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Plasmid profiles, restriction fragment length polymorphisms and O-serotypes among *Vibrio anguillarum* isolates

K. PEDERSEN, T. TIAINEN AND J. L. LARSEN

Laboratory of Fish Diseases, Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Bülowsvej 13, DK-1870 Frederiksberg C, Denmark

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SUMMARY

A total of 279 *Vibrio anguillarum* strains were serotyped and examined for plasmid content. Plasmids were subjected to digestion with restriction enzymes. Most strains belonged to serogroup O1 (39%) and O2 (16%). In total 164 strains (53%) carried plasmids. Of the O1 and O2 isolates, 92% and 30%, respectively, carried one or more plasmids. Restriction fragment length polymorphism (RFLP) analysis of plasmid DNA indicated that plasmids belonged to several groups. Each group seemed to be restricted to a single O-serovar. The largest group was the pJM1-like plasmids among most serovar O1 strains. Most of these plasmids were about 67 kb like the pJM1 plasmid, but various derivatives ranged from 26–77 kb. RFLP studies of the 67 kb plasmids revealed 17 different restriction patterns. Some patterns were dominant among European strains whereas others were dominant among North American strains. The results confirmed the applicability of O-serotyping together with plasmid profile and restriction analysis of plasmids for typing of *V. anguillarum*. They also indicated that plasmids among strains which belonged to the traditional fish pathogenic serogroups, O1 and O2, showed more homology than did strains from most other serogroups, that were usually non-pathogenic, environmental bacteria.

INTRODUCTION

Vibrio anguillarum is one of the most important and wide-spread bacterial fish pathogens. As such, much effort has been put into its isolation, identification, characterization, and typing [1]. Although plasmids have been detected among several *Vibrio* species, including *Vibrio vulnificus* [2], *Vibrio ordalii* [3], *Vibrio cholerae* [4], *Vibrio salmonicida* [5], *Vibrio fischeri* [6], and various other vibrios [7, 8], *Vibrio anguillarum* is maybe the *Vibrio* species most extensively studied with respect to plasmids. This has been done for several reasons: from Japan, several authors have shown the involvement of large plasmids, 80–120 MDa, in resistance to one or more antimicrobial drugs [9–12]. In serogroup O1, an approximately 67 kb (65–70 kb) plasmid has been demonstrated to be common among strains isolated from the kidney of dead fish [13–15]. This plasmid has been shown to encode an iron sequestering system [16, 17] which is an important virulence factor for this bacterium [18]. From Denmark, plasmids among serogroup O1 and O2 isolates have been reported [13, 14, 19], and the study of restriction fragment length polymorphism (RFLP) of the 67 kb virulence plasmid was concluded to be of potential epidemiological value [20]. Other authors have also demonstrated heterogeneity within this plasmid [11, 15, 21], and Mitoma and colleagues [11] used RFLP analysis to study the phylogenetic relationship between the 67 kb plasmid and some larger R-plasmids. Additionally, in a recent study of *V. anguillarum*

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serogroup O1, using plasmid analysis and ribotyping, pathogenic isolates and environmental isolates constituted 2 distinct populations [22]. Thus, the study of plasmids in *V. anguillarum* has relevance to pathogenicity and virulence, antibiotic resistance, and epidemiological as well as ecological investigations. We have demonstrated the existence of several other plasmids but their relationship to the 67 kb plasmid and to each other has not been studied. In the present investigation, we describe the plasmids among *V. anguillarum* isolates belonging to various O-serogroups and study the relationship between plasmids using restriction enzyme fragmentation patterns (REFP).

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 279 *V. anguillarum* isolates were used in the investigation. All strains were stored in broth cultures supplemented with 25–50% sterile glycerol at −80 °C until used and propagated on blood agar plates (Marine agar (Difco) supplemented with 5% sterile, citrate-stabilized calf blood), incubated at 20 °C for 2 days. Strains were isolated in our laboratory or received from various other laboratories.

