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Virulence Studies Based on Plasmid Profiles of the Fish Pathogen *Vibrio salmonicida*†

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Strains of *Vibrio salmonicida* isolated from Atlantic salmon (*Salmo salar*) and rainbow trout (*Salmo gairdneri*) suffering from cold-water vibriosis could be divided on the basis of plasmid profiles into four different categories. Of 32 strains, 19% harbored three plasmids of 24, 3.4, and 2.6 megadaltons (MDa), 69% harbored the 24- and 3.4-MDa plasmids but not the 2.6-MDa plasmid, and 9% harbored only the 24-MDa plasmid. The fourth category, which consisted of only one strain, harbored a plasmid of 10 MDa. In spite of different plasmid patterns, the strains of *V. salmonicida* were very similar with respect to biochemical reactions. The one-third of the *V. salmonicida* strains which were serotyped were of the same type. The 50% lethal doses, which were determined by intraperitoneal injection, ranged from 4×10^6 to 1×10^8 CFU per fish.

In 1979, a new disease appeared on a large scale in Norwegian salmonid farms around the island of Hitra south of Trondheim (8). Since then, the so-called Hitra disease has ravaged fish farms with Atlantic salmon (*Salmo salar* L.) and occasionally with rainbow trout (*Salmo gairdneri* Richardson) all along the western and northern coastline of Norway. The disease has also been reported in Scotland (3). The etiology of Hitra disease has been disputed, as reflected in the two other names used for the same disease: cold-water vibriosis (7, 8, 10) and hemorrhagic syndrome (20, 21). The former name focuses on the proposed causative bacterium, *Vibrio salmonicida*, while the latter focuses on the clinical symptoms of the disease, which were proposed to arise from a nutritional or metabolic disorder. The etiology of the disease may be complex, but a detailed histopathological study of Atlantic salmon during development of the disease (30) strongly supports the bacterial aspect of the disease. We have been able to isolate *V. salmonicida* from the kidney of all the fish we have examined which were showing clinical symptoms of cold-water vibriosis (8, 9), which is the term used in this paper.

The purpose of this study was to categorize strains of *V. salmonicida* with respect to plasmid profiles and test whether different plasmid profiles corresponded to differences in biochemical and serological properties as well as to different degrees of virulence.

MATERIALS AND METHODS

Bacteria and growth conditions. Thirty-one strains of *V. salmonicida* isolated from Atlantic salmon (including the type strain NCMB 2262 [10]) and one strain from rainbow trout (HI 7920) were investigated. The bacterial isolations were carried out in the period 1983 to 1987 from outbreaks of cold-water vibriosis along the greater part of the western Norwegian coast. In addition to the Norwegian isolates, an isolate from a Scottish fish farm where cold-water vibriosis was confirmed was included (MT160). Plasmids in *Escherichia coli* V517 ranging from 35.8 to 1.4 megadaltons (MDa) (16) were isolated as size standards. The virulence plasmid pJM1 in *Vibrio anguillarum* 775 (5), plasmids in *Vibrio ordalii* strains DF_1 K and MSC2-75 (24, 25), and the plasmid pUC18 in *E. coli* K-12 (American Type Culture Collection Catalogue of Recombinant DNA Collections, 1st ed., 1986) were isolated for comparisons.

The general growth substrates used throughout this study were tryptone soy broth (TSB) (Oxoid Ltd., London, England) and nutrient agar (Oxoid) supplemented with 5% sheep blood (NBA); both substrates were supplemented with NaCl to a final concentration of 1.5%.

Generally, the *V. salmonicida* strains were incubated aerobically at 15°C for 48 h, strains of *V. anguillarum* and *V. ordalii* were incubated at 22°C for 18 to 24 h, and *E. coli* was incubated at 37°C for 15 h.

Enzymes for plasmid isolation. Lysozyme (Sigma Chemical Co., St. Louis, Mo.) was dissolved (4 mg/ml) in 0.5 M Tris, pH 8.0, and stored at −20°C until used. RNase A (Sigma) was prepared and stored as described by Maniatis et al. (17).

