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Plasmids in *Vibrio salmonicida* Isolated from Salmonids with Hemorrhagic Syndrome (Hitra Disease)

HENNING SØRUM,[1]* TRYGVE T. POPPE,[2] AND ØRJAN OLSVIK[1]

Department of Microbiology and Immunology, The Norwegian College of Veterinary Medicine, Postbox 8146, Dep.,[1] and National Veterinary Institute,[2] N-0033 Oslo 1, Norway

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***Vibrio*-like isolates from Atlantic salmon (*Salmo salar* Linnaeas) and a few from rainbow trout (*S. gairdneri* Richardson) suffering from hemorrhagic syndrome (Hitra disease), also called cold-water vibriosis, a disease of great importance in Norwegian fish farming, were examined for plasmid content. Of 84 strains isolated from 1982 to 1984, 70 (83.3%) had a common 21-megadalton (MDa) plasmid. A 3.4-MDa plasmid was found in 58 of the strains with the 21-MDa plasmid, and a 2.8-MDa plasmid was found in 23 of the strains with both the 21- and 3.4-MDa plasmids. The strains were isolated from fish farms along the western and northern coasts of Norway. Ten (11.9%) of the strains possessed a 61-MDa plasmid in addition to a 21-MDa plasmid. Two strains had only a 21-MDa plasmid. Of the 84 *Vibrio*-like isolates, 14 did not harbor plasmids identical in mass to any other plasmids found in this material. *Vibrio salmonicida* strains, 257 in all, isolated from salmonids with the same disease from the same areas from July 1986 to July 1987, all possessed a 21-MDa plasmid, either alone or in addition to a 3.4-MDa plasmid, or a combination of 3.4- and 2.8-MDa plasmids. Six of the strains had a 5.5-MDa plasmid instead of the 3.4-MDa plasmid. The restriction endonuclease patterns of the plasmids of similar molecular mass reflected similar nucleotide sequences. The plasmid content detected in isolates of *V. salmonicida* obtained from a coastline of more than 2,000 km and over a period of almost 6 years is stable.**

A disease of great importance in Norwegian salmonid farms, first called hemorrhagic syndrome or Hitra disease and later designated cold-water vibriosis, appeared in 1977. The disease occurred for the first time on a large scale on Hitra, an island off the coast of central Norway, in 1979 but has since ravaged fish farms with Atlantic salmon (*Salmo salar* Linnaeas) and, to a lesser extent, fish farms with rainbow trout (*S. gairdneri* Richardson) all along the western and northern coastlines of Norway (9). This disease has also been reported in Scotland (6). Although the etiology seems to be complex, *Vibrio*-like bacteria are regularly isolated from the kidneys and other internal organs of moribund fish. These vibrios could not be classified into known species of the genus *Vibrio* that cause classical vibriosis (*Vibrio anguillarum* and *V. ordalii*) (17; T. T. Poppe, Dr. Scient. thesis, Norwegian College of Veterinary Medicine, Oslo, 1986).

A new species designation, *V. salmonicida* sp. nov., has been proposed for *Vibrio* isolates from hemorrhagic syndrome (10). The fact that the *Vibrio* isolates from salmonids with hemorrhagic syndrome revealed heterogeneous biochemical properties (Poppe, thesis) resulted in a need for other markers which could help in classifying the strains into one or more homogeneous groups.

The fish pathogen *V. anguillarum* harbors a 47-megadalton (MDa) plasmid as a marker for pathogenic strains (7, 8). Other plasmids which characterize pathogenic strains have been reported in *Yersinia enterocolitica* (12), *Y. pseudotuberculosis* (13), *Shigella* sp. (25, 26), *Salmonella enteritidis* (23), *S. dublin* (27), and *Bacillus anthracis* (22, 30). *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis in salmonids, seems to harbor a pattern of cryptic plasmids which characterize the species (manuscript in preparation; 29). The intention of this study was to identify plasmids that could help in characterizing *Vibrio* isolates from salmonids with cold-water vibriosis.

* Corresponding author.

MATERIALS AND METHODS

Strains. A total of 84 bacterial isolates from Atlantic salmon (*S. salar* Linnaeas) and a few from rainbow trout (*S. gairdneri* Richardson) with hemorrhagic syndrome were investigated in the first part of this study. All of the strains were classified as *Vibrio*-like bacteria distinctly different from *V. anguillarum* and *V. ordalii*. These strains were classified into six groups on the basis of morphology and biochemical tests, in accordance with an earlier study (Poppe, thesis). The isolations were performed from 1982 to 1984, during outbreaks of hemorrhagic syndrome all along the Norwegian coast, from southwest Norway to the northern-most part of the country.