O-serotyping

O-serotyping was carried out using the slide agglutination method as previously described [23, 24]. The strains were tested for reactivity with the 10 antisera, O1–O10, described in the European serotyping system [24], and with seven additional antisera, temporarily designated VaNT1–7 [25; Pedersen and colleagues, unpublished results]. Strains reacting with 2 or more antisera were designated as cross-reacting, whereas strains that agglutinated spontaneously in acetate buffer were called self-agglutinating. Finally, strains that did not react with any of the antisera, and were not cross-reacting or self-agglutinating, were called non-typeable.

Plasmid profiles

Cultures for isolation of plasmids were propagated in Luria–Bertani broth (L-broth, Gibco), agitated vigorously overnight at 20 °C. Plasmid DNA for profiling was extracted by the method of Kado and Liu [26] and subjected to electrophoresis in 0·8% agarose gels (SeaKem GTG, FMC BioProducts, Rockland, ME, USA) in TAE (Tris 40 mM, sodium acetate 5 mM, ethylenediaminetetraacetate (EDTA) 1 mM; pH 8·0). Gels were stained with ethidium bromide and photographed in UV light. Plasmids from *Escherichia coli* 39R861 and V517 were used as molecular weight size markers [27, 28]. Distinction between supercoiled, covalently closed, circular (CCC) plasmids, 'nicked', open circular (OC) plasmids, and linear (L) forms was done using the method of Hintermann and colleagues [29].

Restriction enzyme digestion of plasmids

Plasmid DNA for REFP studies was extracted by the method of Olsen [30] or by the method of Kado and Liu [26]. Plasmid DNA recovered by the Kado and Liu method [26] was purified by precipitation with ethanol, whereafter the pellets were washed with 70% ethanol and resuspended in TE buffer (Tris 10 mM, EDTA 1 mM), pH 8·0. Additionally, some plasmids were isolated using a commercial kit, Wizard Minipreps, following the instruction of the manufacturer (Promega Corp., Madison, WI, USA). Plasmids were digested with *Bam*H I and *Hind* III (Promega) and subjected to electrophoresis in 1·0% agarose gels in TAE buffer.

RESULTS

The majority of the strains belonged to the two serogroups O1 (119/279, 43%) and O2 (49/279, 18%). Remaining serogroups contained minor numbers of strains and some strains were self-agglutinating or cross-reacting with 2 or more antisera. Thirty-one strains (11%) did not react with any of the antisera (Table 1).

The highest frequency of plasmids was recovered in the VaNT7 serogroup. All (6/6) strains carried plasmids. Four of the strains had a 16 kb plasmid alone, while one strain had the 16 kb plasmid together with a 49 and a 68 kb plasmid, and the sixth strains had the 16, 49 and 68 kb plasmids, and additionally an 80 kb plasmid. However, these strains were all isolated in Greece, and may not be epidemiologically unrelated. The second highest frequency of plasmids was found in serogroup O1 (Table 1), where 110 out of 119 (92%) strains carried plasmids. Most of the O1

Table 1. *Distribution of plasmid profiles of* 279 Vibrio anguillarum *strains belonging to various O-serogroups*

Serogroup	Number of strains	Plasmid profiles, kb (number of strains with this profile)
O1	119	Plasmid-free (9)
		26
		38
		67 (85)
		77
		5·3 + 51
		53 + 56
		35 + 60
		5·3 + 67 (2)
		10 + 67
		36 + 67 (2)
		48 + 67
		50 + 67 (4)
		53 + 67 (2)
		67 + 78 (2)
		67 + 84 (2)
		67 + 108
		74 + 84
		29 + 31 + 60
O2	49	Plasmid-free (34)
		20
		64 ~ 200 (4)
		17 + 23 (8)
		5·0 + 5·5 + 11·5
O3	13	Plasmid-free (13)
O4	11	Plasmid-free (9)
		3·9
		13 + 155
O5	7	Plasmid-free (6)
		2·6
O6	7	Plasmid-free (4)
		44
		200
		3·2 + 5·3 + 50 + 70
O7	6	Plasmid-free (6)
O8	4	Plasmid-free (4)
O9	2	Plasmid-free (2)
O10	1	Plasmid-free
VaNT2	5	Plasmid-free (3)
		11·5
		68 + 83
VaNT4	2	Plasmid-free (2)
VaNT5	1	Plasmid-free
VaNT7	6	16 (4)
		16 + 49 + 68
		16 + 49 + 68 + 80
Cross-reacting	12	Plasmid-free (8)
		2·3
		3·3 + 12·3
		15 + 20 + 150
		46 + 68 + 83
Self-agglutinating	3	Plasmid-free (2)
		6·0 + 14
Non-typeable	31	Plasmid-free (28)
		13
		50
		16 + 155
Total	279	