Plasmid screening. Our method for plasmid screening of fish-pathogenic vibrios was developed on the basis of experience with previously described methods (1, 12, 17). Bacteria were grown to late exponential phase (1×10^9 to 3×10^9 cells per ml) in TSB. Biofuge A (Heraeus-Christ GmbH, Osterode am Harz, Federal Republic of Germany) with rotor 1220 was used in all centrifugation steps, which were performed at 6°C. The screening protocol was designed to be performed in 1.5-ml Eppendorf centrifuge tubes. When necessary, the pH was adjusted by addition of HCl or NaOH. Cells were harvested by centrifugation of 1 to 3 ml of culture for 3 min at 5,000 × *g*. The pelleted cells were suspended in 500 μl of freshly prepared 10% sucrose–50 mM Tris (pH 8.0). From this point, the reagents were gently mixed immediately after addition by 5 to 10 inversions of the tube, with the exception of phenol. The tubes were kept on ice except during mixing or when another temperature is specified. A 200-μl amount of 0.5 M disodium EDTA (pH 8.0) was added to the suspended cells, followed by 50 μl of lysozyme solution. The tube was incubated for 5 min at room temperature. Then, 100 μl of 20% (wt/vol) sodium dodecyl sulfate (SDS) in distilled H_2O was added. This treatment was sufficient for complete lysis of the *Vibrio* strains, while *E. coli* strains V517 and K-12 had to pass through a few cycles of heat pulses and mixing (one cycle was 15 s in a 55°C water

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bath and then inversions for 15 s after removal) after addition of SDS. After complete lysis, 90 μl of 3.0 M KCl was added, and the tube was incubated for 30 min. The sample was then centrifuged for 10 min at 12,000 × *g*, and 400 μl of phenol prepared according to Maniatis et al. (17) was added to the supernatant. After 20 to 30 inversions, the sample was incubated for 5 min before centrifugation at 5,000 × *g* for 2 min. The upper phase was removed, extracted once or twice with 400 μl of chloroform-isoamyl alcohol (24:1), and centrifuged at 5,000 × *g* for 2 min. The upper phase was transferred to a fresh tube, and 2 volumes of ice-cold 100% ethanol were added. The tube was incubated at −20°C for 10 min. The precipitated DNA was collected by centrifugation at 12,000 × *g* for 10 min. The supernatant was gently discarded, and the pellet, which often was gelatinous, was dried in a vacuum centrifuge (Speed Vac Concentrator, model SVC-100H; Savant, Farmingdale, N.Y.). The dry pellet was dissolved in 100 μl of TE buffer (0.01 M Tris, 0.001 M disodium EDTA, pH 8.0), and 1.5 μl of RNase A solution was added. The tube was incubated in a 37°C water bath for 30 min. Thereafter, 4 μl of 2.5 M NaCl was added, and DNA was precipitated as described above. The dried pellet was dissolved in 15 μl of TE buffer before addition of 5 μl of loading buffer I (17).

To test the effect of alkaline denaturation and neutralization, two additional steps were included in the method described above. Plasmid isolation from selected strains was performed by addition of 50 μl of 3 N NaOH after SDS addition. For our cultures, this raised the pH to 11.9 (glass electrode). Then, 100 μl of 2 M Tris, pH 7.0, was added to lower the pH to about 8.5, followed by addition of 100 μl of 3 M KCl. The remaining steps of the procedure were performed as described above.

Restriction endonuclease cleavage analysis. As opposed to our plasmid screening method, the plasmid DNA obtained by the method described by Birnboim and Doly (2) was clean enough for direct use in restriction analysis. Plasmid DNA was therefore isolated by the Birnboim and Doly method with some modifications. The volumes of cell culture and solutions I, II, and III were multiplied by a factor of 50. Solution III was prepared according to Maniatis et al. (17). After interfering material was removed by centrifugation at 12,000 × *g* for 15 min at 4°C (Sorvall SS-34; Du Pont Instruments, Newtown, Conn.), the plasmids were precipitated by adding 0.6 volume of isopropanol (17) and recovered by centrifugation at 12,000 × *g* (Sorvall SS-34) for 30 min at room temperature. The pellet was dried in a vacuum desiccator, suspended in 500 μl of TE buffer, and transferred to a 1.5-ml Eppendorf tube. Then, 7.5 μl of RNase A solution was added. One extraction step with chloroform-isoamyl alcohol was introduced before the final ethanol precipitation. Restriction endonuclease digestion with *Hin*dIII, *Eco*RI, and *Pvu*II was performed in accordance with the specifications of the supplier (Amersham International plc, Buckinghamshire, England). Reactions were stopped according to Maniatis et al. (17); the only modification was elimination of xylene cyanol from loading buffer I. Bacteriophage λ DNA (Boehringer Mannheim GmbH) digested with *Hin*dIII was used as a control.