Another group of 257 *Vibrio* strains, isolated from fish farms with outbreaks of hemorrhagic syndrome or cold-water vibriosis (within the geographical areas described above) that occurred from July 1986 to July 1987, was also investigated. All 257 strains were morphologically characterized as *V. salmonicida*. Detection by monoclonal antibodies (clone 2B5) of a specific surface protein of *V. salmonicida* called VS-P1 (11; S. Espelid, K. O. Holm, K. Hjelmeland, and T. Jørgensen, J. Fish Dis., in press; K. Hjelmeland, K. Stensvåg, T. Jørgensen, and S. Espelid, J. Fish Dis., in press) was performed to verify that this group of strains consisted of only *V. salmonicida*.

After isolation, the strains were stored in seawater-yeast-peptone (SWYP) broth with 17% glycerol and 1% inactivated horse serum at −70°C. SWYP broth was prepared with artificial seawater as described by Hendrie et al. (15).

Plasmid profiling. The strains were grown on 5% cattle blood agar with 2% NaCl at 15°C for 3 days before being cultivated in 5 ml of SWYP broth for 2 days at 15°C in a roller drum.

Samples (1.0 ml) of the broth were transferred to 1.5-ml Eppendorf tubes, the bacteria were harvested and lysed, and the plasmids were isolated by a modified Birnboim procedure as described previously (5, 21). In some cases, a

TABLE 1. *Vibrio* isolates from Atlantic salmon and rainbow trout with hemorrhagic syndrome isolated from 1982 to 1984, grouped by plasmid profiles

Group	Plasmid(s) (MDa)	No. of strains	% of *Vibrio* isolates
A	21, 3.4	35	41.7
B	21, 3.4, 2.8	23	27.4
C[a]	61, 21	10	11.9
D	21	2	2.4
E	Heterogenous plasmids	14	16.7

[a] Some of the strains in group C seemed to harbor a small amount of a 3.4-MDa plasmid which could be visualized in some plasmid isolations (Fig. 1) but not in others.

phenol-chloroform extraction step was performed to purify the plasmid DNA before the lysates were subjected to electrophoresis.

Electrophoresis was performed in 1% agarose gel with Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) at 120 V (35 mA) for 2.75 h in a vertical gel apparatus at room temperature. The gels were stained with ethidium bromide for 10 min and destained with destilled water for 30 min. Photographs of the DNA bands were taken under UV light exposure. *Escherichia coli* K-12 strains containing the plasmids pDK9 (140 MDa), RP4 (34 MDa), and Sa (23 MDa) and the plasmids from *E. coli* V517 (1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, and 35.8 MDa) were used as controls and standards in each run. The procedure of Kado and Liu (19) was also performed for plasmid isolation in combination with, or instead of, the modified Birnboim procedure.

Restriction endonuclease characterization. Plasmids from eight strains, representing the main plasmid profile variants in this material, were analyzed by restriction endonuclease digestion. Three extraction steps with phenol, phenol-chloroform, and chloroform were introduced before the alcohol precipitation step in the modified Birnboim procedure.

Restriction endonuclease digestion with *Hin*dIII, *Acc*I, *Cla*I, and *Bam*HI (New England BioLabs, Beverly, Mass.) was performed in accordance with the instructions of the manufacturer. The samples were then subjected to electrophoresis in a 1% agarose gel with Tris-borate-EDTA buffer at 45 V (11 mA) for 8.5 h. Phage lambda (New England BioLabs) were digested and used as standards and controls.

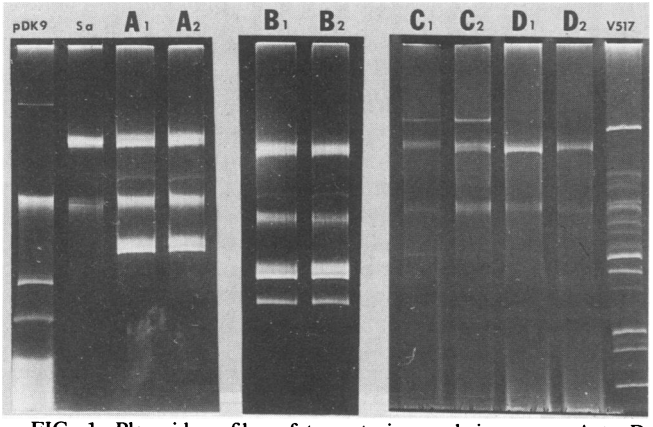

FIG. 1. Plasmid profiles of two strains each in groups A to D (Table 1), isolated by the modified Birnboim procedure. pDK9, Sa, and the plasmids from *E. coli* V517 were used as standards and controls.