strains carried one plasmid (88/119 or 74%) whereas 21 (18%) harboured two plasmids. Only nine O1 strains were plasmid-free and a single isolate harboured three plasmids. Among the O2 strains, 15/49 (31%) carried plasmids while 34/49 (69%) were plasmid-free. The majority of the strains from the remaining serogroups were plasmid-free. In some serogroups, O3, O7, O8, O9, O10, VaNT4, and VaNT5, no plasmids were detected. The highest number of plasmids that was found was 4, detected in one serogroup O6 and in one serogroup VaNT7 strain.

Plasmids smaller than approximately 20 kb usually had no restriction sites for *Hind* III or *Bam*H I. Often, *Hind* III yielded many, closely positioned bands from large plasmids while *Bam*H I was unable to digest the small plasmids. Thus, for comparison of REFPs, best results were obtained when digesting smaller plasmids with *Hind* III and the larger plasmids with *Bam*H I.

Based on the REFP results, plasmids belonged to several groups. Plasmids within a group showed very similar restriction patterns whereas comparison of the patterns between groups usually showed marked difference.

Plasmids smaller than approximately 20 kb were very variable in restriction patterns as well as in size, the smallest being 2·2 kb. Three serogroup O1 strains carried the same 5·3 kb plasmid and six strains, isolated in Greece, harboured the same 16 kb plasmid. These 6 strains did not belong to any of the serogroups O1–O10 [24] but to a new O-serogroup, presently designated VaNT7 [4]. Likewise, eight Japanese O2 strains all carried 2 plasmids, 17 and 23 kb, shared the same restriction pattern. These 2 plasmids were not detected in strains outside Japan, and were found only in O2 strains. Apart from these, none of the small plasmids were identical in size or restriction pattern.

In contrast, most of the larger plasmids formed distinct groups. The largest of these groups was composed of the 67 kb pJM1-like plasmid, contained

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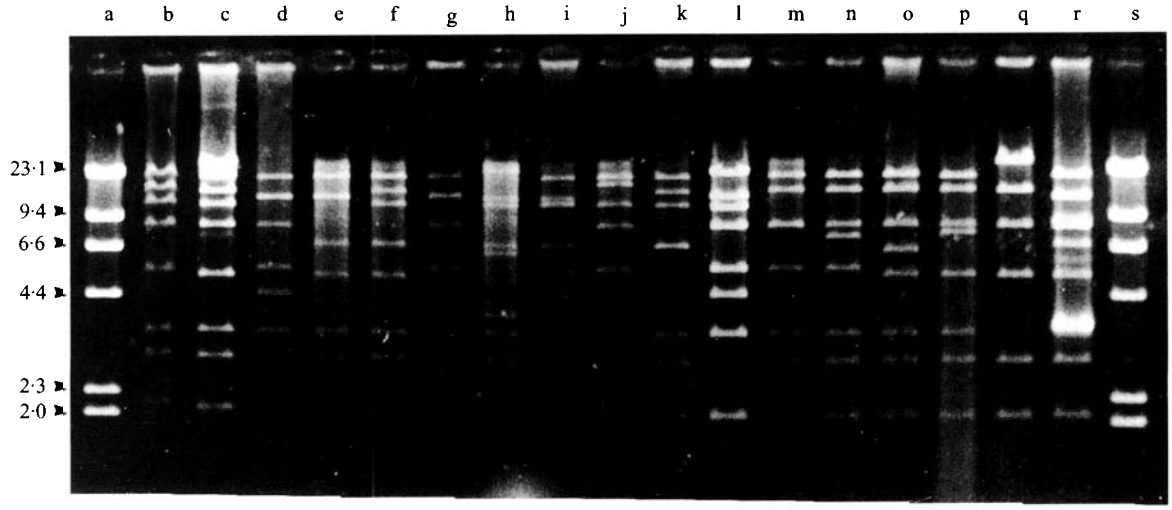

