
HI 9088	IMR	Halibut (*H. hippoglossus*)	Norway
HI 9086	IMR	Halibut (*H. hippoglossus*)	Norway
HI 9973	IMR	Halibut (*H. hippoglossus*)	Norway

[a] NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; IMR, Institute of Marine Research, Directorate of Fisheries, Bergen, Norway; LNP, Laboratoire National de Pathologie des Animaux et Aq., Brest, France; UO, University of Oregon, Aquatiques, Corvallis.

(NCMB 2129 and NCMB 2133) were used as antigens. The strains were cultivated in TSB at 22°C and washed twice in sterile 0.01 M phosphate-buffered saline (PBS), pH 7.2, supplemented with 3.5% formaldehyde. Cells were finally suspended in PBS to 5×10^6 cells per ml. Antibodies were elicited in rabbits (chinchilla bastard CHCHbb [Karl Thomae GmbH, Biberach/Risa, FRG]) by immunization in the marginal ear vein. Injection volumes were increased from 0.1 to 1.0 ml within 3 weeks, and a booster (1.0 ml) was given after 2 more weeks. Blood was allowed to coagulate at room temperature for 1 h and thereafter overnight in a refrigerator. Sera were heated to 56°C for 20 min to inactivate complement proteins and were stored at −20°C.

ELISA procedure. A 96-well microtitration plate (Intermed, Nunc, Roskilde, Denmark) was coated with whole bacterial cells. Cells were grown in TSB, Formalin fixed, and diluted in 50 mM sodium carbonate buffer, pH 9.6, to give 7×10^7 cells per ml. Then, 150 μl of the cell suspension was added to each well and incubated overnight at 4°C. The plate was washed by adding 250 μl with 6 mM PBS, pH 7.2, containing 0.05% (vol/vol) Tween 20 (PBS-Tween) to each well. Subsequently, the PBS-Tween supplemented with 0.5% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) (PBS-Tween-BSA) was added to the wells to block free sites. After 15 min at room temperature, the wells were washed three times as described above. Rabbit sera were diluted in PBS-Tween-BSA (NCMB 2129, 1:8,000; NCMB 2133, 1:4,000). A 50-μl amount of diluted sera was administered to each well and incubated for 1 h at room temperature. After three washing cycles, 50 μl of goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase conjugate (BioRad Laboratories, Richmond, Calif.) diluted 1:3,000 in PBS-Tween-BSA was added to the wells and incubated as described for the sera. After washing, 100 μl of OPD substrate (40 mg of *O*-diaminobenzene dihydrochloride [E. Merck AG, Darmstadt, FRG] in 100 ml of half-strength phosphate-citrate buffer [pH 5.0] with 40 μl of 30% H_2O_2 added immediately before use) was added to each well. The reaction was stopped by adding 50 μl of 2.5 N H_2SO_4 to each well after 10 min, and the optical density was read with a model MR 600 microplate reader (Dynatech Laboratories Inc., Alexandria, Va.).

Biochemical tests. Biochemical activity was tested by using the API 50CH (API 50CHE medium) and API 20B systems (API Systems, S.A., La Balme Les Grottes, Montalieu Vercieu, France) with sterile NaCl added to the fluid to a final concentration of 1.5%. The API 50CH battery was used to determine the fermentative ability of the organisms. The results were read after 48 h. Hemolysis was tested by growing the bacteria on NBA at 22°C for 48 h.

Experimental infections. A 24-h TSB culture of a strain of *V. anguillarum* originally isolated from reared coastal cod suffering from vibriosis was used as the infective agent. Water-borne infection was established by exposing 11 rainbow trout (mean length, 21.5 cm) and 49 cod (mean length, 8.8 cm) to 2.2×10^7 CFU/ml for 1 h. During challenge, the rainbow trout were held in a 30-liter tank and the cod in a 10-liter tank, both with oxygenation. The water temperature was raised from 10°C to 12 to 13°C at the start of bacterial challenge. After challenge, rainbow trout and cod were returned to 200-liter tanks with fresh flowing seawater diluted to 1.5% salinity and full-strength seawater, respectively, and observed for 14 days. The water temperature was kept at 12 to 13°C during the observation period. Vibriosis was diagnosed when the fish showed clinical symptoms of disease (12, 14) and gram-negative curved bacteria which were sensitive to vibriostatic agent O/129 could be reisolated from the kidney.

