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Occurrence of Plasmids in Danish Isolates of *Vibrio anguillarum* Serovars O1 and O2 and Association of Plasmids with Phenotypic Characteristics

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Two hundred and twenty-eight isolates of *Vibrio anguillarum* serovar O1 (125 isolates) and serovar O2 (103 isolates) have been characterized with regard to plasmid contents, biochemical properties, and in vitro hemagglutination and hydrophobic properties. Among 74 *V. anguillarum* isolates from diseased fish, 63 carried only a 67-kb plasmid (pJM1), 9 carried an additional 98-kb plasmid, and 1 isolate carried only the 98-kb plasmid. Only one isolate was without plasmids. In *V. anguillarum* serovar O1 from nondiseased fish (mucus and gills), plasmids of the same sizes were present in 29 isolates (58%), whereas 21 isolates (42%) were plasmid free. Based on hemagglutination and biochemical properties, *V. anguillarum* serovar O1 isolates were divided into eight biovars. The plasmid-carrying strains (102 isolates) all fell within biovars 1 and 2, whereas the 23 strains of biovars 3 to 8 were without plasmids. It was tentatively concluded there are two populations of *V. anguillarum* serovar O1. One population contains plasmid(s), is hemagglutination negative and trehalose negative, and does not form pellicles in broth cultures, whereas the other population is plasmid free and has the opposite characteristics. The former group is the one related to disease in fish. All 20 *V. anguillarum* serovar O2 isolates from the environment were without plasmids, whereas 54 (65%) of the isolates from fish (trout and cod) carried plasmids. The biochemical diversity within serovar O2 was pronounced; 13 different biovars were demonstrated. No correlation between the presence of plasmids and biochemical properties was observed.

Vibrio anguillarum is the most important agent of hemorrhagic septicemias among marine fish. *V. anguillarum* isolates can be subdivided into 10 groups based on O antigens; most isolates from diseased fish are classified as serovar O1 or O2 (22).

In their analysis of highly virulent strains of *V. anguillarum*, Crosa et al. (6) consistently demonstrated a 50-MDa plasmid, designated pJM1, that was not present in weakly virulent strains. Curing of the pJM1 plasmid was correlated with decreased virulence (5). pJM1 encodes a very efficient iron-sequestering system that enables invading *V. anguillarum* to compete with iron-binding proteins in serum (4). Two plasmid-mediated components, a diffusible siderophore that is liberated to the environment and a nondiffusible receptor (presumably the outer membrane protein OM2), are involved in the system (1, 28).

In concert with resistance to the bactericidal activity of normal serum, iron sequestering is considered crucial in the pathogenesis of systemic *V. anguillarum* infections (27); indeed, the diffusible siderophore itself appears to be a virulence factor (30).

Chromosome-mediated production of the siderophore and its receptor in *V. anguillarum* serovar O1 has also been reported (25), and recently it has been shown that *V. anguillarum* serovars O1 and O2 possess a chromosome-regulated iron uptake system that is genetically unrelated to previously described systems (16). Since plasmids have not been demonstrated in *V. anguillarum* serovar O2, it has been concluded that virulence is chromosome encoded in this serovar (26).

In a study of outer membrane proteins, it has been suggested that such components play an important role in the pathogenicity of *V. anguillarum* by mediation of adhesion, penetration, dissemination, association with leukocytes, and serum resistance (2). The Col V plasmid in invasive *Echerichia coli* also encodes an iron-chelating system, and it has been shown that this plasmid confers an increased hydrophobicity to recipient cells, thus allowing stronger adhesion to host cells (23).

The purpose of this work was to study the distribution of pJM1-like and other plasmids in Danish *V. anguillarum* serovar O1 and O2 isolates from different sources to obtain essential ecological and epidemiological data. Furthermore, we wanted information on the relationships between the presence of plasmids and selected phenotypic properties (hemagglutination, hydrophobicity, and biochemical activities).

MATERIALS AND METHODS

Bacterial strains. *V. anguillarum* reference strains with (775) and without (1,800) the pJM1 plasmid and *Vibrio ordalii* DF_3K were obtained from Michael H. Schiewe, Northwest and Alaska Fisheries Centre, Seattle, Wash.