Fig. 1. *Bam*H I restriction patterns of 99 pJM1-like plasmids isolated from *V. anguillarum* serogroup O1. A total of 17 patterns was recognized, arbitrarily designated pattern number 1–17. Lane a and s, *Hin*d III digested λ phage DNA as molecular weight reference molecules (molecular weights are indicated to the left). Lane b–r, REFP 1–17.

by most serogroup O1 strains. Using *Bam*H I, a total of 17 different restriction patterns were demonstrated (Fig. 1). The geographical pattern of these patterns was not uniform: certain patterns were dominant in certain areas (Table 2). This result was statistically significant in a χ^2 test ($P < 0{\cdot}0001$). Pattern 1 and 2 were dominant among European strains whereas pattern 4, 5, 7, 8 and 17 were found among North American strains. All Tasmanian isolates had pattern 5, the most dominant North American pattern, whereas the Japanese strains were more diverse: the five 67 kb plasmids detected among the Japanese strains showed 4 different *Bam*H I restriction patterns. Two of these, pattern 10 and 16, were unique.

Instead of the 67 kb virulence plasmid, some serogroup O1 strains contained a plasmid that, as judged from their restriction pattern, was apparently derived from this plasmid by deletion or insertion. Such plasmids with molecular weights of 26, 38, 56, 60, 74, 77 and 78 kb were identified. *Hin*d III and *Bam*H I patterns of some of these plasmids are shown in Figure 2. All 110 serogroup O1 strains that carried plasmids harboured the 67 kb virulence plasmid or a plasmid derived from it.

Some O1 strains carried a group of plasmids having a restriction pattern very different from that of the 67 kb virulence plasmid. This group varied in size from approximately 56 kb to approximately 84 kb. A plasmid from this group was always present together with a pJM1-like plasmid, never alone. A restriction pattern of one such plasmid from strain 393/91 isolated from sea bass (*Dicentrarchus labrax*) in Italy is shown in Fig. 3.

Four Danish O2 strains carried large plasmids, > 200 kb. These plasmids displayed two different but closely related restriction patterns.

Remaining plasmids seemed to display unique REFPs, not related to other plasmids.

All groups of plasmids that displayed similar REFPs, were only recognized within one O-serogroup. Only one serogroup O2 strain carried a plasmid with a molecular weight and a restriction pattern, comparable to the 67 kb virulence plasmid of the O1 strains, and had several *Hin*d III restriction fragments with the same size as fragments of the 67 kb plasmid (Fig. 2).

DISCUSSION

The high prevalence of plasmids among *V. anguillarum* serogroup O1 strains reported here is in accordance with previous publications [13, 15, 31, 32]. The prevalence of plasmids among serogroup O2 isolates was found to be 15/49 (31%). This figure is somewhat lower than previously reported by Larsen and Olsen [13] who found that 54 out of 103 (52%) O2 strains carried plasmids. Tiainen and colleagues [33] detected plasmids in 48 out of 129 (37%) O2 strains, a figure comparable to that reported in the present study. However, Tiainen and colleagues [33] found a remarkable difference between northern European and southern European strains. Plasmids

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Table 2. Bam*H I restriction patterns of the 67 kb virulence plasmid of* Vibrio anguillarum *serogroup O1. Number of strains from various continents*

Geographic origin	Number of strains	Number of strains with *Bam*H I restriction pattern number															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Europe	64	33	15	1	—	—	3	—	—	5	—	2	1	1	2	1	—	—
North America	15	—	—	—	2	5	—	3	3	—	—	—	—	—	—	—	—	2
South America	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Japan	5	—	2	—	—	1	—	—	—	—	1	—	—	—	—	—	1	—
Tasmania	14	—	—	—	—	14	—	—	—	—	—	—	—	—	—	—	—	—
Total	99	34	17	1	2	20	3	3	3	5	1	2	1	1	2	1	1	2

