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Intraspecific Characterization of *Vibrio tapetis* Strains by Use of Pulsed-Field Gel Electrophoresis, Ribotyping, and Plasmid Profiling

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A total of twenty-two strains of *Vibrio tapetis*, the causative agent of brown ring disease affecting cultured clams, were compared and evaluated in an investigation of strain heterogeneity using pulsed-field gel electrophoresis (PFGE), ribotyping, and plasmid profile analysis. A total of 90.9% of the *V. tapetis* strains tested by using *Not*I showed the same PFGE pattern, consisting of 15 bands. In contrast, the *V. tapetis* strains showed a low degree of similarity with six reference *Vibrio* species tested. All *V. tapetis* strains harbored a large plasmid of 74.5 kb. This plasmid was not detected in any of the other *Vibrio* species. In addition, endonuclease restriction analysis of the plasmid content of the strains using *Eco*RI and *Hin*dIII clearly showed that all the strains of *V. tapetis* possessed the same cleavage pattern. The three enzymes used for ribotyping, *Pvu*II, *Sma*I, and *Sal*I, yielded patterns with 8 to 12 bands ranging in size from 2 to 23 kb. The application of the *Sal*I and *Sma*I endonucleases rendered the separation of the strains tested into two ribotypes, while all the *V. tapetis* strains belonged to the same ribotype when the enzyme *Pvu*II was used.

Brown ring disease is the first pathology of bacterial origin described to occur in adult bivalves cultured in Europe. The causative agent of this disease has been recently described as a new species within the genus *Vibrio*, designated *Vibrio tapetis* (5). Primary identification of *V. tapetis* can be performed by using biochemical criteria, such as those described by Castro (7) and Borrego et al. (5). Further characterization of *V. tapetis* isolates is usually performed by serological procedures (10). All these works have demonstrated that *V. tapetis* strains are very homogenous, sharing antigenic characteristics. Therefore, other techniques must be employed to subtype this new species.

Plasmid profiling has proved to be of limited value as a method for epidemiological studies with several *Vibrio* species (19). This contrasts with results obtained with members of the family *Enterobacteriaceae*, in particular *Salmonella* spp. (4), and other pathogenic bacteria. In previous investigations, we described the presence of only a 74.5-kb plasmid in all the *V. tapetis* strains tested (6). In similar cases, restriction fragment length polymorphism analysis of the plasmids has been employed to establish several subtypes in a clonal line, and therefore, it has been useful as an epidemiological marker and as an intraspecific classification method (22, 23, 35).

Chromosomal DNA fingerprinting allows the typing of most bacterial strains. Pulsed-field gel electrophoresis (PFGE), a technique for separation of large DNA fragments, has been successfully employed for both epidemiological investigations and differentiation of several bacterial types within a number of species, such as *Clostridium difficile* (11), *Enterobacter cloacae* (17), *Enterococcus faecalis* (15), *Leptospira* spp. (18), *Salmonella* spp. (24, 25), *Staphylococcus aureus* (28), and *Vibrio anguillarum* (30).

In recent years, rRNA gene restriction analysis (ribotyping) has become widely used in the typing of bacteria (16) and in the differentiation of isolates of a single serovar (23, 26). This technique involves the hybridization of rRNA or genes coding for rRNA with genomic DNA cleaved with restriction endonucleases. These enzymes cleave the DNA within or between the rRNA operons, and following hybridization with a specific rRNA gene probe, the technique results in the formation of various banding patterns (1). Ribotyping has been used to differentiate strains in a variety of species including fish and shellfish pathogens like *V. ordalii* (32), *V. anguillarum* (23, 30, 32), *Pasteurella piscicida* (21), and *V. cholerae* non-O1 (13).

In this study the genetic methods listed above were compared and evaluated in the investigation of strain heterogeneity within the population of *V. tapetis* isolated from different epizootic outbreaks and different clam beds over a 5-year period.

