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Evidence for the Existence of Distinct Populations of *Vibrio anguillarum* Serogroup O1 Based on Plasmid Contents and Ribotypes

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A total of 103 *Vibrio anguillarum* serogroup O1 strains displaying 15 different plasmid profiles were characterized with respect to biochemical properties and ribotypes. The results confirmed that *V. anguillarum* O1 is a biochemically homogeneous group. The 103 strains could be allocated to three main clusters with high similarity coefficients. None of the biochemical properties were connected with the presence of plasmids. In total, 12 different ribotypes were demonstrated, with *Hin*dIII being used as the restriction enzyme. Forty of the strains were isolated from the same Danish fish farm, some from the kidneys of diseased fish and some from the environment, and some strains were isolated from the mucus, gills, and feces of healthy fish. Nineteen of these isolates possessed the 67-kb virulence plasmid alone or in combination with other plasmids, while 21 had no plasmids. All strains isolated from the kidneys of diseased fish on this farm had plasmids. Irrespective of their origin (kidneys, gills, or mucus), all 19 strains carrying the 67-kb virulence plasmid had the same ribotype, profile 1, while isolates without plasmids belonged to five different profiles, all different from profile 1. These results suggest that pathogenic *V. anguillarum* O1 strains possessing a virulence plasmid and nonpathogenic strains without plasmids from a small geographical area and even from the same fish may constitute two essentially distinct populations. Thus, it may be suggested that an exchange of virulence plasmids among strains is unlikely to occur in vivo.

Vibrio anguillarum has been studied by several authors, and various methods have been developed for its characterization and typing: biochemical analysis (2–4), serological analysis (11, 22), plasmid analysis (10, 15, 25–27), restriction enzyme digestion of plasmids (13), ribotyping (16, 17), and pulsed-field gel electrophoresis (21). API 20E has previously been used to identify and characterize *Vibrio* species. Overman et al. (14) found that API 20E correctly identified 60 isolates belonging to nine species of the family *Vibrionaceae*, and they concluded that API 20E would be a valid system for the identification of the more commonly occurring members of this family. However, *V. anguillarum* was not among the species tested, and *V. anguillarum* is not (yet) included in the database from the manufacturer. Santos et al. (19) tested the API 20E system on various fish pathogenic bacteria. They reported that a certain proportion of the strains were misidentified. All *V. anguillarum* strains were wrongly identified, with some of them being identified as *Aeromonas hydrophila*. The authors concluded that API 20E could be a useful tool for field diagnosis of fish pathogenic bacteria, provided that some important species, among them *V. anguillarum*, were added to the database and that the gallery was extended with some additional tests. Grisez et al. (7) used the API 20E system for the identification of *V. anguillarum* and *Vibrio ordalii*. They found that those two species were well separated from each other and from other *Vibrio* species included in the study. In that study, the *V. anguillarum* strains were subdivided into six phena. However, no information on the serotypes of the strains was given. In the present study, we used API 20E supplemented with a few additional tests for the subdivision of the *V. anguillarum* serogroup O1.

The presence of an approximately 67-kb virulence plasmid in *V. anguillarum* serogroup O1 strains pathogenic to fish has been described (6, 10, 27), and it has been shown that curing of this plasmid reduces the virulence dramatically (5). The virulence is believed to be caused by a plasmid-encoded iron sequestering system. In addition, the 67-kb plasmid seems to mediate a restriction system that prevents conjugal entry of plasmid DNA into the cell (20). Hitherto, no other phenotypic properties associated with this plasmid have been identified with certainty. Recently, it was suggested that *V. anguillarum* serogroup O1 isolates containing plasmids were arabinose positive and trehalose negative and did not agglutinate erythrocytes or form a pellicle and that strains without plasmids were more variable but generally were positive with regard to these four characteristics (9, 10). However, whether the phenotypic differences for these properties were associated with the plasmid itself or were due to the presence of different populations of bacteria has not been studied. In the present article, we present API 20E results for a group of *V. anguillarum* serogroup O1 strains and demonstrate that none of the examined phenotypic properties could be ascribed to the presence or absence of plasmids and, on the basis of ribotyping, that populations of *V. anguillarum* with and without plasmids may constitute distinct populations of bacteria, even within a very small area.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 103 *V. anguillarum* serogroup O1 strains were used in the study. The strains were isolated in our own laboratory or received as gifts from other laboratories. The origins of the strains are listed in Table 1. All strains were stored at −80°C until used. The strains were propagated on blood agar plates (Marine agar; Difco) to which 5% sterile