Storing of bacterial pellets by freezing. We tested whether storing bacterial pellets at −20°C for 1 to 4 days could alter the observed plasmid profile. The plasmid DNA was isolated according to our plasmid screening method.

Plasmid amplification experiment. TSB (250 ml) was inoculated with 5 ml of a late-exponential-phase culture of *V. salmonicida*. The culture was incubated at 15°C with continuous shaking until a bacterial concentration of approximately 10^8 cells per ml (OD_{600}, ≈0.5) was reached. Chloramphenicol (Norsk medisinaldepot, Oslo, Norway) was added to a final concentration of 170 μg/ml, and the culture was incubated for further 12 h. The plasmid amplification experiment was tried on two strains of *V. salmonicida*. As a reference, these two strains were cultivated under the conditions described above except for the addition of chloramphenicol. Plasmid DNA was isolated according to our plasmid screening method.

Agarose gel electrophoresis. DNA samples were electrophoresed on 0.7% agarose minigels (GNA 100; Pharmacia, Uppsala, Sweden) at 60 V for 2 h or 0.7% agarose (20 by 20 cm gel) (GNA 200, Pharmacia) at 120 V for 4 h in Tris-borate buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA [pH 8.2]). Gels were stained with ethidium bromide (0.5 μg/ml) incorporated in both the gel and running buffer and photographed through an orange filter on Polaroid type 665 film.

Molecular mass. Molecular mass of the plasmids was determined by the use of a gel scanner (Ultroscan XL, Laser and Densitometer; LKB, Bromma, Sweden) equipped with the program LKB 2400 GelScan XL.

Biochemical tests. Biochemical activity was tested by using the API 50CH and API 20B systems (API System S.A., La Balme Les Grottes, Montalieu Vercieu, France) according to Wiik et al. (33). The galleries were incubated at 15°C. Hemolysis was tested by growing *V. salmonicida* on NBA for at least 48 h.

Serology. Serotyping was done by an enzyme-linked immunosorbent assay (33). Whole Formalin-fixed cells of *V. salmonicida* NCMB 2262 (10) were used as the antigen for production of sera in rabbits.

LD_{50} determination. The virulence of strains of *V. salmonicida* having different plasmid patterns was assessed by determination of the 50% lethal dose (LD_{50}; bacterial dose at which 50% of the animals die) (22). Apparently healthy Atlantic salmon post-smolts were anesthetized with 60 mg of ethyl-*p*-aminobenzoate (benzocaine) per liter (Norsk medisinaldepot). Groups of five fish received intraperitoneally 0.5 ml of bacterial cell suspensions which were serially diluted 10-fold with saline solution (0.9% NaCl). The cells were grown on NBA for 48 h before being suspended in saline. Control fish were injected with sterile saline. The fish were returned to 200-liter sea-water tanks to recover and observed for at least 3 weeks. Water temperature was recorded daily. Cold-water vibriosis was confirmed when *V. salmonicida* could be reisolated from the kidney of moribund or dead fish, and the spleen imprint, fixed and stained with M+D Diff-Quik (Merz+Dade AG, Düdingen, Switzerland), was positive for curved bacteria. The fish were measured (i.e., length in centimeters) at the end of the experiments.

RESULTS

Plasmid profiles. On the basis of plasmid composition, strains of *V. salmonicida* could be divided into four categories. Of a total of 32 strains, 19% harbored three plasmids of 24, 3.4, and 2.6 MDa (category 1), 69% harbored the 24- and 3.4- MDa plasmids but not the 2.6-MDa plasmid (category 2), and 9% harbored only the 24-MDa plasmid (category 3). The fourth category, which consisted of only one strain (3%), harbored a plasmid of 10 MDa. The plasmid profiles are shown in Fig. 1. Bands corresponding to molecular masses of 6.8 MDa and approximately 10 MDa were often, but not always, observed in the plasmid profiles of *V. salmonicida* strains harboring the 3.4-MDa plasmid, nor