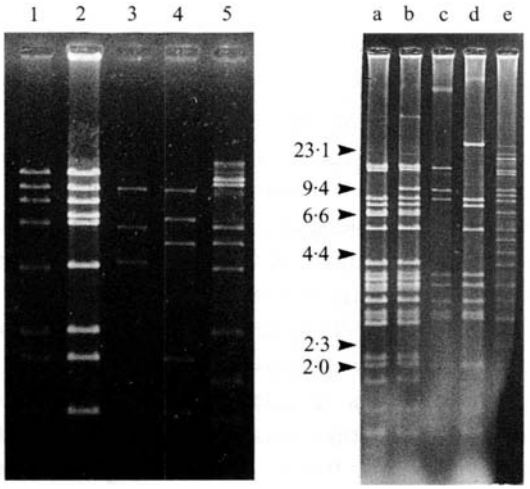

Fig. 2. (*a*) *Bam*H I and (*b*) *Hin*d III restriction patterns of pJM1 and plasmids derived from it and the *Bam*H I and *Hin*d III restriction pattern of a 64 kb plasmid isolated from a serogroup O2 strain. Its DNA homology with the pJM1-like plasmid is at present unknown. Lane 1 and a, pJM1 from *V. anguillarum* strain 775 (67 kb); lane 2 and b, plasmid from strain VIB 79 (77 kb); lane 3 and c, plasmid from strain VIB 64 (26 kb); land 4 and d, plasmid from strain VIB 704 (38 kb); lane 5 and e, plasmid from strain VIB 96, serogroup O2 strain carrying a 64 kb plasmid.

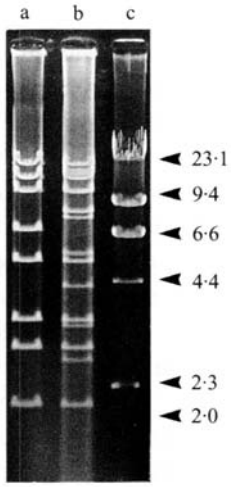

Fig. 3. *Bam*H I restriction pattern of an approximately 84 kb plasmid, harboured by a *V. anguillarum* serogroup O1 strain, 393/91, isolated from a sea bass in Italy. Plasmids with similar restriction patterns were recognized in five serogroup O1 strains, isolated from Denmark, Norway, and Italy. Lane a, *Bam*H I digested pJM1 plasmid, pattern 1; lane b, *Bam*H I digested 84 kb plasmid + pJM1, harboured by the same strain; lane c, *Hin*d III digested λ phage DNA as molecular weight reference molecules.

were almost exclusively found in the northern European isolates, and since in the study by Larsen and Olsen [13] only Danish isolates were included, this may very well account for the reported difference in

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plasmid content. The present study included O2 strains from all over the world. Toranzo and colleagues [34] found plasmids in 5 of 6 *V. anguillarum* strains but, since this paper was published before the serotyping system for *V. anguillarum* was developed [24], no information on serotypes was given.

Mitoma and colleagues [11] described phylogenetic relationships among some large *V. anguillarum* plasmids by using restriction endonuclease analysis and Southern blotting. They reported the existence of at least 4 homology groups but the results of the Southern blottings revealed that certain fragments hybridized with fragments of plasmids from other homology groups. In the present study we demonstrated several groups of plasmids. However, plasmids belonging to the same group were only with certainty recovered from strains belonging to the same O-serogroup. The reason for this is at present not known, and it is unknown if this is general for *V. anguillarum*. On one occasion, a plasmid detected in one serogroup O2 strain had a molecular weight comparable to the serogroup O1 virulence plasmid, 65–67 kb, and by restriction analysis this plasmid was shown to share several fragments with the serogroup O1 virulence plasmid (Fig. 2). However, since we did not carry out any hybridization experiments, we cannot, in contrast to the study of Mitoma and colleagues [11], speculate on any possible DNA homology between plasmids within or between groups. Unfortunately, the work of Mitoma and colleagues [11] was carried out before the O-serotyping system for *V. anguillarum* was developed, so apart from strain 775 which belonged to serogroup O1, we know nothing about the serotypes of the strains studied. The possible transfer of plasmid DNA from *V. anguillarum* to other bacterial species and *vice versa* or the transfer of plasmids from strains belonging to one serogroup to another has at present not been investigated, although it has been shown that the pJM1 plasmid is non-conjugative [11]. However, it was recently described that strains of *V. anguillarum* serogroup O1 containing the 67 kb virulence plasmid were clearly distinct from strains without the virulence plasmid based on ribotypes [22], suggesting that exchange of plasmid DNA between O1 strains is uncommon.