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TABLE 1. *V. anguillarum* strains with various plasmid profiles and ribotypes

Strain no.	Strain	Isolated from	Country	Plasmid(s)	Ribotype
1	UB 909/89	Sea bass	Italy	Empty	1
2	LMG 13187	Sea bass	France	Empty	1
3	RVAU PT24	Ayu	Japan	Empty	1
4	RVAU PT213	Ayu	Japan	Empty	1
5	850610-1/5A	Rainbow trout	Denmark	Empty	1
6	V1 3/2	Water	Denmark	Empty	7
7	S1 1/2	Mucus, rainbow trout	Denmark	Empty	9
8	S4 5/7	Mucus, rainbow trout	Denmark	Empty	7
9	S2 2/9	Mucus, rainbow trout	Denmark	Empty	10
10	S2 3/9	Mucus, rainbow trout	Denmark	Empty	10
11	S2 4/9	Mucus, rainbow trout	Denmark	Empty	10
12	S3 3/9	Mucus, rainbow trout	Denmark	Empty	10
13	S3 4/9	Mucus, rainbow trout	Denmark	Empty	10
14	S4 1/9	Mucus, rainbow trout	Denmark	Empty	10
15	S4 2/9	Mucus, rainbow trout	Denmark	Empty	10
16	G2 3/9	Gills, rainbow trout	Denmark	Empty	11
17	G2 4/9	Gills, rainbow trout	Denmark	Empty	10
18	G2 5/9	Gills, rainbow trout	Denmark	Empty	11
19	G3 3/9	Gills, rainbow trout	Denmark	Empty	11
20	G4 2/9	Gills, rainbow trout	Denmark	Empty	10
21	F2 1/9	Feces, rainbow trout	Denmark	Empty	10
22	F2 2/9	Feces, rainbow trout	Denmark	Empty	10
23	F2 3/9	Feces, rainbow trout	Denmark	Empty	10
24	F2 4/9	Feces, rainbow trout	Denmark	Empty	10
25	F2 5/9	Feces, rainbow trout	Denmark	Empty	10
26	90-11-286	Rainbow trout	Denmark	4.5 kb	2
27	V1 1/2	Water	Denmark	7.0 kb	8
28	UB AO23	Turbot	Spain	26 kb	1
29	LMG 12101	Unknown	Unknown	77 kb	5
30	9014/8	Rainbow trout	Denmark	90 kb	1
31	UB 191/90	Sea bass	Italy	78 kb, 84 kb	1
32	RVAU T 265	Atlantic salmon	United Kingdom	51 kb, 11.5 kb, 5.3 kb	1
33	6018/1	Rainbow trout	Denmark	pJM1	1
34	HWU 63	Atlantic salmon	Chile	pJM1	1
35	HWU BA52	Rainbow trout	Japan	pJM1	1
36	UB 861/89	Sea bass	Italy	pJM1	3
37	UB AO24	Turbot	Spain	pJM1	1
38	RVAU 820901-2/2	Rainbow trout	Sweden	pJM1	12
39	RVAU 775	Salmon	United States	pJM1	4
40	RVAU VA41	Atlantic salmon	Canada	pJM1	4
41	RVAU VA61	Sockeye salmon	Canada	pJM1	4
42	IP G83.01	Turbot	France	pJM1	1
43	IP 19.22	Sea bream	France	pJM1	1
44	IP 408F	Rainbow trout	France	pJM1	5
45	IP 07.84	Rainbow trout	Italy	pJM1	3
46	IP 04.88	Rainbow trout	Italy	pJM1	3
47	IP V62	Bar	France	pJM1	1
48	IP 10283	Turbot	France	pJM1	1
49	IP 10466	Turbot	France	pJM1	1
50	HWU BA37	Rainbow trout	Japan	pJM1	1
51	HWU BA136	Brown trout	Italy	pJM1	3
52	VIB 247	Rainbow trout	Finland	pJM1	5
53	HWU 85/3954-4	Rainbow trout	Not known	pJM1	1
54	HWU 89/4068	Rainbow trout	Not known	pJM1	1
55	HWU 86/3674	Atlantic salmon	Not known	pJM1	1
56	HWU RIP	Chinook salmon	Canada	pJM1	1
57	LMG 13583	Chinook salmon	United States	pJM1	1
58	LMG 13635	Chanos chanos	Not known	pJM1	1
59	89-12-199	Rainbow trout	Denmark	pJM1	1
60	1359A	Rainbow trout	Italy	pJM1	3
61	VA 12	Rainbow trout	Canada	pJM1	4
62	408F	Rainbow trout	France	pJM1	5
63	R62	Turbot	Spain	pJM1	1
64	28/89	Sea bass	Italy	pJM1	1
65	261/91	Sea bass	Italy	pJM1	1
66	49/92	Sea bass	Italy	pJM1	1
67	143/93	Sea bass	Italy	pJM1	1
68	860611-3	Rainbow trout	Denmark	pJM1	1