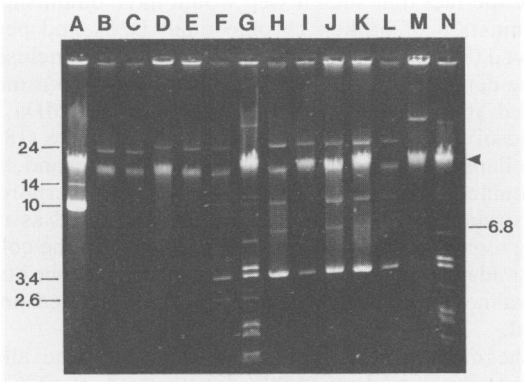

FIG. 1. Demonstration of plasmids in selected strains of *V. salmonicida* by use of our plasmid screening method. Lanes: A, strain with the 10-MDa plasmid (TEO 83.001 in category 4); B, *V. ordalii* MSC2-75; C, *V. ordalii* DF_1K; D and E, two strains in category 3; F, one strain in category 1; G, *E. coli* V517; H–L, five strains in category 2; M, *V. anguillarum* 775; N, *E. coli* V517. Molecular masses (in megadaltons) are indicated. The bands corresponding to molecular masses 6.8 and 10 MDa in lanes H to L most probably represent a dimer and the OC state, respectively, of the 3.4-MDa plasmid. Similarly, the band of 14 MDa in lane A most probably represents the OC state of the 10-MDa plasmid. Arrowhead indicates chromosomal bands.

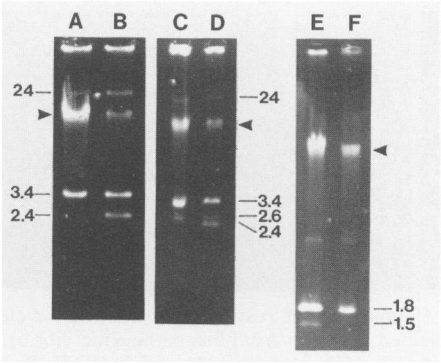

FIG. 2. Demonstration of denaturation of CCC plasmids into collapsed circles by alkaline treatment. Lanes: A, plasmids of *V. salmonicida* (category 2), isolated without alkaline treatment; B, plasmid profile of the same strain as in lane A after alkaline treatment; C, plasmids of *V. salmonicida* (category 1), isolated without alkaline treatment; D, plasmid profile of the same strain as in lane C after alkaline treatment; E, pUC18 isolated with alkaline treatment; F, pUC18 isolated without alkaline treatment. Arrowheads indicate chromosomal bands. Molecular masses (in megadaltons) are indicated.

could these bands always be observed when plasmids from the same strain were isolated repeatedly by the same method. The 6.8-MDa band most probably represented a dimer of the 3.4-MDa plasmid, while comparison with the 3.4-MDa plasmid of *E. coli* V517 indicated the 10-MDa band represented the open circular (OC) state of the 3.4-MDa plasmid. That the 10-MDa band did not represent a covalently closed circular (CCC) plasmid was shown by cutting out the 3.4-MDa band from a low-melting-temperature agarose gel and electrophoresing it separately on a conventional agarose gel (data not shown). Both the 3.4-MDa band and the 10-MDa band appeared on this gel, which shows that the 10-MDa band originated from the 3.4-MDa band. By the same method, the band corresponding to a molecular mass of 14 MDa in the plasmid profile of strain TEO 83.001 (category 4) probably represented the OC state of the 10-MDa plasmid. The *V. ordalii* strains both had a plasmid of 23 MDa.

The additional steps of alkaline denaturation and neutralization led to irreversible denaturation of some CCC plasmid molecules into collapsed circles, a phenomenon which is described by Orberg and Sandine (19). This denaturation leads to artificial bands, which may be mistaken for real plasmids (Fig. 2). The method of Birnboim and Doly (2) also led to denaturation of CCC plasmids into collapsed circles. For *V. salmonicida*, we observed this phenomenon for the plasmids of 10 and 3.4 MDa. Denaturation into collapsed circles was not observed for our plasmid screening method.

Freezing of the bacterial pellet before plasmid isolation implied that the 24-MDa plasmid could not be detected on the gel (Fig. 3).

