Vibrio tapetis sp. nov., the Causative Agent of the Brown Ring Disease Affecting Cultured Clams

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A taxonomic characterization was carried out on strains of the bacteria that cause the brown ring disease of clams. On the basis of their phenotypic and genotypic characteristics, these strains can be considered to constitute a new taxonomic unit, distinct from other *Vibrio* species. The guanine-plus-cytosine content of the strains ranged between 42.9 and 45.5 mol% (43.2 mol% for the proposed type strain). DNA-DNA hybridization studies showed 100% intragroup relatedness, but levels of genetic relatedness to the reference strains of different *Vibrio* species tested ranged between 15 and 58%. The strains have all the properties characteristic of the genus *Vibrio* and can be clearly differentiated from other species of this genus by their growth at 4°C and their negative responses for growth at 30°C and in 6% NaCl, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, and Voges-Proskauer reaction. The name *Vibrio tapetis* is proposed for the new species; strain B1090 (CECT 4600) is the type strain.

Since 1980, the genus *Vibrio* has been subject to an extensive taxonomic revision, and 15 new species have been described (12, 22). At present, the genus *Vibrio* includes more than 35 species, most of which are of aquatic origin. Some of them have been demonstrated to be pathogenic for aquatic animals, including fish (*Vibrio anguillarum*, *V. alginolyticus*, *V. damsela*, *V. fischeri*, *V. ordalii*, *V. salmonicida*, *V. splendidus*, and *V. vulnificus*) and shellfish (*V. harveyi*, *V. pelagius*, *V. splendidus*, and *V. tubiashii*) (1, 6, 9–11, 15, 18, 25, 30, 31).

Brown ring disease is the first epizootic infection described in Europe which affects cultured and wild populations of adult clams, mainly manila clams (*Tapes philippinarum*) and fine clams (*Tapes decussatus*). This disease was firstly described in France by Paillard et al. (21), although further epidemiological studies have demonstrated its prevalence in other locations, mainly southwestern and northwestern Spain (2, 3, 23).

The biochemical characterization of the causative agent of brown ring disease indicated that it belonged to the genus *Vibrio*, and it was tentatively named *Vibrio* group P1 (20). However, on the basis of the response to routine biochemical tests, *Vibrio* group P1 does not match any of the presently described species of the genus *Vibrio* (4).

We carried out a phenotypic and genetic characterization of several strains belonging to *Vibrio* group P1 and compared them with other *Vibrio* strains frequently isolated from affected clams. The results obtained have allowed us to propose a new species of the genus *Vibrio*, *V. tapetis*, on the basis of its physiological, biochemical, and genetic features.

MATERIALS AND METHODS

Bacterial strains. Twenty-four strains of *Vibrio* group P1 constituting the phenon 6 described by Castro (2), isolated from cultivated and natural populations of diseased clams between 1988 and 1991, were studied (Table 1). They were maintained as working stocks at –20°C in basal medium (peptone, 0.4%;

yeast extract, 0.1%; NaCl, 2%) supplemented with 20% glycerol (vol/vol). For comparative purposes, 15 culture collection strains of the genus *Vibrio* were used (Table 2). All of them are halophilic microorganisms frequently isolated from marine environment and/or from poikilotherms, including fish and shellfish (4), and they shared several biochemical and physiological characteristics with the strains of *V. tapetis*. All strains were cultured on marine agar (Difco Laboratories, Detroit, Mich.) at 20 ± 2°C for 24 to 36 h.

Phenotypic characterization. All biochemical tests were performed at 20 ± 2°C using media supplemented with NaCl at a final concentration of 2%, except growth tests in NaCl. The isolates were identified by standard morphological, physiological, and biochemical tests (5, 14, 26, 33). Sensitivity to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) was determined after 24 h on marine agar plates by using O/129 discs (150 µg) (Oxoid Ltd., Basingstoke, Hampshire, England). The utilization of substrates as sole carbon and energy