RESULTS

Plasmid composition and restriction endonuclease cleavage patterns. All the strains of *V. anguillarum* isolated from local rainbow trout, three of six strains isolated from local salmon, and the only strain isolated from char harbored a plasmid of 47 MDa (Table 2), which is the molecular mass of the pJM1 virulence plasmid present in *V. anguillarum* 775 (28). Figure 1 shows the plasmid bands of selected strains of *V. anguillarum*. The plasmid screening methods described by Kado and Liu (20) and Wiik et al. (33) gave identical plasmid profiles for the strains tested. The method of Wiik et al. (33), however, generally led to the most well-defined plasmid bands. The relationship between the pJM1 plasmid and the 47-MDa plasmid of strains isolated from local fish was assessed by comparison of restriction endonuclease cleavage patterns. Cleavage of the 47-MDa plasmids from *V. anguillarum* strains 775 and NCMB 2129 showed that the plasmids were very similar but not completely identical (Fig. 2). The *Hin*dIII cleavage patterns of pJM1 of strain 775 and the 47-MDa plasmid of NCMB 2129 were only slightly different, with an additional fragment of 4.8 MDa for the NCMB 2129 plasmid. The *Eco*RI patterns of these plasmids appeared to differ slightly in the region from 4.6 to 2.6 MDa. The *Pvu*II patterns of pJM1 and the NCMB 2129 plasmid were quite similar; the only difference was that the NCMB

TABLE 2. Presence of 47-MDa plasmid, serotype, and differential biochemical properties of strains identified as *V. anguillarum*

Test	NCMB 6	408	775	NCMB 2129	NCMB 2131	NCMB 2132	HI 9199	HI 9084	HI 9829	HI 10067	HI 9984	HI 9588	HI 7400	HI 9485	HI 4791	HI 9260	HI 10116	HI 9552	HI 10101	NCMB 2130	NCMB 2133	HI 9976	HI 9977	HI 9088	HI 9086	HI 9973
47-MDa plasmid	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Serotype	2	1	1	1	1	1	1	1	1	ND[a]	ND	2	2	2	2	2	2	2	ND	2	2	ND	ND	ND	ND	ND
Fermentation tests																										
L-Arabinose	+	+	+	+	+	+	+	+	+	ND	+	−	−	−	−	−	ND	−	ND	−	−	−	−	−	+	−
Ribose	+	+	−	+	+	+	+	+	+	ND	+	+	+	+	+	+	ND	+	ND	+	+	+	+	+	+	−
D-Mannose	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	ND	+	ND	+	+	+	+	+	−	+
Mannitol	+	+	+	+	+	+	+	+	+	ND	+	+	+	−	+	+	ND	+	ND	+	+	+	+	+	+	−
Sorbitol	+	+	+	+	+	+	+	+	+	ND	+	+	+	−	+	+	ND	+	ND	+	+	−	−	−	−	−
Cellobiose	+	+	+	+	+	+	+	+	+	ND	+	−	−	−	−	−	ND	−	ND	−	−	+	+	+	+	−
Lactose	−	−	+	−	−	−	−	−	−	ND	−	−	−	+	−	−	ND	−	ND	−	−	−	−	−	−	−
Trehalose	+	+	+	−	+	−	−	−	−	ND	−	+	+	+	+	+	ND	+	ND	+	+	+	+	+	+	−
L-Fucose	−	−	−	−	−	−	−	−	−	ND	−	−	−	−	−	−	ND	−	ND	−	−	−	−	+	−	−
Gluconate	+	−	+	+	+	+	+	+	+	ND	+	+	+	−	+	+	ND	+	ND	+	+	+	+	−	−	−
API 20B tests																										
Gelatin	+	−	+	+	ND	+	+	+	+	ND	+	+	+	−	ND	+	+	+	ND	+	+	+	+	+	+	−
Nitrites	−	+	−	+	ND	−	+	+	+	ND	+	−	+	+	ND	−	+	−	ND	+	+	+	+	+	+	+
β-Galactosidase	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	ND	+	+	+	+	+	−	+
Saccharose	+	+	+	+	ND	+	+	+	+	ND	+	+	−	+	ND	+	+	+	ND	−	+	−	−	−	+	−
L-(+)-Arabinose	+	+	+	+	ND	+	+	+	+	ND	+	−	−	−	ND	−	−	−	ND	−	−	−	−	−	+	−
Mannitol	+	+	+	+	ND	+	+	+	+	ND	+	+	+	−	ND	+	+	+	ND	+	+	+	+	+	+	−
Starch	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	ND	−	+	+	+	+	+	−
Rhamnose	−	−	−	−	ND	−	−	−	−	ND	−	−	−	−	ND	−	−	−	ND	+	+	−	−	−	−	−
Sorbitol	+	+	+	+	ND	+	+	+	+	ND	+	+	+	−	ND	+	−	+	ND	+	+	−	−	−	−	−
Glycerol	+	+	+	+	ND	+	−	−	+	ND	+	+	+	+	ND	+	+	−	ND	+	+	+	+	+	+	−
Indole	+	+	−	+	ND	+	+	+	+	ND	+	+	−	−	ND	+	+	+	ND	−	−	+	+	+	+	−
Acetoin	+	+	+	+	ND	+	+	+	+	ND	+	+	+	−	ND	+	−	+	ND	+	+	−	−	−	−	+
Simmons citrate	−	+	+	+	ND	+	+	+	+	ND	+	+	−	−	ND	+	−	−	ND	+	+	−	−	−	−	−
Catalase	+	+	−	+	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	ND	+	+	+	+	+	+	+