Amplification of plasmids by the use of chloramphenicol was tried on two strains of *V. salmonicida* representing categories 2 and 4. The results were negative. This indicated that plasmid replication was under stringent chromosomal control (31).

Restriction endonuclease characterization. Possible relationships between the 24-MDa plasmid from a *V. salmonicida* strain harboring this plasmid only (HI 9535) and the 10-MDa plasmid from the only *V. salmonicida* strain with this plasmid (TEO 83.001), as well as with the pJM1 plasmid of *V. anguillarum* and the 23-MDa plasmid of *V. ordalii*, were assessed by restriction endonuclease analysis. The cleavage patterns obtained after digestion of the plasmids with the enzymes *Hin*dIII, *Eco*RI, and *Pvu*II were compared. The cleavage patterns of the plasmids compared were clearly different (Fig. 4).

Biochemical and serological reactions. The strains differed in 10 of 71 API tests (Table 1). We could not find any correspondence between biochemical reaction patterns and plasmid patterns (Table 1). All the strains were positive for the following fermentative tests (API 50CH): D-fructose, D-glucose, gluconate, maltose, *N*-acetylglucosamine, and trehalose, and all the strains were positive for the following API 20B tests: catalase, cytochrome oxidase, and galactose. Hemolysis was not observed for any of the *V. salmonicida* strains.

Ten of the 32 strains of *V. salmonicida*, representing the categories harboring two and three plasmids, were found to be the same serotype.

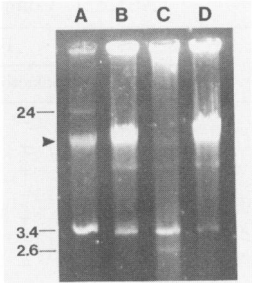

FIG. 3. Demonstration of the effect of freezing (−20°C) the bacterial pellet before plasmid isolation. Lanes: A, plasmid profile of *V. salmonicida* (category 2) without freezing; B and D, plasmid profile of two strains of *V. salmonicida* (category 2) after freezing; C, plasmid profile of *V. salmonicida* (category 1) after freezing. Arrowhead indicates chromosomal bands. Molecular masses (in megadaltons) are indicated.

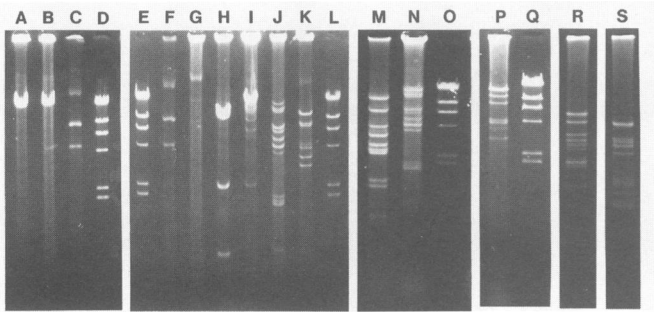

FIG. 4. Comparison of restriction endonuclease cleavage patterns of selected plasmids from fish-pathogenic vibrios. Lanes: A, *Pvu*II digest of 23-MDa plasmid of *V. ordalii* DF_1K; B, *Pvu*II digest of 24-MDa plasmid of *V. salmonicida*; C, *Pvu*II digest of 10-MDa plasmid of *V. salmonicida*; D, E, L, O, and Q, *Hin*dIII digest of λ DNA; F, *Eco*RI digest of 10-MDa plasmid of *V. salmonicida*; G, *Eco*RI digest of 24-MDa plasmid of *V. salmonicida*; H, *Eco*RI digest of 23-MDa plasmid of *V. ordalii* DF_1K; I and M, *Hin*dIII digest of 23-MDa plasmid of *V. ordalii* DF_1K; J and N, *Hin*dIII digest of 24-MDa plasmid of *V. salmonicida*; K, *Hin*dIII digest of 10-MDa plasmid of *V. salmonicida*; P, *Pvu*II digest of pJM1 of *V. anguillarum* 775; R, *Eco*RI digest of pJM1 of *V. anguillarum* 775; S, *Hin*dIII digest of pJM1 of *V. anguillarum* 775. Molecular masses of fragments of *Hin*dIII digested λ DNA are 15.3, 6.2, 4.4, 2.9, 1.5, 1.3, and 0.4 MDa. The smallest fragment of 0.4 MDa is barely visible on the gel.