TABLE 1. Designation and geographical sources of *V. tapetis* strains tested

Strain designation	Source	Origin
B1090 ^T	Cultured <i>Tapes philippinarum</i>	Landeda, France
IS-1	Cultured <i>Tapes philippinarum</i>	Landeda, France
IS-5	Cultured <i>Tapes philippinarum</i>	Landeda, France
IS-7	Cultured <i>Tapes philippinarum</i>	Quiberon, France
IS-8	Natural <i>Venerupis aurea</i>	Quiberon, France
IS-9	Natural <i>Cerastoderma edule</i>	Quiberon, France
2.3	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.1	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.2	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.3	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.4	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.5	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.6	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.7	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.17	Cultured <i>Tapes philippinarum</i>	Landeda, France
8.19	Cultured <i>Tapes philippinarum</i>	Landeda, France
9.3	Cultured <i>Tapes philippinarum</i>	Landeda, France
9.4	Cultured <i>Tapes philippinarum</i>	Landeda, France
9.5	Cultured <i>Tapes philippinarum</i>	Landeda, France
9.7	Cultured <i>Tapes philippinarum</i>	Landeda, France
11.1	Cultured <i>Tapes philippinarum</i>	Landeda, France
11.2	Cultured <i>Tapes philippinarum</i>	Landeda, France
11.3	Cultured <i>Tapes philippinarum</i>	Landeda, France
11.4	Cultured <i>Tapes philippinarum</i>	Landeda, France

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TABLE 2. Phenotypic characteristics of *Vibrio* species studied, including *V. tapetis*

Test	Result for species ^a :															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Swarming	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Indole production	+	+	+	+	—	—	—	+	—	—	—	+	—	+	+	+
Acetoin production	—	+	+	—	—	+	—	—	—	—	—	—	—	—	—	—
Growth at (°C):																
4	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35	—	+	+	+	+	+	—	—	+	+	+	+	+	—	+	+
Growth in NaCl (%):																
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	—	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
8	—	+	—	—	+	—	—	—	—	+	+	+	—	—	—	—
10	—	+	—	—	+	—	—	—	—	—	+	+	—	—	—	—
Production of:																
Alginase	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
Amylase	+	+	+	+	—	—	+	+	—	—	—	+	—	+	+	+
Arginine dehydrolase	—	—	+	—	+	+	—	—	—	—	+	—	—	+	+	—
Catalase	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
β-Galactosidase	+	—	+	—	—	+	—	+	—	+	—	—	—	+	+	+
Gelatinase	+	+	+	+	—	—	—	+	—	+	—	+	—	+	+	+
Lipase	+	+	+	+	—	—	+	+	+	+	—	+	+	+	+	+
Lysine decarboxylase	—	+	—	+	—	—	+	+	+	—	—	+	—	—	—	+
Nitrate reduction	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	—	—	—	—	—	—	—	+	—	—	—	+	—	—	—	+
Hydrolysis of:																
Esculin	—	—	—	—	+	—	+	—	+	+	—	—	+	—	—	—
Casein	+	+	+	—	—	+	—	+	—	—	+	+	—	+	+	+
Urea	—	—	—	—	—	+	+	—	—	+	—	—	—	—	—	—
Utilization of:																
Acetate	—	+	+	—	+	—	—	+	+	+	+	+	+	—	+	—
Aconitate	—	+	+	+	—	—	—	+	—	+	+	+	+	+	—	+
D-Alanine	—	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+
L-Alanine	—	+	—	+	+	—	—	+	+	+	+	+	+	+	+	+
γ-Aminobutyrate	—	—	—	—	—	—	—	—	—	+	+	—	—	—	+	—
Amygdalin	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Arabinose	—	—	+	—	—	—	—	—	—	+	—	+	—	—	—	—
L-Aspartate	—	—	+	—	—	+	+	+	+	+	+	+	+	+	—	+
Cellobiose	—	—	+	+	—	—	+	+	+	+	—	—	—	—	+	+
Citrate	—	+	+	+	—	—	+	+	+	+	+	+	—	+	+	+
D-Fructose	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+
D-Galactose	—	—	—	—	—	+	—	+	+	+	—	+	+	—	+	+
D-Galacturonate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D-Gluconate	—	+	+	—	+	—	—	+	—	+	+	+	+	—	+	+
α-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucuronate	—	—	—	—	—	—	—	+	—	+	—	+	—	—	—	+
L-Glutamate	—	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+
Glycerol	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycine	—	+	—	—	—	—	—	+	—	+	+	+	+	+	+	—
L-Histidine	—	+	+	—	+	+	—	+	+	+	+	+	+	—	+	—
β-Hydroxybutyrate	—	—	—	—	—	—	—	—	—	+	+	—	—	—	+	—
myo-Inositol	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—
α-Ketoglutarate	—	+	+	+	—	—	—	+	—	+	+	+	—	+	+	+
DL-Lactate	—	+	+	+	—	+	—	+	+	+	+	+	+	+	+	+
Lactose	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
L-Leucine	—	+	—	—	—	—	—	—	—	+	+	+	—	—	—	—
DL-Malate	—	+	+	+	—	—	—	+	+	+	+	+	—	+	+	—
Maltose	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	—	+	+	+	+	—	+	+	+	+	—	+	+	+	+	—
D-Mannose	+	+	+	—	—	+	+	+	+	—	—	+	+	+	+	+
Melibiose	—	—	—	—	—	—	—	—	—	+	—	—	—	—	+	—
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Ornithine	—	—	—	—	—	—	—	—	—	+	+	—	+	—	+	—
L-Proline	—	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	—	—	+	—	+	+	+	+	+	—	+
Putrescine	—	—	—	—	—	—	—	—	+	+	+	+	+	—	+	—