Continued on following page

TABLE 1—*Continued*

Strain no.	Strain	Isolated from	Country	Plasmid(s)	Ribotype
69	S1 2/3	Mucus	Denmark	pJM1	1
70	S3 4/3	Mucus	Denmark	pJM1	1
71	G2 1/3	Gills, rainbow trout	Denmark	pJM1	1
72	840530-1/1	Rainbow trout	Denmark	pJM1	1
73	9014/12	Rainbow trout	Denmark	pJM1	1
74	S1 1/3	Mucus, rainbow trout	Denmark	pJM1	1
75	S1 3/3	Mucus, rainbow trout	Denmark	pJM1	1
76	S1 5/3	Mucus, rainbow trout	Denmark	pJM1	1
77	S2 2/3	Mucus, rainbow trout	Denmark	pJM1	1
78	S2 4/3	Mucus, rainbow trout	Denmark	pJM1	1
79	S3 2/3	Mucus, rainbow trout	Denmark	pJM1	1
80	G1 1/3	Gills, rainbow trout	Denmark	pJM1	1
81	G1 3/3	Gills, rainbow trout	Denmark	pJM1	1
82	G1 5/3	Gills, rainbow trout	Denmark	pJM1	1
83	840523-2/1	Kidney, rainbow trout	Denmark	pJM1	1
84	850827-3/2	Kidney, rainbow trout	Denmark	pJM1	1
85	HWU 53	Rainbow trout	Denmark	pJM1, 50 kb	6
86	RVAU 91-8-178	Turbot	Norway	pJM1, 53 kb	6
87	RVAU RG 75-834	Salmon	United States	pJM1, 36 kb	1
88	IP P09.80	Rainbow trout	France	pJM1, 83 kb	5
89	IP 10482	Bar	France	pJM1, 36 kb	1
90	348/93	Sea bass	Italy	pJM1, 90 kb	1
91	90/93	Sea bass	Italy	pJM1, 90 kb	1
92	G1 2/5	Gills, rainbow trout	Denmark	pJM1, 90 kb	1
93	840606-2/4	Rainbow trout	Denmark	pJM1, 90 kb	1
94	9013/3	Rainbow trout	Denmark	pJM1, 90 kb	6
95	G2 2/3	Gills, rainbow trout	Denmark	pJM1, 90 kb	1
96	G4 2/3	Gills, rainbow trout	Denmark	pJM1, 90 kb	1
97	G4 4/3	Gills, rainbow trout	Denmark	pJM1, 90 kb	1
98	S1 4/5	Mucus, rainbow trout	Denmark	pJM1, 90 kb	1
99	S4 1/5	Mucus, rainbow trout	Denmark	pJM1, 90 kb	1
100	840523-2/2	Rainbow trout	Denmark	pJM1, 90 kb	1
101	840627-2/2	Rainbow trout	Denmark	pJM1, 90 kb	1
102	HWU BA35	Sockeye salmon	United States	pJM1, 11.5 kb, 5.3 kb	1
103	HWU C1	Chum salmon	Canada	pJM1, 11.5 kb, 5.3 kb	1

citrate-stabilized calf blood had been added and were incubated at 20°C for 2 days.

Serotyping. O serotyping was performed by slide agglutination as previously described (11, 22).