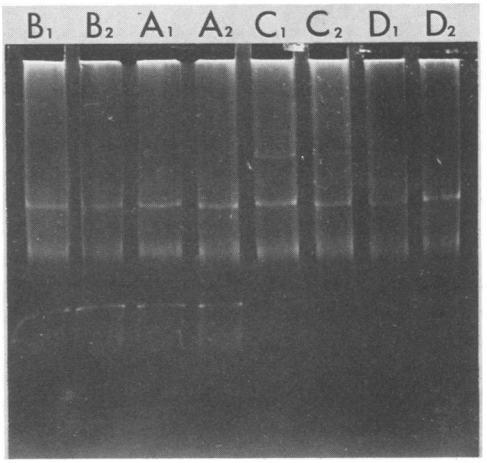

FIG. 2. Plasmids from groups A to D (Table 1) visualized after performance of the Kado and Liu procedure.

RESULTS

Plasmid profiling. Apart from 14 strains with completely heterogeneous profiles regarding both size and number of plasmids, four plasmids of different size were found in similar profiles, consisting of one to three plasmids, in the 70 remaining strains in the material of *Vibrio*-like isolates from 1982 to 1984. On the basis of the plasmid profile, the 70 strains (83.3%) could be placed into four groups according to plasmid patterns (Table 1; Fig. 1 and 2). Group A contained 35 strains (41.7%) with a 21-MDa plasmid and a 3.4-MDa plasmid. Group B contained 23 strains (27.4%) with a 2.8-MDa plasmid in addition to both the 21- and 3.4-MDa plasmids. Group C contained 10 strains (11.9%) with a 21-MDa plasmid and a 61-MDa plasmid, and group D had two strains (2.4%) with only a 21-MDa plasmid.

A 21-MDa plasmid was detected in all of the 257 *V. salmonicida* strains isolated from July 1986 to July 1987. One hundred and seventy-five strains (68%) had one extra plasmid of 3.4 MDa in addition to the 21-MDa plasmid. Fifty-two (20%) of the strains had a third plasmid of 2.8 MDa in addition to the first two mentioned. Twenty-four strains (9.3%) harbored only the 21-MDa plasmid. The six remaining strains (2.4%) had a 5.5-MDa plasmid instead of the 3.4-MDa plasmid. Four of these six isolates had a 2.8-MDa plasmid in addition to the 21- and 5.5-MDa plasmids. A 61-MDa plasmid was not seen in the isolates from 1986 to 1987 (Table 2).

The modified Birnboim procedure often resulted in conformational changes of the plasmid DNA in that gels often showed more than one band for the same plasmid. The degree of these conformational changes of plasmid DNA often varied from one preparation to another, even if care was taken to reproduce the procedure accurately.

TABLE 2. Plasmids of 257 *V. salmonicida* strains isolated from salmonids with hemorrhagic syndrome or cold-water vibriosis from July 1986 to July 1987

Plasmid size(s) (MDa)	No. of strains	% of *V. salmonicida* isolates
21, 3.4	175	68.1
21, 3.4, 2.8	52	20.2
21	24	9.3
21, 5.5, 2.8	4	1.6
21, 5.5	2	0.8

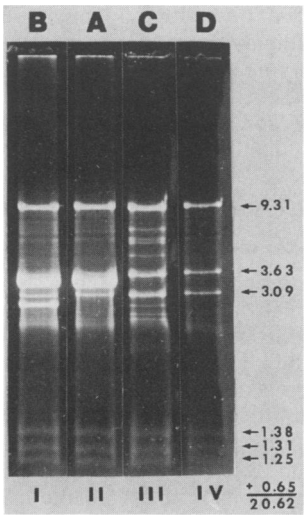

FIG. 3. *Hin*dIII endonuclease digestion of one strain each from groups A to D (Table 1). The lanes are marked from I to IV. Major digestion products from the 21-MDa plasmid in a group D strain are indicated in megadaltons in lane IV. The 0.65-MDa fragment is not visible in the gel. Additional weaker bands are products of partial digestion.

The Kado and Liu method for extraction of plasmids (19) resulted in sharper bands for all except the smallest plasmid (2.8 MDa) than did the modified Birnboim procedure (5). The small 2.8-MDa plasmid was not always visualized by the Kado and Liu procedure (Fig. 2).