LD_{50}s. The LD_{50}s were determined for four strains of *V. salmonicida* representing the four different plasmid profiles. The LD_{50}s ranged from 4×10^6 to 1×10^8 CFU per fish (Table 2). The bacterial strains all led to cold-water vibriosis, irrespective of plasmid profile. The 10^{-2} dilution of strain NCMB 2262 had to be disregarded, which implied that the respective LD_{50} (4×10^6 CFU per fish) was comparatively uncertain. It should also be noted that the water temperature was about 2°C lower for experimental infection with NCMB 2262 than for the remaining strains. Due to these uncertainties and to the fact that there were only five fish in each group, the LD_{50} values were not estimated as being significantly different. No deaths occurred among the controls. Bacteria could not be isolated from surviving fish in any group.

DISCUSSION

The plasmid screening method preferred in this study was very gentle and did not include an alkaline denaturation step despite the fact that such a step would have diminished the contamination of broken chromosomal DNA and perhaps improved the DNA yield for some plasmids (12). Inclusion of alkaline denaturation and neutralization steps in our method involved irreversible denaturation of the 3.4-MDa CCC plasmid of *V. salmonicida* into a collapsed circle (18, 19). This collapsed CCC plasmid led to an additional band, which was situated near the 2.6-MDa plasmid band harbored by some strains, and could easily be misinterpreted as representing a separate CCC plasmid (Fig. 2). Due to the collapse of plasmids from both *V. salmonicida* and reference strains, an alkaline denaturation step was not included in our method.

In the method of Birnboim and Doly (2), the alkaline treatment involved irreversible denaturation of CCC plasmids into collapsed circles. Artificial bands were observed for the 10-MDa and 3.4-MDa plasmids of *V. salmonicida*. H. Sørum (D.Sc. thesis, The Norwegian College of Veterinary Medicine, Oslo, Norway, 1986) has also described the double-band effect on plasmids of *V. salmonicida* with the method of Birnboim and Doly (2). According to Sørum, the problem was omitted by using the plasmid detection method of Kado and Liu (15). When the Kado and Liu procedure was employed, however, the smallest plasmid of *V. salmonicida* could not be detected. According to our results and knowledge available from the literature (19; Sørum, D.Sc. thesis), the method of plasmid screening used in this study gave reliable information about the total plasmid composition of *V. salmonicida*.

The categorization of *V. salmonicida* based on plasmid profiles is on the whole in accordance with the results of Sørum (D.Sc. thesis). The slight differences in molecular masses of two plasmids between the two studies is most probably due to different measuring methods. Disregarding category 4, consisting of only one strain harboring a 10-MDa plasmid, Sørum's material included the three remaining plasmid profiles reported in this paper. In addition, Sørum detected *V. salmonicida* strains harboring a 21-MDa plasmid (24-MDa plasmid) in combination with a 61-MDa plasmid. In some of these strains a faint band of 3.4 MDa was observed. These strains originated from an area in the most northern part of Norway. Isolates from this area were not incorporated in our study. Within our material, there seemed to be no correlation between any plasmid profile and the geographic origin of the respective strain, nor could we detect any correlation between any plasmid profile and the year of isolation of the respective strain.

TABLE 1. Comparison of differential biochemical reactions and plasmid profiles

Category	No. of strains[a]	Fermentation tests					Oxidation tests				
		D-Mannose	Galactose	Glycerol	Mannitol	Sorbitol	Mannose	Glucose	Glycerol	Maltose	Mannitol
1	1	+	−	(+)[b]	+	(+)	−	−	−	−	−
2	1[c]	−	+	+	+	−	−	+	+	+	+
	1	+	+	+	+	+	−	+	+	+	+
	1	+	−	−	+	−	−	−	−	+	+
	3	+	+	(+)	+	(+)	−	(+)	−	(+)	(+)
	3	−	+	−	−	−	−	+	+	+	+
1 + 2	2	+	−	−	+	−	(+)	+	(+)	+	+
	4	+	−	−	+	+	−	(+)	(+)	+	(+)
2 + 3	2	+	−	+	+	−	+	+	+	+	+
2 + 4	2	−	−	−	+	−	−	−	(+)	+	+
1 + 2 + 3	10	+	−	−	+	−	−	+	(+)	(+)	+

[a] Values for one strain each in categories 1 and 2 are missing.
[b] (+), Weakly positive.
[c] Type strain NCMB 2262.