Biochemical characterization. Biochemical tests were carried out with API 20E strips (Biomérieux, Marcy l'Étoile, France) according to the instructions of the manufacturer, except that the bacteria were suspended in 0.9% saline and the incubation was done at 20°C for 48 h. The API 20E tests were supplemented with fermentation of trehalose and the formation of a pellicle in broth cultures. Clustering analysis was performed with the computer program TAXAN, version 4.0 (Sea Grant College, University of Maryland), by simple matching coefficient and unweighted average linkage.

Plasmid profiling. Plasmids were extracted by the method of Kado and Liu (8) and separated by electrophoresis in 0.8% agarose gels (SeaKem GTG; FMC BioProducts, Rockland, Maine) in Tris-acetate-EDTA (TAE) buffer, pH 8.0. Gels were stained with ethidium bromide and photographed in UV light. Plasmids from *Escherichia coli* 39R861 (23) and V517 (12) were used as molecular weight size markers.

Ribotyping. The method described by Pedersen and Larsen (17) was used. Chromosomal DNA was isolated by the method of Pedersen and Larsen (17), digested with *Hin*dIII, electrophoresed in 0.8% agarose gels in TAE buffer, transferred to nylon hybridization membranes (Hybond-N; Amersham), and fixed to the membranes by incubation at 80°C for 1 h. Hybridization was carried out with a digoxigenin-labeled probe complementary to 16S and 23S rRNA of *E. coli*, and the hybridized fragments were visualized with alkaline phosphatase-labeled antidigoxigenin (Boehringer, Mannheim, Germany) and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer).

RESULTS

On the basis of the clustering analysis, which is based on the biochemical properties of the strains, the strains were divided into three major groups (Fig. 1). Groups 1 and 2 had a similarity of 82%, while group 3 was separated from groups 1 and

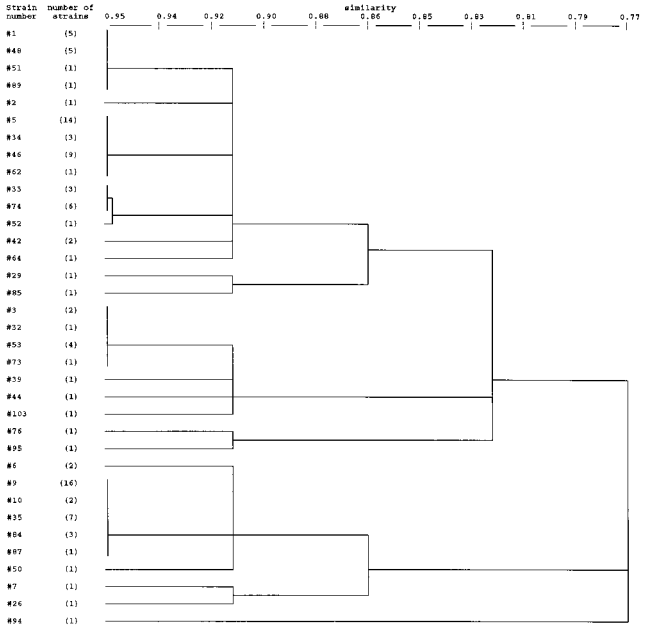

FIG. 1. Dendrogram generated on the basis of the phenotypic properties of 103 *V. anguillarum* serogroup O1 strains with the TAXAN computer program version 4.0, by simple matching coefficient and unweighted average linkage. The strain numbers are the same as those listed in Table 1, while the numbers in parentheses refer to the number of strains with identical properties.

TABLE 2. Biochemical reactions of 103 *V. anguillarum* serogroup O1 strains

Reaction	No. of positive strains
ONPG	102
Arginine dihydrolase	101
Lysine decarboxylase	2
Ornithine decarboxylase	0
Citrate utilization	89
H_2S production	0
Urease	0
Tryptophane deaminase	0
Indole	102
VP	99
Gelatinase	92
Acid from:	
Glucose	103
Mannitol	103
Inositol	72
Sorbitol	100
Rhamnose	0
Saccharose	103
Melibiose	0
Amygdalin	84
Arabinose	77
Trehalose	40
Pellicle	54

2, with the three groups having a similarity of only 77%. In addition, a few single strains that did not cluster in any of these three main groups were found. The strains of the first group were mainly gelatinase positive, inositol positive, arabinose variable, pellicle negative, and trehalose positive. The strains belonging to the second group were mainly gelatinase positive, inositol negative, arabinose variable, pellicle positive, and trehalose negative, while strains from the third group were usually gelatinase negative, inositol positive, arabinose positive, pellicle negative, and trehalose positive.