A total of 228 Danish *V. anguillarum* serovar O1 and O2 isolates were tested. The sources of the strains are listed in Table 1. The strains from diseased rainbow trout comprised own isolates and isolates kindly supplied by Inger Dalsgaard, Department of Fish Diseases, Royal Veterinary and Agricultural University, Copenhagen.

Biochemical tests and serotyping. All strains were preliminarily examined as described previously (3) and then tested in the decarboxylase tests (arginine, lysine, and ornithine), for vibriostatic agent sensitivity and growth on TCBS agar (Difco) (11). The serovar was determined by using the slide

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TABLE 1. Sources of *V. anguillarum* serovar O1 and O2 isolates

Source	No. of isolates	
	O1	O2
Seawater	2	2
Sediment		2
Healthy rainbow trout[a]		
Mucus	25	2
Gills	18	5
Feces	5	9
Rainbow trout with vibriosis	74[b]	35
Cod with ulcus syndrome	1	48

[a] Isolates from one mariculture.
[b] Isolates from 10 different maricultures producing rainbow trout of 2 to 5 kg.

agglutination test of Sørensen and Larsen (22). The following important variable characteristics were tested as described previously (11): pellicle formation; formation of acid from arabinose, cellobiose, galactose, glycerol, D-inositol, and ribose; citrate (Simmons test); and Tween 80.

Hemagglutination and hydrophobic properties. Testing for hemagglutination with erythrocytes from humans (type O), poultry, guinea pigs, horses, pigs, and rainbow trout and measurement of hydrophobic properties were performed as previously described (15).

DNA isolation and agarose gel electrophoresis. Plasmid DNA was isolated as described by Kado and Liu (8). Samples of 15 μl of plasmid preparations mixed with 5 μl of sample buffer (30% glycerol, 1 mM EDTA, 0.1% bromophenol blue [Merck]) were separated in 0.7% agarose gels at approximately 3 V/cm for 2 h along with the plasmids (147, 63, 36, and 7 kb) of *E. coli* 39R861 (24). The gels were stained in 2-mg/ml ethidium bromide (Sigma), destained in water, and photographed under 254-nm light. Molecular weights were estimated from the migration in the gels as described by Rochelle et al. (18).

RESULTS

Occurrence of plasmids in *V. anguillarum* serovar O1 and O2 isolates. Plasmids were found in 73 of 74 *V. anguillarum* serovar O1 isolates from rainbow trout with vibriosis. An approximately 67-kb plasmid, which comigrated with plasmid pJM1 in reference strain 775, was present in 72 isolates; among these, 9 isolates also harbored an approximately 98-kb plasmid. Agarose gel electrophoresis profiles of representative plasmids are shown in Fig. 1. The results of the plasmid analysis are shown in Table 2 with the distribution of isolates among 10 marine fish farms.

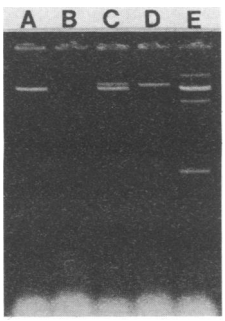

FIG. 1. Plasmid profiles of Danish *V. anguillarum* serovar O1 isolates. Lanes A: A, 6018/1 (ATCC 43305); B, 9007/7; C, 9013/3; D, 901418; E, markers from *E. coli* 39R861 (147, 63, 36, and 7 kb).

TABLE 2. Presence of plasmids in *V. anguillarum* serovar O1 isolates from rainbow trout in Danish and Swedish maricultures

Mariculture	No. of strains			
	Total	With plasmids		Without plasmids
		67 kb	98 kb	
Dan-Marin[a]	26	25		1
DAI-Ensted	18	18		
Asnæs	1	1		
Ebeltoft	2	2		
Hvide Sande	2	2	2	
Skærbæk	12	11	2[b]	
Tærø	12	12	6	
Sweden	1	1		

[a] Dan-Marin provided fish from maricultures at Alssund, Bågø, and Begtrup Vig.
[b] One strain harboured only the 98-kb plasmid.