MATERIALS AND METHODS

Bacterial strains. Twenty-two strains of *V. tapetis* were used in this study. Their geographical origins and isolation years have been specified previously (5). They were maintained as working stocks at −20°C in basal medium (0.4% peptone, 0.1% yeast extract, 2% NaCl) supplemented with 20% glycerol (vol/vol). For comparative purposes, six culture collection strains including *V. anguillarum* O1 (R82), *V. anguillarum* O3 (6062A), *V. splendidus* I CECT 528^T, *V. splendidus* II ATCC 25914, *V. pelagius* ATCC 25916^T, and *V. tubiashii* CECT 631 were used.

PFGE. Strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl for 16 to 18 h and then were centrifuged at 4,000 × *g* for 10 min at 4°C. The pellet was washed twice in TEN buffer (10 mM Tris, 100 mM EDTA, 150 mM NaCl [pH 7.2]), and the cells were resuspended in the same buffer to achieve a bacterial concentration of about 10^9 CFU/ml. The bacterial suspension was mixed with an equal volume of a 2% agarose solution in TEN buffer (InCert agarose; FMC Bioproducts, Rockland, Maine), deposited in molds, and stored at 4°C for 30 min. Agarose plugs were treated with bacterial lysis solution (10 mM Tris-HCl [pH 7.5], 0.1 M EDTA, 500 mM NaCl, 0.2% sodium deoxycholate, 0.5% sarcosyl, 0.1% lysozyme, 200 μg of RNase I/ml) for 5 to 6 h at 37°C. Bacterial lysis was completed by 48 h of treatment at 50°C with a solution containing 0.4 M EDTA (pH 8.0), 1% sarcosyl, and 1 mg of proteinase K/ml. Afterwards, the blocks were thoroughly washed with TE buffer (10 mM Tris [pH 7.5], 0.1 mM EDTA) at 37°C and incubated in the same buffer overnight. Finally, the blocks were equilibrated in Dummy buffer (100 mM Tris [pH

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8.0], 5 mM $MgCl_2$) at room temperature. All the chemicals were supplied by Sigma Chemical Co., St. Louis, Mo.

For DNA digestion, fragments of the agarose blocks were first incubated in restriction enzyme buffer for 1 h at 4°C and then with 30 to 50 U of *Not*I (Promega) in the same buffer (supplemented with 0.1 mg of RNase/ml) for 18 h at 37°C. DNA fragments were separated in a 1% pulsed-field agarose gel (Bio-Rad Lab., Richmond, Calif.), which was prepared and run in 0.5 × TBE buffer (45 mM Tris-borate [pH 8.0], 1 mM EDTA) on a contour-clamped homogeneous electric field machine (CHEF-DR II; Bio-Rad). The running conditions were 200 V for 20 to 22 h with pulse times ranging from 5 to 8 s. Lambda DNA PFGE markers (Pharmacia) were used as size standards. After electrophoresis, gels were stained in ethidium bromide (2 μg/ml) (Sigma) for 45 min, destained in distilled water for 1 h, and photographed with 254-nm UV transillumination.

Plasmid analysis. The alkaline extraction method described by Birnboim and Doly (2) as modified by Sambrook et al. (29) was used for DNA plasmid isolation. DNA was concentrated by addition of 2.5 volumes of absolute ethanol and incubation at −30°C for 10 min. After ethanol removal by centrifugation (12,000 × *g* for 15 min), the pellet was resuspended in 20 μl of TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 7.9]) and supplemented with 6 μl of stabilized solution (0.25% bromocresol purple and 50% glycerol in 50 mM Tris-acetate [pH 7.9]).

DNA was electrophoresed by using 0.7% agarose gels in TAE buffer. DNA bands were visualized by staining with ethidium bromide as described above. *Escherichia coli* V517 and R40a were used as molecular mass markers in the same gel.

Purified plasmid bands of the *V. tapetis* strains were digested with restriction endonucleases *Eco*RI and *Hin*dIII following the instructions of the manufacturer (Boehringer, Mannheim, Germany). Lambda DNA digested with *Hin*dIII was used as a molecular weight marker.

Ribotyping. Total DNA from each isolate was extracted by sequential treatments with sodium dodecyl sulfate, pronase (Sigma) (final concentration 25 μg/ml), and ammonium acetate (7.5 M). DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Concentration and purity of the total DNA were spectrophotometrically evaluated (at 260 nm).