Restriction endonuclease characterization. Plasmids from two strains in each plasmid group in Table 1 were digested with *Hin*dIII (Fig. 3; Table 3). Only the 21- and 61-MDa plasmids were digested. The 21-MDa plasmid was digested into seven fragments of equal size in all strains, while the 61-MDa plasmid was digested into eight fragments. Restriction endonuclease digestion with *Acc*I gave essentially the same results as *Hin*dIII digestion (Table 3).

A similar restriction endonuclease digestion with *Cla*I revealed minor difference in the 21-MDa plasmid in groups A and B, compared with groups C and D. These latter groups showed one single smaller fragment which deviated very slightly in the light direction in relation to the comparable fragment in groups A and B (Table 3). Of the enzymes used, *Cla*I was the only one that digested the small 2.8-MDa plasmid, the result being two or more fragments.

Restriction endonuclease digestion with *Bam*HI produced only one fragment from the 21-MDa plasmid. The other plasmids were not digested by *Bam*HI (Table 3).

A 21-MDa plasmid was common to all four plasmid groups in Table 1 (83.3% of the strains) and showed only minor variation in nucleotide sequence in groups C and D (14.3% of the strains), compared with groups A and B (69.1% of the strains). A 3.4-MDa plasmid was common to groups A and B, and this plasmid was not digested by any of the four restriction endonucleases used. A 61-MDa plasmid occurred only in plasmid group C (11.9% of the strains), while a 2.8-MDa plasmid was found only in group B (27.4% of the strains).

TABLE 3. Numbers and patterns of fragments from plasmids of *Vibrio* strains in Table 1 after digestion with different restriction enzymes[a]

Group	Plasmid size (MDa)	No. of fragments			
		*Hin*dIII	AccI	*Cla*I	*Bam*HI
A	21	7	6	7	1
	3.4	—	—	—	—
B	21	As group A	As group A	As group A	1
	3.4	—	—	—	—
	2.8	—	—	—	—
C	61	8	2	5	—
	21	As group A	As group A	7[b]	1
D	21	As group A	As group A	As group C	1

[a] —, Undigested plasmids.
[b] One single fragment smaller than the corresponding fragment from the 21-MDa plasmid in group A.

DISCUSSION

The fact that more than 83% of the *Vibrio*-like isolates from salmonids with hemorrhagic syndrome (Hitra disease) proved to possess a common 21-MDa plasmid is interesting. The coexistence of a 3.4-MDa plasmid with the 21-MDa plasmid in 69% or more of the *Vibrio* isolates from diseased fish makes the two plasmids important markers of a homogeneous group of vibrios associated with hemorrhagic syndrome in salmonids. One intention of this study was thus fulfilled, in that specific markers of the isolates which could be used to classify the isolates better than traditional tools, such as biochemical tests, were detected. The presence of a 2.8-MDa plasmid together with the 21- and 3.4-MDa plasmids in about 27% of the *Vibrio* isolates further indicates the genetic homology of the bacterial isolates from diseased fish.

The conclusions regarding the central position of this homogeneous *Vibrio* group are further supported by the results of plasmid screening of 12 non-*Vibrio* isolates belonging to the genus *Photobacterium*, isolated from salmonids with hemorrhagic syndrome, which was performed in parallel with this study (data not shown). There was no similarity between these non-*Vibrio* strains regarding the plasmids they possessed or their plasmid profiles. Of the 14 *Vibrio*-like strains found to have only heterogeneous plasmids, 7 strains were the only clear hemolytic ones found in this material. On the basis of hemolytic and biochemical properties, they were placed in a distinct group in a system of six groups of vibrios isolated from hemorrhagic syndrome introduced by Poppe (thesis). If this heterogeneous *Vibrio* group is excluded from the present *Vibrio* isolate material, as many as 91% of the strains possess a common 21-MDa plasmid, a particularly high percentage in this connection.

There is, however, no statistical correlation between the occurrence of the different plasmid profiles described in this study and the remaining five biochemical groups of strains outlined by Poppe. All 257 *V. salmonicida* strains investigated from July 1986 to July 1987 harbored a 21-MDa plasmid, an interesting finding which shows that the 21-MDa plasmid is characteristic for the species *V. salmonicida*. The conclusion can thus be drawn that the 83% of unselected *Vibrio* strains isolated from salmonids with hemorrhagic syndrome or cold-water vibriosis from 1982 to 1984 which possessed this plasmid in fact belonged to the species later named *V. salmonicida* (10).