[a] ND, Not determined.

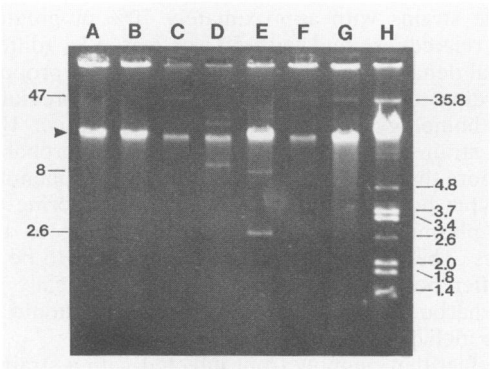

FIG. 1. Demonstration of plasmids in selected strains of *V. anguillarum* by the method of Wiik et al. (33). Lanes: A, NCMB 2130; B, HI 7400; C, HI 9084; D, HI 9485; E, NCMB 6; F, NCMB 2129; G, 775; H, *E. coli* V517. Arrowhead indicates chromosomal bands. Molecular masses are indicated (in megadaltons).

2129 plasmid was cleaved in an additional band of 3.5 MDa. Comparatively weak intermediate bands probably resulted from incomplete cleavage. Because small fragments bind less ethidium bromide than larger fragments, one should expect constantly decreasing band intensity as the molecular masses of the fragments decrease.

Comparatively small plasmids were detected in strains NCMB 6, HI 9485, and HI 9973. The bands of NCMB 6 corresponded to 2.6 and 8 MDa. One of the three bands of HI 9485 corresponded to 9 MDa, while the other ones were approximately 20 MDa. The two bands of HI 9973 were approximately 4 and 10 MDa.

The plasmid amplification experiment was negative. This strongly indicates that plasmid replication is under stringent chromosomal control (31).

Serology. As shown in Table 2, the *V. anguillarum* strains were divided into two serotypes. Strains reacting with the serum against NCMB 2129 were characterized as serotype 1, and strains reacting with the serum against NCMB 2133 were characterized as serotype 2. There was little cross-reaction between strains of the two serotypes. All the strains harboring the 47-MDa plasmid were serotype 1. Except for the plasmidless strain 408, which was serotype 1, the strains without the 47-MDa plasmid were serotype 2.

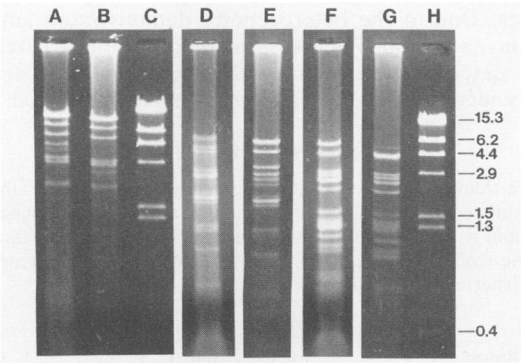

FIG. 2. Restriction endonuclease cleavage patterns of the 47-MDa plasmids in *V. anguillarum* strains 775 and NCMB 2129. Lanes: A and B, *Pvu*II digests of NCMB 2129 plasmid and pJM1, respectively; C and H, *Hin*dIII digest of λ DNA; D and E, *Eco*RI digests of NCMB 2129 plasmid and pJM1, respectively; F and G, *Hin*dIII digest of NCMB 2129 plasmid and pJM1, respectively. Molecular masses are indicated (in megadaltons).