V. anguillarum serovar O1 isolates from the gills, mucus, and intestines of healthy cod and rainbow trout and two isolates from water were also investigated (Table 3). Plasmids were demonstrated only in isolates from the mucus and gills; 29 (67, 4%) of these 43 isolates harbored the 67-kb plasmid, and 11 also harbored the 98-kb plasmid. One isolate from a cod was found to be without plasmids.

V. anguillarum serovar O2 isolates contained either no plasmids (49 of 103 strains) or plasmids with different molecular sizes as shown in Fig. 2 together with the profile of *V. ordalii* reference strain DF_3K. *V. ordalii* was previously named *V. anguillarum* biotype 2 serotype II, and the strain was included for comparison. An approximately 190-kb plasmid was present in 12 cod isolates and 2 rainbow trout isolates. An approximately 70-kb plasmid occurred in three cod isolates, and an approximately 21-kb plasmid was found in three rainbow trout isolates. Small plasmids (approximately 6 kb) were present in both trout and cod isolates, whereas environmental serovar O2 isolates did not contain any plasmids (Table 4). *V. ordalii* DF_3K harbored a 36-kb plasmid that was not seen in *V. anguillarum* serovar O1 and O2 isolates.

Biochemical reaction in variable characters of *V. anguillarum* serovars O1 and O2. Table 5 shows the biochemical behavior and selected characteristics of all isolates of *V. anguillarum*. Serovar O1 strains were generally arabinose positive (97%) and cellobiose positive (100%) and did not form pellicles in broth (86%), as opposed to serovar O2

TABLE 3. Presence of plasmids in *V. anguillarum* serovar O1 strains from apparently healthy rainbow trout and from water[a]

Isolate source	No. of strains			
	Total	With plasmids		Without plasmids
		67 kb	98 kb	
Mucus	25	16	4[b]	9
Gills	18	13	7[b]	5
Feces	5			5
Water	2			2

[a] All isolates were from the mariculture at Tærø.
[b] Isolates harbored a 67-kb plasmid as well as a 98-kb plasmid.

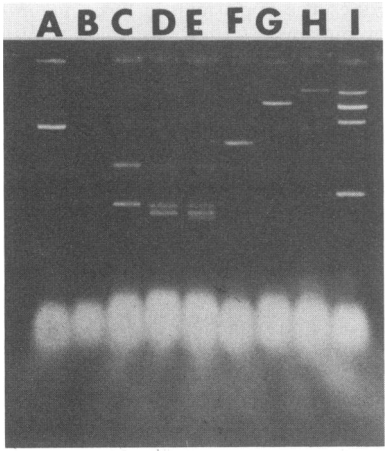

FIG. 2. Plasmid profiles of Danish *V. anguillarum* serovar O2 isolates and *V.* ordalii DF_3K (lane A). Other lanes: B through H, *V. anguillarum* 45.0 (B), 1094/1 (C), 1047/2 (D), 1049/2 (E), 820723-2/8 (F), 1098/3 (G), and 1223/1 (H); I, markers from *E. coli* 39R861 (147, 63, 36, and 7 kb).

strains, which were arabinose variable (35%), cellobiose variable (54%), and formed pellicles when growing in broth (94%). Also, serovar O2 strains were trehalose positive (100%), whereas only 18% of serovar O1 strains fermented this sugar.

Hemagglutination properties of *V. anguillarum* serovars O1 and O2. It appears from Table 6 that most (82.4%) of the 103 *V. anguillarum* serovar O1 strains were hemagglutination negative. Most hemagglutination-positive isolates belonged to type A (91%), whereas a minor proportion of strains (9%) were type D_2. *V. anguillarum* serovar O2 strains were more heterogeneous, but 49 (47, 6%) belonged to type A, whereas 32 (31.1%) were hemagglutination negative.

