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Restriction Fragment Length Polymorphism of the *Vibrio anguillarum* Serovar O1 Virulence Plasmid

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Seventy-eight strains of *Vibrio anguillarum* serovar O1, all harboring one 65- to 70-kilobase plasmid, were typed according to restriction fragment length polymorphism of the plasmid. Six types, three of which comprised 96% of the strains examined, were produced with the restriction endonuclease *Bam*HI. The fragment length polymorphism type did not correlate to any of 12 different phenotypic properties tested.

Vibrio anguillarum serovar O1 is associated with vibriosis in mariculture, and it is considered a major pathogen from an economic point of view; e.g., vibriosis has been estimated to cause annual losses exceeding £ 11,000,000 ($18,000,000) per year in Japan (1).

To understand and control the disease, it is relevant to develop typing systems that make detailed epidemiological studies possible. Plasmid profiling is of limited value; e.g., Danish isolates have been shown to have only two different plasmid profiles, and 62% of the strains carry only one 65- to 70-kilobase plasmid (unpublished data). Discrimination based on biotyping has been used for *V. anguillarum* (16), but most Danish isolates of serovar O1 fall within the same biogroup (unpublished data), so this method is also of limited value.

Restriction fragment length polymorphism (RFLP) of plasmids has been used in epidemiological studies to subtype other bacteria, e.g., otherwise untypable isolates of *Salmonella typhimurium* (2, 10, 11). In this report we show that the *V. anguillarum* serovar O1 plasmid pJM1 shows polymorphy when digested with the restriction endonuclease *Bam*HI, and we suggest that this can be used to subtype isolates that are biochemically and serologically similar. We also demonstrate that different RFLP types have come to dominate different maricultures but that some types are present in several different Danish maricultures.

MATERIALS AND METHODS

Bacterial strains and phenotypic characterization. *V. anguillarum* strains were isolated from diseased fish as previously described (7). Isolates were serotyped (13) and tested for 12 different biochemical characteristics (acid from arabinose, cellobiose, galactose, glycerol, inositol, ribose, trehalose, sorbitol; growth on citrate [Simmons and Christensen]; the formation of a pellicle in broth cultures; decomposition of Tween 80) as described by Larsen (6). Hemagglutination properties were tested as described by Larsen et al. (7).

Plasmid isolation. Plasmids were isolated for profiling as described by Kado and Liu (5). Plasmid DNA for restriction fragment length analysis were isolated and digested with the restriction enzymes *Bam*HI and *Hin*dIII (Boehringer Biochemicals Mannheim) as described by Olsen (9). Strains were grown overnight with vigorous shaking at room temperature in LB medium (8) containing 1% NaCl.

Agarose gel electrophoresis. Native plasmids were electrophoresed in 0.7% agarose gels (Litex LSL) at a current of 3 V/cm, and restriction fragments were electrophoresed in 0.9% agarose gels at a current of 2 V/cm; both preparations were run in 1× TAE buffer (8). Gels were stained in 2 mg of ethidium bromide (Sigma Chemical Co.) per liter and photographed under 254-nm light. Approximate molecular sizes were estimated from the migration during gel electrophoresis as recommended by Rochelle et al. (12); plasmids in *Escherichia coli* 39R861 (14) served as reference molecules for native plasmids, and restriction fragments of phage lambda cut with *Hin*dIII served as reference molecules for restriction fragments.

RESULTS

A total of 125 *Vibrio anguillarum* serovar O1 strains were isolated, and plasmid profiles were determined. Seventy-eight strains carried only one plasmid of 65 to 70 kilobases; these strains were picked for further analysis (details on the plasmid contents of all 125 strains will be given elsewhere).

Six different RFLP types were demonstrated when the plasmids were digested with *Bam*HI (Fig. 1). The different types had four to six bands in common, besides one or two additional type-specific bands. One plasmid from each of the six types was digested with restriction enzyme *Hin*dIII. The

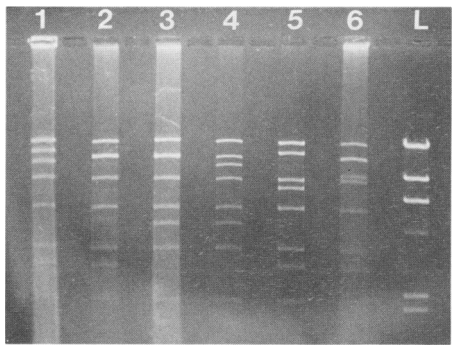

FIG. 1. RFLP types of *V. anguillarum* serovar O1 virulence plasmids digested with *Bam*HI. Lanes: 1, type 1, strain 6018/1; 2, type 2, strain 820617-1/6; 3, type 3, strain S2-1/3; 4, type 4, strain 850610-1/6A; 5, type 5, strain 820901-2/2; 6, type 6, strain 860618-3/4; L, phage lambda cut with *Hin*dIII.

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TABLE 1. RFLP of the *V. anguillarum* serovar O1 virulence plasmid

RFLP type	No. of isolates	% of isolates
I	13	17
II	41	53
III	20	26
IV	1	1
V	2	3
VI	1	1

patterns produced thereby were similar to the *Hin*dIII pattern previously described for the virulence plasmid pJM1 (16).