Biochemical reactions. All the strains were positive for the following fermentation tests (API 50CH): amidon, D-fructose, D-glucose, galactose, glycerol, glycogen, maltose, *N*-acetylglucosamine, and saccharose. All the strains were positive for the following API 20B tests: cytochrome oxidase, fructose, galactose, glucose, maltose, and mannose. The strains differed in 24 of 71 tests (Table 2). Bacterial strains isolated from local fish that were serotype 1, which invariably harbored the 47-MDa plasmid, had very similar biochemical properties. The reference strain *V. anguillarum* 775 diverged from the local strains harboring the 47-MDa plasmid by fermenting lactose, not fermenting ribose, not producing indole from tryptophan, and being catalase negative. The plasmidless strain 408 of serotype 1 differed from the strains harboring the 47-MDa plasmid by being negative for gluconate and gelatin.

The plasmidless strains isolated from Norwegian marine fish and occasionally from Atlantic salmon (serotype 2) had similar biochemical properties, which differed clearly from those of the group harboring the virulence plasmid. Main differential tests between the plasmid-harboring and plasmidless strains were cellobiose and L-arabinose (API 50CH) and L-(+)-arabinose (API 20B). Strains NCMB 6, HI 9485, and HI 9973, harboring plasmids with molecular masses of approximately 20 MDa and less, did not fit into either of the two categories described above. In spite of lacking the 47-MDa plasmid and being serotype 2, strain NCMB 6 was biochemically similar to the group harboring the 47-MDa plasmid. The only diverging reaction was the inability of NCMB 6 to use citrate as the only source of carbon. The biochemical reactions of strains HI 9485 and HI 9973 were both unique. All the strains had hemolytic ability.

Experimental infections. Forty-eight cod died from vibriosis within 4 days after challenge. Bacteria were not isolated from the only surviving individual at day 14. Three rainbow trout died from vibriosis within 8 days after challenge. These fish showed clinical symptoms similar to those usually seen in cod, with the main lesions in the tail, but quite different from those usually seen in rainbow trout (12, 14). In rainbow trout and Atlantic salmon, the predominant symptoms of vibriosis are superficial skin lesions, hemorrhages of the fin bases and the intestine, and a characteristic swollen spleen, while in cod the principal sites of damage are the head and tail regions. Bacteria were not isolated from the eight surviving rainbow trout at day 14.

DISCUSSION

Vibrio anguillarum 775, isolated from coho salmon (*Oncorhynchus kisutch*) in the Pacific Northwest of the United States (18), carries a plasmid, pJM1, associated with the virulence of the strain (10, 11). This plasmid mediates a very efficient iron-sequestering system which allows bacteria to grow at the low concentrations of available iron imposed by high-affinity iron-binding proteins in fish serum (7, 9, 35).

In the present study, a plasmid with the same molecular mass as the virulence plasmid pJM1, 47 MDa, was found in the majority of the bacterial strains isolated from salmonids (rainbow trout, Atlantic salmon, and char). Although showing minor differences, the plasmid exhibited extensive similarity with pJM1, as demonstrated by restriction endonuclease analysis. This result indicates that the 47-MDa plasmid of the Norwegian isolates is involved in the pathogenicity of these organisms in a similar way as described for pJM1 (10, 11, 27).

The absence of plasmids in *V. anguillarum* strains isolated from marine fish and partly from Atlantic salmon strongly

indicates that their virulence properties are chromosome mediated. Demonstration of iron uptake genes both in virulence plasmids and in chromosomes (8, 21, 29), however, suggests the possibility of similar pathogenicity mechanisms in both plasmidless and plasmid-containing strains.

Strains NCMB 2129 and NCMB 2133 were serotyped as O1 and O2, respectively, by Bolinches and Egidius (3) by the method and with the sera of Skov Sørensen and Larsen (24). In the present study, the reference strains NCMB 6, 775, and 408 were serotyped in accordance with the results of Skov Sørensen and Larsen (24). Although our sera were produced against Formalin-fixed whole cells which were not heated to eliminate antigens other than O antigens, they differentiate *V. anguillarum* serologically according to the typing system of Skov Sørensen and Larsen. We therefore conclude that our serotypes 1 and 2 correspond to serotypes O1 and O2, respectively. This conclusion is supported by the fact that serotype O1 of Skov Sørensen and Larsen (24) was the most common type causing vibriosis in cultured salmonids, which was also the origin of our strains described as serotype 1. Similarily, serotype O2 was the predominant type isolated from diseased marine fish, which were the most common hosts of our strains described as serotype 2. Strains of serotype 2 were also isolated from Atlantic salmon, however, which is in agreement with the results of Skov Sørensen and Larsen (24) pertaining to serotype O2. Our two isolates from turbot belonged to serotype O2. Toranzo et al. (30), however, found that most of the *V. anguillarum* strains isolated from turbot reared on the Atlantic coast of Spain belonged to serotype 1 and only a few isolates belonged to serotype 2. The strains from turbot belonging to serotype 1 harbored a 47-MDa plasmid similar to the virulence plasmid pJM1, while the isolates of serotype 2 were plasmidless.