Hydrophobic properties of *V. anguillarum* serovars O1 and O2. It was possible to separate the isolates into different HIC and SAT groups (15), and *V. anguillarum* serovar O1 strains generally were more hydrophobic than were serovar O2 strains (Table 7), but these tests were not able to give any definite separation within the serovars. No correlation between the presence of plasmids and hydrophobicity was demonstrated.

TABLE 4. Presence of plasmids in *V. anguillarum* serovar O2 strains from trout in maricultures and from cod

Isolate source	No. of strains					
	Total	With plasmid				Without plasmids
		190 kb	68 to 70 kb	21 kb	5 to 10 kb	
Mariculture						
Dan-Marin	1				1	
DAI-Ensted	2				1	1
Asnæs	11				6	5
Skærbæk	9				6	3
Samsø	2				2	
Tærø	6	2			2	2
Sweden	4			3		1
Cod	48	12	3	0	16	17
Environment[a]	20	0	0	0	0	20

[a] Strains were from mucus (two), gills (five), and feces (nine) from trout at Tærø and from water (two) and sediment (two isolates).

TABLE 5. Biochemical reactions of *V. anguillarum* serovar O1 and O2 isolates

Test	O1 isolates		O2 isolates	
	Reaction	% Positive	Reaction	% Positive
Formation of acid from:				
Arabinose	+	97	V[a]	35
Cellobiose	+	100	V	54
Galactose	+	100	+	97
Glycerol	+	100	+	96
Inositol	−	1	−	2
Ribose	+	99	+	100
Sorbitol	+	98	+	84
Trehalose	V	18	+	100
Citrate (Simmons test)	+	100	+	99
Tween 80	+	100	+	99
Pellicle formation	−	14	+	94

[a] V, Variable reaction.

Relationship between plasmids and phenotypic characteristics of *V. anguillarum* serovars O1 and O2. An obvious difference was observed between strains of *V. anguillarum* serovar O1 with plasmids and those without plasmids (Table 8). All plasmid-containing isolates were hemagglutination negative, did not produce pellicles and were trehalose negative; furthermore, they were all arabinose positive. Most of the plasmid-free isolates were hemagglutination positive, produced pellicles, and were trehalose positive, and they were most frequently arabinose positive.

The serovar O2 isolates (13 different biovars) showed a pronounced biochemical diversity (Table 9). However, 83.5% of the isolates belonged to biovars 1 through 4.

DISCUSSION

Subdivision of *V. anguillarum* by serotyping and biotyping has led to some understanding of the epidemiology of vibriosis provoked by this organism in both wild fish and fish in maricultures.

It has been suggested that environmental strains, favored by carbohydrate-polluted water, could induce disease under stress conditions (7, 10, 12, 14, 20, 21). However, strains

TABLE 6. Hemagglutination properties of *V. anguillarum* serovar O1 and O2 strains

Agglutination type[a]	Reaction of isolates from:						No. of isolates of indicated type:	
	Human	Poultry	Guinea pig	Trout	Horse	Pig	O1	O2
A1	+	+	+	+	+	+	19	21
A2	+	+	+	+	+	−	1	28
B1	+	+	+	−	+	+	0	1
B2	+	+	+	−	+	−	0	2
B4	+	+	+	−	−	−	0	3
C1	−	+	+	+	+	+	0	1
C2	−	+	+	+	+	−	0	9
D2	−	+	+	−	+	−	2	5
D4	−	+	+	−	−	−	0	1
G4	−	−	−	−	−	−	103	32

[a] Typing system of Larsen et al. (15).

TABLE 7. Groupings of *V. anguillarum* serovar O1 and O2 isolates with HIC and SAT tests[a]

Test group	No. of isolates in indicated group	
	O1	O2
HIC		
1 (<50%)	0	98
2 (50 to 75%)	11	4
3 (>75%)	114	1
SAT		
1 (<0.1 M)	0	0
2 (0.1 to 0.9 M)	123	1
3 (0.1 to 1.5 M)	2	2
4 (>1.5 M)	0	100

[a] Tests were done as described by Larsen et al. (15).

isolated from diseased fish belonged to serovars O1 and O2, whereas most environmental strains were nontypable (22), and strains of *V. anguillarum* isolated from diseased fish and from the environment are distinct in biochemical characteristics (9, 11, 13). This suggests the existence of two populations of *V. anguillarum* with different virulence properties.