TABLE 2. Virulence of four strains of *V. salmonicida* representing the four different plasmid profiles

Bacterial strain[a]	No. of plasmids	Molecular mass(es) (MDa)	Mean length of fish (cm) ± SD	Mean water temp (°C) ± SD	LD_{50} (CFU/fish)	Time to death (range in days)
HI 9962	3	24, 3.4, 2.6	20 ± 2	10.5 ± 0.3	7×10^7	5–11
NCMB 2262	2	24, 3.4	35 ± 5	8.7 ± 0.2	4×10^6	6–22
HI 9535	1	24	19 ± 2	10.8 ± 0.8	1×10^8	5–19
TEO 83.001	1	10	21 ± 3	10.8 ± 0.8	3×10^7	12–17

[a] Strains HI 9962 and HI 9535 are from the Institute of the Marine Research, Directorate of Fisheries, N-5024 Nordnes, Norway. NCMB 2262 (10) was donated to the National Collection of Marine Bacteria, Aberdeen, Scotland. Strain TEO 83.001 was supplied by the University of Tromsø, Tromsø, Norway.

We were not able to find any correspondence between different biochemical reaction patterns and plasmid patterns. Strains with identical biochemical reaction patterns most often differed in geographic origin or year of isolation. In spite of some interstrain variation, the general reaction pattern obtained for the strains in the API batteries is very characteristic for the species *V. salmonicida* and generally in accordance with previously published results (3, 10, 14). The API reaction patterns of *V. salmonicida* clearly differed from the patterns of the fish pathogens *V. anguillarum* (14, 33) and *V. ordalii* (14).

Strains of *V. salmonicida* having two and three plasmids were the same serotype. Strains having the 24-MDa plasmid only (category 3) and the strain with the 10-MDa plasmid (category 4) were not serotyped in this study. However, monoclonal antibodies against the surface layer antigen VS-P1 (13) isolated from strain LFI 83.001 (TEO 83.001) reacted with starved cells of strain NCMB 2262 (category 2) (K. A. Hoff, submitted for publication). Moreover, Espelid et al. (11) report that their monoclonal antibodies reacted equally against all the *V. salmonicida* strains tested. Strains LFI 84.031 (category 3) and LFI 83.001 (category 4) were included in their study. These results, together with the serological testing done by Holm et al. (14) and Egidius et al. (10), clearly indicate a serologically homogeneous species, different from *V. anguillarum* and *V. ordalii*. Espelid et al. (11), however, found that monoclonal antibodies against undefined antigens of *V. salmonicida* cross-reacted with strains of *V. fischeri*. Thus, *V. salmonicida* is serologically related to *V. fischeri*.

On the basis of the present investigation and previous studies of chromosomal DNA (14, 32), *V. salmonicida* must be characterized as a very homogeneous species. The only property which made it possible to differentiate between the strains was plasmid profiles.

Interestingly, all the isolates except one harbored a plasmid of 24 MDa. The most characteristic plasmid profile of *V. salmonicida* was coexistence of the 24-MDa plasmid with a 3.4-MDa plasmid, a finding which is in agreement with the results of Sørum (D.Sc. thesis). The type strain NCMB 2262, the strain from Scotland (MT160), and the strain isolated from rainbow trout belong to this category. Certain strains of *V. salmonicida* which were kept on NBA were tested repeatedly for plasmid composition during a period of 3 years. No changes could be observed for any of the four plasmid categories, nor did passage through fish or freezing in 20% glycerol in TSB involve any changes. Our observations, together with the observations of Sørum, indicate that the plasmid composition of *V. salmonicida* is stable. Compared with the plasmid profiles of other fish-pathogenic bacteria (24, 28, 33) the plasmid profiles of *V. salmonicida* seem to be specific for the species. Although the bands of the 24-MDa plasmid of *V. salmonicida* and the 23-MDa plasmid of *V. ordalii*, which was determined to have a molecular mass of approximately 20 MDa by Schiewe and Crosa (24), are situated near each other on the gel, they can be readily distinguished when both plasmids are present on the gel.