Prior to the restriction enzyme treatment, DNA (200 μl) was adjusted to the appropriate concentration by precipitation with ethanol and resuspended in TAE buffer. DNA samples (2 μg) were digested separately with *Pvu*II, *Sma*I, and *Sal*I following the recommendations of the manufacturers, and the reaction was stopped by addition of 10 μl of stabilized solution. Digested DNA was electrophoresed in 0.8 to 1% agarose gels (Sigma type II-A) in TAE buffer at 70 V for 6 h.

After electrophoresis, gels were washed twice with 0.25 M HCl (15 min each) and twice with 0.5 M NaOH. Then, DNA was denaturalized by two treatments with 1.5 M NaCl (20 min) and neutralized with Tris buffer (0.5 M Tris, 3 M NaCl [pH 7.0]). After naturalization, DNA was transferred under vacuum (Hybaid VacuAid) to a 0.45-μm-pore-size nylon membrane (Boehringer). After a fixation step by UV light (UV cross-linker) for 2 min, the DNA was hybridized with a digoxigenin-labelled probe synthesized from a mixture of 16S and 23S rRNA from *E. coli* (Sigma). The blot hybridizations were visualized colorimetrically with a DIG DNA detection kit (Boehringer).

Data analysis. Similarity between the *V. tapetis* strains was estimated from the number of matching bands in the ribotype patterns by using the Dice coefficient (S_d) (14) as a measure of homology according to the following equation: $S_d = [2A/(2A + B + C)] \times 100$, where A is the number of matching bands and B and C are the numbers of bands present in one strain but not in the other.

RESULTS

PFGE of genomic restriction fragments. Analysis of the restriction digest patterns resolved by PFGE showed that a similar distribution of patterns was observed between *Xba*I and *Not*I, although *Xba*I presented patterns which were more complex to compare (data not shown). For this reason, *Not*I was chosen for analyses of PFGE of all strains. A strain was considered to be different if its PFGE pattern differed by one or more bands. All the strains belonging to *V. tapetis* presented the same PFGE pattern, consisting of 15 bands, except the strain IS-5 (Fig. 1). On the contrary, *V. tapetis* strains showed a low degree of similarity with other *Vibrio* species, including *V. anguillarum* O1, *V. anguillarum* O3, *V. pelagius*, *V. splendidus* I, *V. splendidus* II, and *V. tubiashii* (Fig. 1).

Plasmid profiles. All *V. tapetis* strains harbored the same plasmid profile, consisting of the presence of two bands of 52.8 and 40.2 MDa in the electrophoretic gels (data not shown). However, the presence of two bands may be due to the plasmid methodology used, since one plasmid can suffer conformational changes in the supercoiled DNA and the bands of approximately 52.8 and 40.2 MDa correspond to open circular and closed circular forms, respectively (36).

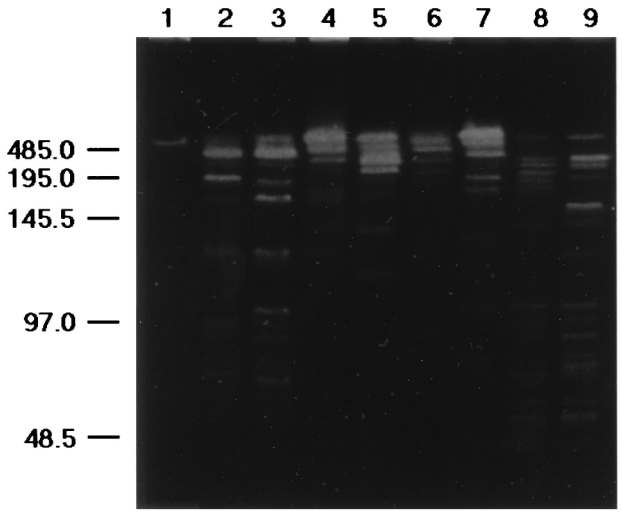

FIG. 1. PFGE patterns of selected *Vibrio* strains after DNA digestion with *Not*I. Lane 1, molecular size markers (in kilobases); lane 2, PFGE pattern observed in *V. tapetis* strains except *V. tapetis* IS-5; lane 3, *V. tapetis* IS-5; lane 4, *V. splendidus* II (ATCC 25914); lane 5, *V. tubiashii* (CECT 631); lane 6, *V. splendidus* I (CECT 528); lane 7, *V. pelagius* (ATCC 25916); lane 8, *V. anguillarum* O3 (6062A); lane 9, *V. anguillarum* O1 (R82).