The present study showed that all but two strains of *V. anguillarum* serovar O1 from diseased rainbow trout carried an approximately 67-kb plasmid corresponding in size to the virulence plasmid pJM1 (4) and that some strains (13%) carried an additional 98-kb cryptic plasmid (Table 2). In contrast to this, 42% of the strains isolated from healthy fish and from the environment were without plasmids (Table 3). Based on in vitro pathogenicity tests, Larsen et al. (15) have suggested that the skin, mucus, and gills of healthy fish are in particular colonized by potentially pathogenic *V. anguillarum*. In support of this, pJM1-like plasmids were demonstrated in our study in most isolates (67%) from the skin, mucus, and gills of healthy fish, whereas the few fecal isolates that we examined were without plasmids. Further studies are needed to elucidate whether pJM1-like plasmids confer affinity for specific receptors on the fish or whether other mechanisms are involved in the affinity.

Strains of *V. anguillarum* serovar O1 that carried the pJM1-like plasmids were hemagglutination negative and did not form pellicles in broth cultures (Table 8). Based on in vivo studies, two populations of *V. anguillarum* serovar O1 isolates with different virulence properties have been suggested; the more virulent population is hemagglutination negative and does not form pellicles in broth cultures (15). From the present results it may be suggested that the difference between the populations noted by Larsen et al. (15) was correlated with the presence or absence of pJM1-like plasmids in the isolates.

Only one strain isolated from a diseased fish did not carry any plasmids (Table 2); this strain was hemagglutination positive. In the light of the high correlation demonstrated between the presence of the virulence plasmid, negative hemagglutination properties, and diseased fish, it might be assumed that the plasmid(s) was lost from this strain after infection. However, chromosomally encoded virulence genes substituting for pJM1 genes have been reported (16), and this may explain why a plasmid-free strain was isolated from a diseased fish.

Virulence has been suggested to be chromosomally encoded in *V. anguillarum* serovar O2 strains (16, 26). This study confirms that no single plasmid can be demonstrated in *V. anguillarum* serovar O2 isolated from diseased fish, although 65% of the isolates carried plasmids. However, isolates from the environment were without extrachromosomal DNA molecules (Table 4). We have not disclosed any phenotypic properties encoded by the plasmids. The phenotypic heterogeneity of *V. anguillarum* serovar O2 isolates was pronounced, with no clear indication of correlation between certain plasmids and any specific biochemical characteristics (Table 9). One can only speculate whether the presence of plasmids adds to the virulence of the *V. anguillarum* serovar O2 strains or whether the selection for the presence of plasmids in isolates from diseased fish, as demonstrated in this study, is brought about by other mechanisms.

Approximately 75% of *V. anguillarum* serovar O2 isolates from diseased fish belonged to biovars 1 and 2 (Table 9). Environmental authorities and fish farmers are often concerned about the spread of pathogenic bacteria in the environment, and subtyping systems for use in epidemiological studies concerning *V. anguillarum* serovar O2 are needed. The present results suggest that biotyping and plasmid profiling should be used together as epidemiological markers in such investigations.

Strains of *V. anguillarum* serovars O1 and O2 have previously been stated to differ in the production of acid from arabinose (26), but it has also been suggested that this characteristic together with the production of acid from cellobiose can be used to distinguish between *V. anguillarum* serovar O1 isolates with and without pJM1 (29). From the present study it appeared that strains of serovars O1 and O2 indeed differed in the production of acid from arabinose and cellobiose and also in hemagglutination properties, pellicle formation in broth cultures, and production of acid from trehalose; however, a 100% correlation between serovars and biochemical reactions was not demonstrated (Table

TABLE 8. Correlation between biovar and characteristics of *V. anguillarum* serovar O1 isolates