The isolates of *V. anguillarum* harboring the virulence plasmid had nearly identical biochemical properties. A high degree of general similarity between these strains is supported by DNA-DNA hybridization studies (34). Except for strains NCMB 6, HI 9485, and HI 9973, the strains of serotype O2 were quite similar in their biochemical reaction patterns, which clearly differed from those of the strains belonging to serotype O1. Our result, that the strains of serotype O1 were positive for arabinose and that the strains of serotype O2 were negative, is in accordance with the results of Toranzo et al. (30). On the basis of plasmid content, serotype, and biochemical characteristics, the majority of the strains included in this study could be divided into two groups. One group, which consisted of strains harboring the virulence plasmid, originated invariably from salmonids, while strains in the other group, which were plasmidless, were mainly isolated from marine fish species. Unlike our finding that strains of *V. anguillarum* harboring the virulence plasmid exclusively originated from anadromous salmonids, strains with a plasmid nearly identical to pJM1 have also been isolated from turbot (*S. maximus*) on the Atlantic coast of northwest Spain (27).

Generally, the division of *V. anguillarum* strains isolated from marine versus anadromous fish is supported by DNA-DNA hybridization studies (34). As an example, strains NCMB 2130 and NCMB 2133 isolated from saithe and NCMB 6 isolated from cod showed more than 80% DNA similarity with each other, while these strains showed only 53 to 67% DNA similarity with strain NCMB 2129, isolated from rainbow trout. Strains which in the present study were found to harbor the virulence plasmid showed more than 80% DNA similarity with strain NCMB 2129. A phylogenetic definition of a bacterial species, which generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m (difference in thermal denaturation temperature), has been proposed (32). According to this proposed definition and previous DNA-DNA homology studies (34), the presumptive *V. anguillarum* strains included in the present study probably represent more than one species. Due to the great phenotypic and genotypic homogeneity among strains harboring the virulence plasmid, these strains should belong to the same species. The plasmidless strains determined to be serotype O2 differ both phenotypically and genotypically from the group harboring the virulence plasmid and should probably not be included in that species.

The fact that rainbow trout infected with a strain isolated from cod showed clinical symptoms similar to those in cod but rather different from those usually seen in rainbow trout (12, 14) indicates that plasmidless strains interact with the host in a different way than strains harboring the virulence plasmid. The infection experiment also indicated that the strain isolated from cod was more virulent to cod than to rainbow trout. The infection experiments of Egidius and Andersen (13) indicate that strains isolated from rainbow trout are rather virulent to salmonids but only slightly virulent to saithe. Similarly, strains isolated from saithe are virulent to saithe but seem to be avirulent to rainbow trout. In conclusion, it seems that strains with the virulence plasmid, which in our material invariably originated from salmonids, interact with the host in a different way than the plasmidless strains from marine fish. According to Toranzo et al. (30), however, *V. anguillarum* strains of serotype 2 isolated from turbot and strains of serotype 1 isolated from rainbow trout were equally virulent to rainbow trout. It should be mentioned that their experimental conditions differed from ours and those of Egidius and Andersen (13) by involving much smaller rainbow trout (weight, 5 to 8 g) and higher water temperatures.

Strains NCMB 6 (ATCC 19264), which is the type strain of *V. anguillarum* (1), and 775 (10, 11) are both used as reference strains for the species *V. anguillarum* (21, 24–26, 30). According to the present study and the DNA-DNA hybridization data (34), it can be questioned whether these strains belong to the same species. Our findings are not in harmony with the results of Schiewe et al. (23), however, who found a high degree of genetic similarity between these strains. Conspicuous phenotypic differences between our strains, however, generally correspond to genotypic differences. Due to the heterogeneity demonstrated among our strains, a thorough and systematic genetic characterization, i.e., determination of 16S rRNA sequences, of strains initially identified as *V. anguillarum* is recommended.

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