The 10 isolates with the 61-MDa plasmid originated from six fish farms in three local areas in northern Norway (group

C; Table 1; Fig. 1 and 2). This plasmid was not found in other parts of Norway nor was it found among the 257 *V. salmonicida* strains isolated from July 1986 to July 1987. The genetic message of this plasmid is also cryptic.

The strains with the 61-MDa plasmid seem mainly to contain only the 21-MDa plasmid in addition. In some plasmid isolations performed on the 10 strains, it was possible to see a faint band of the 3.4-MDa plasmid (Fig. 1). The reason for the presence of the 3.4-MDa plasmid in a lower copy number in group C strains than in the other strains may be that the 61- and 3.4-MDa plasmids belong to the same incompatibility group and therefore cannot exist harmoniously together in the same bacterial cell (4, 14).

Among the 257 *V. salmonicida* strains isolated from July 1986 to July 1987, more than half showed resistance to tetracycline and the vibriostatic agent O/129 (unpublished data). Tetracycline and sulfa-trimethoprim have been regularly used in treatment of cold-water vibriosis since the disease first appeared in the late 1970s, so isolation of the first *V. salmonicida* strain resistant to one or more of these commonly used drugs was not unexpected (16). O/129 has, however, not been used for treatment of fish diseases. It is interesting that we could not detect additional plasmids in the antimicrobial agent-resistant *V. salmonicida* strains compared with the drug-susceptible isolates from the earlier period of 1982 to 1984 or compared with susceptible strains isolated in parallel with the resistant ones. Resistance plasmids are normally found in drug-resistant fish pathogens (2, 3). However, the methods used for plasmid isolation and the conditions provided for growth of *V. salmonicida* in this study might not be optimal for detecting very low-copy-number or very large-molecular-mass R plasmids. There is no statistical correlation between the occurrence of the different plasmids reported in this study and the resistance to antibacterial drugs found in the strains.

The plasmids of the *V. salmonicida* strains included in this material showed no mobility during repeated subcultivations and isolations over 2 years. This stability makes plasmids a useful characterizing feature in this group of bacteria. All four restriction endonucleases used in this study revealed extensive homology between the nucleotide sequences of the plasmids in the strains examined. Plasmids of the same size within each plasmid group seem to be identical. The fact that the 3.4-MDa plasmid was not digested by any one of the restriction enzymes used indicates that this plasmid also has a homological polynucleotide sequence. Only between the 21-MDa plasmid of the strains of groups C and D and the 21-MDa plasmid of the strains of groups A and B were minor differences detected (Table 3). Further restriction endonuclease characterization with other restriction enzymes and, especially, complete sequencing of the plasmids of the different groups should reveal the precise difference in nucleotide sequence between the groups. A divergence of the nucleotide sequences of the 47-MDa plasmid in *V. anguillarum* has been shown (28), whereas in *Y. enterocolitica*, a divergence of the nucleotide sequence of the 40- to 50-MDa pathogenicity plasmid between different serogroups, and even within the same serogroup, has been demonstrated (24).

The modified plasmid isolation procedure of Birnboim (5) gave a double banding of both the 21- and 3.4-MDa plasmids (Fig. 1), which could be mistaken as representing two plasmids with nearly identical masses instead of one. Though this problem was relatively constant with this procedure, the procedure of Kado and Liu (19) gave sharp single bands. The mechanical manipulation of the DNA in the modified Birnboim procedure is much more pronounced than with the Kado and Liu procedure, and it seems that the plasmids of *V. salmonicida* are more sensitive to this than, for instance, the plasmids of *E. coli* (20) and *S. typhimurium* (18). Degrees of conformational changes of the plasmids from the supercoiled form may alter the speed of the plasmids in the gel during electrophoresis (31), perhaps explaining the appearance of the confusing plasmid bands when the modified Birnboim procedure was used in this study.

When the Kado and Liu procedure for plasmid isolation was used, the small 2.8-MDa plasmid could not always be isolated. This is not surprising, since Toranzo et al. (29) have mentioned that the Kado and Liu method is more effective in detecting high-molecular-weight plasmids.

The present study revealed that the plasmid contents of isolates of *V. salmonicida* obtained from a coastline of more than 2,000 km and over a period of almost 6 years is stable. Plasmids therefore constitute a valuable marker of the species. The 47-MDa plasmid of *V. anguillarum* is a pathogenicity plasmid with specific encoding of an iron-sequestering system which allows the bacterium to grow inside the fish and cause disease (1, 8). It would be of great interest to investigate the importance of the genetic message of the cryptic plasmids described in this study, especially the widespread 21-MDa plasmid.

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