Endonuclease restriction analysis of the plasmid content of the strains using *Eco*RI and *Hin*dIII clearly shows that all the strains of *V. tapetis* possessed the same cleavage patterns (data not shown), consisting of 9 fragments by *Eco*RI digestion and 10 fragments by *Hin*dIII digestion, and confirms the existence of only one plasmid, which had an estimated size of 74.5 kb.

Ribotyping. The three enzymes tested, *Pvu*II, *Sma*I, and *Sal*I, yielded a sufficient number of appropriately sized fragments to perform an accurate analysis of the strains. When ribotyping was carried out, these enzymes gave patterns with 8 to 12 bands ranging in size from 2 to 23 kb.

As was expected from comparisons with other methods (5, 10), *V. tapetis* strains showed a high degree of homogeneity. In fact, all the isolates tested belonged to the same ribotype when the enzyme *Pvu*II was employed (Fig. 2). Application of the *Sal*I and *Sma*I restriction enzymes resulted in the separation of the strains into two ribotypes. *Sma*I discriminated the strains into a group which comprised strains 8.1, 8.3, 8.7, and IS-1 and another which comprised strains IS-5, B1090^T, and 9.4. On the other hand, *Sal*I grouped the strains into a major ribotype comprising strains 8.1, 8.3, 8.7, IS-1, IS-5, and B1090^T and another with only the 9.4 strain (Fig. 2).

The results of the application of the Dice coefficient (S_d) as an indicator of homology among the strains tested are given in Table 1. Only the use of the *Sma*I and *Sal*I endonucleases allowed the discrimination of the *V. tapetis* strains into two groups, with S_ds of 66.6 and 18.2% similarity, respectively.

DISCUSSION

V. tapetis has been demonstrated to be the causative agent of brown ring disease, which is the first epizootic infection described in Europe that affects cultured and wild populations of adult clams, mainly manila clams (*Tapes philippinarum*) and fine clams (*Tapes decussatus*) (7, 8, 20). However, intraspecific discrimination among the strains belonging to this species was

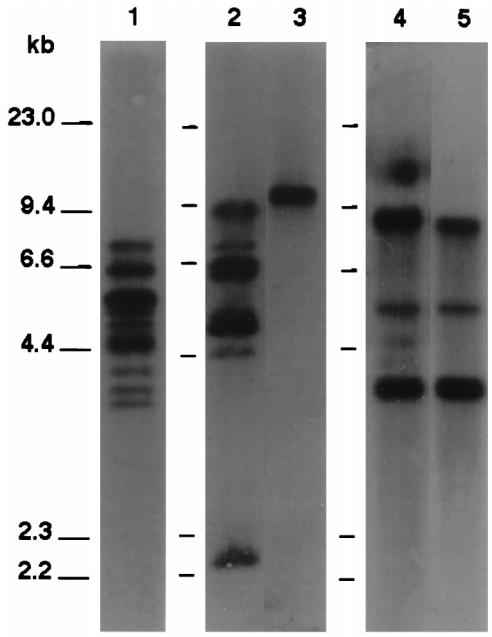

FIG. 2. Ribotypes observed among *V. tapetis* strains after DNA restriction with *Pvu*II (lane 1), *Sal*I (lanes 2 and 3), and *Sma*I (lanes 4 and 5). Molecular size standards are given on the left. Lane 2, *V. tapetis* 8.1, 8.3, 8.7, IS-1, IS-5, and B1090^T; lane 3, *V. tapetis* 9.4; lane 4, *V. tapetis* 8.1, 8.3, 8.7, and IS-1; lane 5, *V. tapetis* IS-5, B1090^T, and 9.4.

not possible by means of the classical typing methods used in epidemiological and pathobiological studies, such as biotyping and serotyping (5, 9), since *V. tapetis* strains have been shown to be homogenous in their antigenic characteristics as well as in their lipopolysaccharide and outer membrane protein patterns (10). For these reasons, in the present study we have investigated the intra- and interspecific relationship of *V. tapetis* by genetic methods, including DNA fingerprinting by PFGE, ribotyping, and plasmid profiling.