The results of the plasmid profiling are shown in Table 1. Twenty-five strains carried no plasmids. Apart from strains 1 to 5, these were all isolated at the same fish farm at Tærø, Denmark, from water samples and from the mucus and gills of various rainbow trout. These 20 isolates, strains 6 to 25, were all without plasmids upon isolation. For strains 1 to 5, no information about their plasmid contents was available at the time of isolation. Seven strains (26 to 32) contained one, two, or three plasmids that were not the 67-kb virulence plasmid; 52 strains (33 to 84) harbored the 67-kb plasmid alone; while 19 isolates (85 to 103) had the 67-kb plasmid together with one (17 strains) or two (2 strains) other plasmids.

None of the biochemical characteristics could be ascribed to the presence or absence of plasmids. However, the group of strains that were from the Tærø fish farm and that possessed the 67-kb plasmid were mainly pellicle and trehalose negative and arabinose positive. Most of these strains clustered with strain 5 (Table 2). Most of the plasmid-free strains from this fish farm clustered in another group with strain 9.

A total of 12 different ribotypes were identified (Fig. 2). Profile 1 was by far the largest (63 strains), and strains both with and without the virulence plasmid fell into this category. However, for strains without plasmids, this profile applied only to strains isolated from the kidneys of fish. Strains with the profile 1 ribotype were retrieved from several countries and fish species. Profile 2 fit only one strain. This was isolated from the kidney of a rainbow trout in Denmark. This strain had a

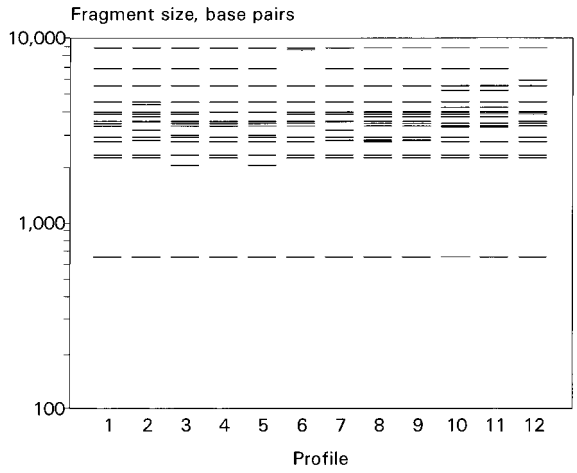

FIG. 2. Twelve different ribotypes of *V. anguillarum* serogroup O1, with *Hin*dIII being used as the restriction enzyme. Lane 1, 909/89 (profile 1); lane 2, 90-11-286 (profile 2); lane 3, HWU BA136 (profile 3); lane 4, RVAU VA41 (profile 4); lane 5, IP 408F (profile 5); lane 6, 9013/3 (profile 6); lane 7, V1 3/2 (profile 7); lane 8, V1 1/2 (profile 8); lane 9, S1 1/2 (profile 9); lane 10, S2 2/9 (profile 10); lane 11, G2 3/9 (profile 11); lane 12, RVAU 820901-2/2 (profile 12).

small plasmid, 4.5 kb. Profiles 3, 4, 6, and 12 applied only to isolates containing the 67-kb virulence plasmid, while profile 5 fit strains possessing the 67-kb virulence plasmid and one strain containing a 77-kb plasmid. However, restriction enzyme analysis of this 77-kb plasmid showed that it was very closely related to the virulence plasmid (data not shown). Profiles 7 to 11 applied exclusively to environmental isolates without the virulence plasmid. Strains 6 to 25 and 27 were all isolated from the same fish farm at Tærø, Denmark. Nineteen isolates that came from the same fish farm but that contained the 67- or 90-kb plasmid or both plasmids or an approximately 80-kb plasmid (strain 30) closely related to the 67-kb plasmid all belonged to ribotype 1. The ribotype 1 strains were isolated from the kidneys of farmed rainbow trout as well as from mucus and the gills. In some cases, plasmid-containing strains belonging to ribotype 1 and empty strains belonging to other ribotypes were isolated from the same fish.