Digestion of this 67 kb plasmid with *Bam*H I yielded a suitable number of bands, well separated and easy to compare, while digestion with *Hin*d III resulted in too many and too closely positioned bands to compare restriction patterns properly from one gel to another. All pJM1-like plasmids were therefore digested with *Bam*H I. With this enzyme, we found 17 different restriction patterns of the 67 kb plasmid, some patterns being dominant in certain areas of the world. This indicated that REFP analysis may be used in epidemiological investigations. The results give evidence to suggest that the virulence plasmid is widespread among *V. anguillarum* O1 strains over the world and has been so for a long time. It is noteworthy that all virulence plasmids from Tasmanian strains had identical restriction patterns, indicating a clonal spread in Tasmania. Additionally, this pattern was identical to the most prevalent pattern in North American strains (Table 2). However, a clonal connection between Tasmanian strains and North American strains needs further verification using other methods, e.g. pulsed-field gel electrophoresis [35]. In a recent study of *V. ordalii* [36], it was demonstrated that all Australian isolates had the same ribotype and the same restriction pattern of their 32 kb plasmid, and that this ribotype was identical to the most prevalent ribotype of North American *V. ordalii* strains. This observation also indicates a clonal connection between Australian and North American fish pathogenic bacteria. Minor differences in restriction patterns within the 67 kb plasmid were previously reported by Wiik and colleagues [15] who had studied two plasmids. Also Crosa and colleagues [18] reported minor differences and Olsen and Larsen [20] detected 6 RFLP types among 79 Danish *V. anguillarum* serogroup O1 virulence plasmids.

Plasmids similar to the approximately 85 kb plasmid (Fig. 3) has previously been detected in Italian as well as Danish strains of *V. anguillarum* serogroup O1, although in previous publications the molecular weights have been estimated to 90 or 98 kb [13, 22]. This plasmid has never been detected alone, only together with a pJM1-like or pJM1-derived plasmid. The restriction pattern of this plasmid together with pJM1 is shown in Fig. 3. The pattern of the 84 k plasmid is thus obtained by subtracting pattern 1 of the pJM1-like plasmid from the restriction pattern of both plasmids together. At present, it is not known whether this plasmid can exist alone, nor do we know anything about the properties it is coding for. In a recent publication [37], a restriction pattern almost identical to that shown in Fig. 3 was presented. However, the authors did not show a plasmid profile for this strain, so we suggest that it may in fact have carried 2 plasmids, almost identical to the strain in this study.

Tsai and colleagues [21] described the plasmids of three serovar C strains (= serovar O1 in the European serotyping system) and their restriction profiles. They claimed to have detected 2 plasmids, 18·7 and 44 kb, respectively, in each of them. However, from their photographs we conclude that the 18·7 kb band was most likely the chromosomal band while the 44 kb plasmid was really the 67 kb (approximately 45 MDa) virulence plasmid. These authors found two different *Hin*dIII restriction patterns of these plasmids, both identical to patterns recognized among some of the 67 kb virulence plasmid in our investigation.

At present, very little is known about the function of plasmids among *V. anguillarum*. The 67 kb virulence plasmid is known to encode an iron sequestering system [17] and some large plasmids have been shown to carry antibiotic resistance factors [9, 10, 12]. Further studies are needed to describe the function of these plasmids as well as the possible homology between them by using southern hybridization and similar techniques.

Polymorphism of restriction patterns was not only detected among the pJM1-like plasmid but also among other plasmid groups. This suggests that REFP studies may be a general method, applicable for epidemiological studies of *V. anguillarum*.

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