PFGE and ribotyping have been shown to be valuable typing methods for epidemiological investigations of several pathogenic bacteria (11, 15, 25, 28), including fish pathogens (21, 23, 30). On the other hand, plasmid profiling has been successfully used as an epidemiological marker (3, 4, 8, 32).

In our investigation, ribotyping did not separate isolates that had originated from different time periods and shellfish species, since all the strains were included into only one ribotype with the *Pvu*II restriction enzyme and into two ribotypes by use of *Sma*I and *Sal*I. The latter endonucleases grouped 57.1 and 85.7% of the strains, respectively, into a single ribotype. Therefore, ribotyping does not seem to be suitable when a high degree of discrimination is needed.

TABLE 1. Number of ribotypes of *V. tapetis* related to previously established groups

Group[a]	No. of strains	No. of ribotypes			S_d		
		*Sma*I	*Sal*I	*Pvu*II	*Sma*I	*Sal*I	*Pvu*II
B1090^T	1	1	1	1	100	100	100
8	9	1	1	1	100	100	100
IS	5	2	1	1	66.6	100	100
9	4	1	2	1	100	18.2	100

[a] See reference 7.

Olsen and Larsen (23) found that different *Vibrio* species had very different ribotypes, whereas ribotypes of different serovars of *V. anguillarum* were quite similar. Thus, ribotypes of serovar O1 strains (26) as well as serovar O2 strains (32) appear to have similarities with the ribotypes of *V. anguillarum* O1 to O10 strains examined by Olsen and Larsen (23). This indicates a close genetic relationship among all *V. anguillarum* strains regardless of serovar.

The ribotype patterns were compared by using a simple S_d based on the presence of the same hybridization-positive restriction fragments in the strains analyzed. This coefficient has previously been used for comparison of electrophoretic patterns of *Salmonella* virulence plasmid restriction fragments (27) and for determination of ribotype similarity of *V. anguillarum* serovars (23). In the case of *V. tapetis* strains, high similarity between strains, as reported previously by Castro et al. (10), was confirmed. Strain 9.4 was the only strain shown to belong to a different ribotype by both the *Sma*I and *Sal*I restriction enzymes. However, Borrego et al. (5) and Castro et al. (10) demonstrated that this strain has not shown phenotypic and serological intraspecific differences from other *V. tapetis* strains.

PFGE subdivided the *V. tapetis* strains into two groups, one of which included 75% of the strains tested. However, this typing method has allowed us to clearly discriminate *V. tapetis* strains from other *Vibrio* species, such as *V. anguillarum* O1 and O3, *V. tubiashii*, *V. pelagius*, and *V. splendidus* I and II.

Recently, both typing methods, ribotyping and PFGE, have been used to establish clonal lineages among *V. anguillarum* serovar O1 strains isolated from different locations (30). Only PFGE could be used to demonstrate the existence of at least two clonal lineages from different isolation locations in Europe. In the case of *V. tapetis* strains, the same PFGE pattern was shared by strains isolated from different locations over several years.

An analysis for plasmid content was also performed. All the *V. tapetis* strains harbored a highly conserved 74.5-kb plasmid, which suggests that it may be related to important physiological and/or pathological functions of these strains (31). Several virulence factors of pathogenic bacteria are encoded in plasmid genes, such as adhesive properties, toxin production, and iron-sequestering systems (12, 19, 33, 34). However, it cannot be definitively confirmed that the 74.5-kb plasmid harbored by all *V. tapetis* strains is implicated in the virulence of these strains (6).

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