Biovar	Hemagglutination	Pellicle	Formation of acid from:					No. of strains	
			Arabinose	Inositol	Ribose	Trehalose	Sorbitol	Total	With plasmids
1	−	−	+	−	+	−	+	101	100
2	−	−	+	−	−	−	+	1	1
3	+	+	+	−	+	+	+	13	0
4	+	−	+	−	+	+	+	5	0
5	+	+	−	−	+	+	−	2	0
6	+	+	−	−	+	+	+	1	0
7	+	+	−	+	+	+	+	1	0
8	−	−	+	−	+	+	+	1	0

TABLE 9. Relationship between biovar and characteristics of *V. anguillarum* serovar O2 isolates

Biovar	Hemagglutination	Pellicle	Formation of acid from:							Tween 80 test	No. of isolates									
											With plasmids						Without plasmids		From diseased fish	From environment[a]
											190 kb		68 to 70 kb (cod)	21 kb (trout)	5 to 10 kb					
			Arabinose	Cellobiose	Citrate	Galactose	Glycerol	Inositol	Sorbitol		Cod	Trout			Cod	Trout	Cod	Trout		
1	V[b]	+	−	−	+	+	+	−	+	+	11	0			5	3	11	4	34	6
2	+	+	+	+	+	+	+	−	+	+	0	1			9	13	2	3	28	1
3	+	+	−	+	+	+	+	−	−	+								2	2	9
4	+	+	−	+	+	+	+	−	+	+				1	1			2	4	2
5	+	−	+	+	+	+	+	−	+	+				2	1				3	
6	−	+	−	−	+	−	−	−	+	+	1						2		3	
7	V	−	−	−	+	+	+	−	+	+			3						3	
8	V	+	−	+	+	+	+	+	−	+		1					1		2	
9	+	+	+	+	+	+	+	−	−	+										9
10	+	+	−	−	+	+	+	−	+	+							1		1	
11	+	+	−	+	−	+	+	−	+	−								1	1	
12	+	+	+	+	+	+	−	−	+	+						1			1	
13	+	+	+	+	+	+	+	−	+	+						1			1	

[a] Environment isolates had no plasmids.
[b] V, Variable reaction.

5). From the testing of hydrophobic properties it also appeared that *V. anguillarum* serovar O1 strains were the more hydrophobic, although a few hydrophobic serovar O2 strains were demonstrated (Table 7). The hydrophobicity of a bacterium is normally correlated with the ability to adhere to mucosal membranes (19). This may mean that within *V. anguillarum* serovar O1 there is a selection toward very hydrophobic strains; if so, serovar O1 strains should be considered to be normally associated with fish, not with fish and the environment.

Arabinose-positive *V. anguillarum* serovar O1 strains were demonstrated, irrespective of whether the strain carried pJM1-like plasmids (Table 8), whereas negative hemagglutination properties and the absence of acid formation from trehalose (with the exception of one isolate) were seen only in serovar O1 strains carrying pJM1-like plasmids. The strain that did not carry a pJM1-like plasmid but that did carry the 98-kb cryptic plasmid appeared to be like the other plasmid-carrying strains in biochemical properties.

Trehalose-positive, plasmid-free *V. anguillarum* serovar O1 isolates have been reported (29), and thus pJM1 is not likely to be the reason for all plasmid-carrying strains in this study being trehalose negative. We suggest that certain clones of *V. anguillarum*, in this case a trehalose-negative clone, have the ability to accept and maintain plasmids in a stable manner. Due to the selective advantage brought about by the plasmids (e.g., pJM1), and perhaps due to as yet unknown chromosomally encoded virulence genes, such clones become those normally involved in disease.

Others, like us, have noted the extreme homology among epizootic *V. anguillarum* serovar O1 strains (26). However, subdivision of such strains can be performed on the basis of restriction length polymorphy of the pJM1 plasmid itself (17), suggesting that different clones of bacteria that have evolved separately for a period of time do exist. However, further studies on the clonal connections of *V. anguillarum* serovar O1 and O2 strains from diseased fish are needed to bring about an understanding of the interactions between fish in the environment and in maricultures and an understanding of the connections between bacterial isolates from diseased fish and isolates from the environment.

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