It has been demonstrated that the virulence of certain strains of *V. anguillarum* is related to the presence of a plasmid having a molecular mass of 47 MDa (5, 6, 28). This plasmid mediates an effective iron uptake system which enables *V. anguillarum* to grow under conditions of iron limitation (4, 5, 26, 27). Whether the virulence of *V. salmonicida* is encoded by plasmidal or chromosomal genes or a combination of the two is not known. The infection experiments indicated, however, that if the virulence is completely or partly plasmid mediated, the 24-MDa plasmid is important. This plasmid is the only one which is harbored by all the strains except the strain in category 4.

Previous restriction endonuclease characterization of the plasmids of *V. salmonicida* indicates that plasmids of the same size within each plasmid category are identical, while some very minor differences could be observed between the 24-MDa plasmids from different categories (Sørum, D.Sc. thesis). In the present study, the restriction endonuclease cleavage patterns of the 24-MDa and 10-MDa plasmids of *V. salmonicida*, the 23-MDa plasmid of *V. ordalii*, and the virulence plasmid (pJM1) of *V. anguillarum* were all clearly different from each other. If the virulence of *V. salmonicida* is coupled to a plasmid, homologous base sequences may exist between the 24-MDa plasmid, harbored by all but one of the strains studied, and 10-MDa plasmid, harbored by the one exceptional strain. It should be mentioned that the whole-cell DNA similarity between the type strain NCMB 2262 and the strain harboring the 10-MDa plasmid (TEO 83.001) was 100% (32).

Sakai (23) determined the LD_{50}s for a virulent strain of *Aeromonas salmonicida* and its protease-deficient mutant. These strains were intraperitoneally injected in rainbow trout. The LD_{50} for the virulent strain was 1.3×10^5 viable cells per fish, while the LD_{50} for the mutant was more than 10^8 cells per fish. This mutant was described as nonpathogenic. Toranzo et al. (29) found LD_{50}s ranging from 10^2 to 3×10^5 cells (in most cases below 10^3) for *V. anguillarum* strains intraperitoneally injected into rainbow trout weighing 5 to 8 g. In the present infection experiments, the LD_{50} values for *V. salmonicida* (4×10^6 to 1×10^8 CFU per fish) were considered very high. The facts that the individuals which died after infection had characteristic clinical symptoms of cold-water vibriosis (8, 9, 20) and that *V. salmonicida* was reisolated from the dead individuals showed that the bacterial strains were capable of provoking cold-water vibriosis. The high lethal bacterial injection doses do not seem to be compatible with the lower bacterial charges which are expected to provoke disease in fish farms. However, certain environmental conditions may lead to increased abundance or virulence of the bacterium. Such factors must have an important influence on the outcome of

a situation in which fish are challenged with a pathogen. It is also important to note that we do not yet know the infection route for *V. salmonicida* under farming conditions. The natural entry mechanisms may represent a more harmful infection route than intraperitoneal injection. In addition, a possible decline in virulence of *V. salmonicida* after being isolated from fish must be considered. Bacteria were isolated from the kidney of the fish and directly transferred to NBA. The strains used in the experimental infections had been kept in the laboratory on NBA at 4°C or in 20% glycerol at −70°C for periods varying from a few weeks to about 4 years. The strain NCMB 2262 had been passed through fish (Atlantic salmon) several times since isolation in 1983, which could be the cause of the lower LD_{50} calculated for this strain. In our laboratory, however, we have not been able to observe any clear increase in virulence by passing a strain of *V. salmonicida* through fish several times over a short period.

Both the degree of virulence of the infective agent and the degree of susceptibility of the host are important in determining whether pathogenic challenge will result in disease. The high LD_{50}s determined in the present experiments may be due to the fish being exposed to less stress (e.g., changing temperature, crowding, and pollution) than farmed fish. Recent observations in our laboratory indicate that fish which do not thrive are more susceptible to infection with *V. salmonicida*. In conclusion, *V. salmonicida* exposure seems to be necessary but possibly not sufficient for the outbreak of cold-water vibriosis in fish farms.

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ADDENDUM IN PROOF

The recent paper by Sørum et al. (25a) is highly relevant to the present study.

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