DISCUSSION

The API 20E results showed that *V. anguillarum* serogroup O1 is a relatively homogeneous group but shows variation in some characteristics. The strains clustered irrespective of plasmid content and ribotype. One strain, number 94, deviated considerably from the other isolates by being *o*-nitrophenyl-β-D-galactopyranoside (ONPG), arginine, citrate, Voges-Proskauer (VP), and gelatinase negative. However, this strain was isolated in 1981, and it is not known whether these properties were absent upon isolation or whether they were lost by subculturings in the laboratory. This strain carried the 67-kb virulence plasmid and had the common ribotype 1. Strains 7 and 26 formed a small group and deviated somewhat from all other strains. These two strains had unique ribotypes, 9 and 2, respectively, carried no virulence plasmid, and were the only two lysine decarboxylase-positive strains. Our API 20E results did not form the same clusters as those described by Grisez et al. (7), although the results showed many similarities. Significant differences were found in the ability to ferment inositol. While Grisez et al. (7) found only 2 of 68 isolates to be positive, we found 72 of 103 strains to be positive. Additionally, in our study, only two strains were arginine dihydrolase negative,

while Grisez et al. (7) found a cluster of four strains and a single strain belonging to another cluster to be negative for arginine dihydrolase. In our study, we found 11 strains to be negative for gelatinase, while Grisez et al. (7) found all strains to be positive. Wiik et al. (29) described how *V. anguillarum* could be divided into two biochemical groups, with one group carrying a 47-MDa plasmid. Serological examination showed that the plasmid-carrying group was serotype O1 while the other was O2. Therefore, the O1 strains all belonged to the same cluster. The authors reported only minor differences in biochemical reactions among O1 strains. However, one serotype O1 strain was reported to be gelatinase negative, one was indole negative, and some variation in the ability to ferment trehalose was recorded. Grisez et al. (7) reported that the Scandinavian strains formed an indole-negative phenon. This was not confirmed by our results or the results of Wiik et al. (29) or Tiainen et al. (24). In a study by Bryant et al. (4), all (75) *V. anguillarum* strains were arginine positive, one strain was lysine positive, and one was ornithine positive. All strains in that study were ONPG, gelatinase, mannitol, and trehalose positive, while most strains were indole (88%) and VP (97%) positive. Some (9%) of the strains were inositol positive. However, no information on serotypes was given.

Various phenotypic characteristics have been associated with plasmids in bacteria, among them lactose and saccharose fermentation, degradation of proteins, and utilization of citrate (for a review, see Tschäpe [28]). None of these properties correlated with the plasmid contents of *V. anguillarum* O1, and recent research results obtained in our laboratory indicate that antibiotic resistance factors are not carried by the 67-kb virulence plasmid (18). Aoki et al. (1) demonstrated the presence of R plasmids in Japanese isolates of *V. anguillarum* and showed that these had molecular weights higher than those of the virulence plasmid.

In previous studies of the ribotypes of *V. anguillarum* O1, six different ribotypes were detected (16, 17), with both *Hin*dIII and *Eco*RI being used as restriction enzymes. The strains in these studies were all isolated from dead fish. In the present investigation, environmental strains without plasmids were included. Environmental strains are defined as strains originating from the skin, gills, mucus, feces, etc., of healthy fish and from water, sediment, etc. Some of these strains were shown to have hitherto undescribed ribotypes. These strains were never isolated from the kidney of a dead fish, and their pathogenic properties remain to be studied in more detail. Some strains without the virulence plasmid but having the common ribotype 1 were isolated from the kidneys of fish and should be considered pathogenic to fish. These strains may have had the plasmid upon isolation and lost it in the laboratory, but to the authors' knowledge, no studies on the stability of plasmids in *V. anguillarum* have been conducted so far. Our results also confirm the applicability of ribotyping in epidemiological and ecological studies of *V. anguillarum*. Further investigations of environmental *V. anguillarum* serogroup O1 isolates without plasmids should be carried out to study their prevalence, ecological significance, and possible role as pathogens and their similarity to the fish-pathogenic plasmid-containing strains. Also, experiments should be performed to investigate if environmental isolates are able to receive a virulence plasmid in in vitro models.

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