The overall distribution of the six types among the 78 strains is given in Table 1. Type I (17%), type II (53%), and type III (26%) were common, whereas types IV, V, and VI were represented by only one or two strains each.

The distribution of the types within each of the nine maricultures from where the bacteria had been isolated is shown in Table 2. Type II was the common type in six of the maricultures; types I and III were common in one mariculture each; and type III was isolated from only one mariculture, where it was shown in 20 of the 24 strains examined.

Of the 12 biochemical properties and the hemagglutination properties tested, the 78 *V. anguillarum* serovar O1 strains varied in three criteria (pellicle formation in broth, fermentation of ribose, and decomposition of Tween 80). Based on this variation, the strains could be separated into four biovars (Table 3). RFLP types II, IV, and VI (the two latter only representing one strain each) all belonged to the major biovar group, whereas strains of RFLP types I, III, and V also were seen in other biovar groups.

DISCUSSION

The 65- to 70-kilobase plasmid, noted to be the only plasmid in 62% of the *V. anguillarum* strains we isolated, corresponds in size to the pJM1 plasmid, first noted by Crosa et al. (3, 4) in highly virulent strains of *V. anguillarum* and later described in detail with respect to plasmid-encoded iron-sequestering and iron-binding proteins (15). The 65- to 70-kilobase plasmids in Danish isolates of *V. anguillarum* have *Hin*dIII profiles similar to that previously published for pJM1 by Wiik et al. (16), and we assume that they belong to the same class of virulence plasmids.

In their initial work on the virulence association of pJM1, Crosa et al. (4) noted that the plasmids were not 100% identical. Later, Wiik et al. (16) observed polymorphy in pJM1 when comparing restriction profiles of their plasmid isolates with that of pJM1 in *V. anguillarum* strain 775, which was one of the strains originally used by Crosa et al. (4) for virulence studies. It is in the light of these observations that we decided to classify individual isolates according to the RFLP types of the plasmids. We noted by chance that the restriction profiles produced by *Bam*HI digestion showed an RFLP that was easily interpreted, and this enzyme was chosen for further use.

TABLE 2. Distribution of RFLP types of *V. anguillarum* serovar O1 plasmids in nine different maricultures

Mariculture	No. of isolates of RFLP type:						
	I	II	III	IV	V	VI	Total
Alssund	1	18	0	1	0	0	20
Ensted	2	13	0	0	0	0	15
Taroe	2	0	20	0	1	1	24
Skaerbaek	7	0	0	0	0	0	7
DanMarin	1	4	0	0	0	0	5
Ebeltoft	0	2	0	0	0	0	2
Baagoe	0	1	0	0	0	0	1
DAI	0	3	0	0	0	0	3
(Sweden)	0	0	0	0	1	0	1

TABLE 3. Biochemical and surface properties in relation to RFLP type of *V. anguillarum* virulence plasmids

Biovar[a]	No. of isolates of RFLP type:						
	I	II	III	IV	V	VI	Total
1	11	41	18	1	1	1	73
2	0	0	2	0	1	0	3
3	1	0	0	0	0	0	1
4	1	0	0	0	0	0	1

[a] 1, Arabinose positive, cellobiose positive, galactose positive, glycerol positive, iniositol negative, ribose positive, trehalose negative, sorbitol positive, citrate (Simmon) positive, citrate (Christensen) positive, no pellicle formation in broth, hemagglutination negative, and Tween 80 positive. 2, Like biovar 1, but Tween 80 negative. 3, Like biovar 1, but ribose negative. 4, Like biovar 1, but Tween 80 negative and positive for pellicle formation in broth.

Six clearly related and yet easily distinguishable RFLP types of virulence plasmid were seen; three of these types were dominant (Table 1 and Fig. 1). None of the types correlated to the biochemical and surface properties we have tested (Table 3). This observation means that we have no easy marker system for the RFLP types but must go through digestion in each analysis. The RFLP typing gave better discrimination between the strains than the biotyping (four groups, one of which enclosed 94% of the isolates), and it was therefore considered for use in epidemiological analysis.

We have carried out a preliminary investigation into the usefulness of the typing system by determining the prevalence of each RFLP type in different maricultures. In each of the cultures one RFLP type had become dominant (Table 2). This observation is in accordance with a clonal spreading of bacteria once they are introduced. In several maricultures, however, more than one RFLP type was present. Since maricultures are based on smolt from freshwater and since *V. anguillarum* can be introduced in the stock from carrier fish in the environment, when the smolt is transferred into the sea, we think that these other types represent different clones of bacteria introduced from a different source. They may, however, represent a change of type by a mutation, in which case the RFLP typing is less useful in epidemiological studies; further investigations are needed.

It is a matter of interest how the RFLP types have evolved from each other, and studies on the genetic relationships between the plasmid types must be performed. The use of specific typing systems may facilitate the understanding of how *V. anguillarum* serovar O1 organisms are spread among populations of fish in maricultures and to these from fish in the wild population and vice versa; high priority must be given to the development of such typing systems.

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