Continued on following page

TABLE 2—Continued

Test	Result for species ^a :															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Pyruvate	—	+	+	+	+	+	—	+	—	+	+	+	+	+	+	+
Quinate	—	—	—	—	—	+	—	—	—	+	—	—	—	—	+	—
L-Rhamnose	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
L-Serine	—	+	+	+	+	—	—	+	+	+	+	+	+	+	+	—
D-Sorbitol	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—
Succinate	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+
Sucrose	—	+	+	—	+	—	—	+	+	+	+	—	+	—	+	—
D-Trehalose	+	+	+	+	+	—	—	+	+	+	+	+	+	+	+	+
L-Threonine	—	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+
Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	—	+	+	+	+	+	—	+	—	+	—	+	+	+	+	+

^a 1, *V. tapetis* B1090^T; 2, *V. alginolyticus* CECT 521^T; 3, *V. anguillarum* CECT 522^T; 4, *V. campbellii* CECT 523^T; 5, *V. costicola* NCIMB 1281^T; 6, *V. damsela* ATCC 33539; 7, *V. fischeri* CECT 524^T; 8, *V. harveyi* CECT 525^T; 9, *V. mediterranei* CECT 621^T; 10, *V. natriegens* CECT 526^T; 11, *V. nereis* CECT 595^T; 12, *V. parahaemolyticus* CECT 588; 13, *V. pelagius* ATCC 25916^T; 14, *V. splendidus* CECT 528^T; 15, *V. tubiashii* CECT 631; 16, *V. vulnificus* CECT 529^T.

sources was performed by using GN Microplates (Biolog, Inc., Hayward, Calif.). All the biochemical tests were repeated twice.

Clustering analysis. Tests were coded as 1 (positive result), 0 (negative result), and 9 (no growth or equivocal result). Test error was determined by the method of Sneath and Johnson (28). Clustering analysis was performed using 73 traits determined for 15 reference strains of known *Vibrio* species and 10 strains of *Vibrio* group P1. The simple matching similarity coefficient (27) and unweighted pair group method with arithmetic averages were used for clustering analysis (29). The analyses were performed with the NTSYS-PC package (version 1.70) (24).

DNA base composition. DNA was isolated by the method of Marmur (16) from cells grown in tryptone soya broth (Oxoid) supplemented with 2% NaCl. The melting temperature (T_m) of DNA was determined by the method described by Marmur and Doty (17) using a Perkin-Elmer UV-Vis 551S spectrophotometer at 260 nm, programmed for temperature increases of 1.0°C/min. The T_m of DNA was calculated graphically (8), and the guanine-plus-cytosine (G+C) content was calculated from this temperature with 0.1 standard saline citrate (SSC) (0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.0). DNA from *Escherichia coli* NCTC 9001 was used as the standard (19).

DNA labelling. DNA was labelled by the multiprimer system, a commercial kit (kit RPN 1601 Y), and tritiated dCTP (TRK 621) (the last two were supplied by Amersham International, Amersham, Buckinghamshire, United Kingdom). The average specific activity obtained by this procedure was 8.4×10^6 cpm/μg of DNA. The labelled DNA was denatured before hybridization by being heated at 100°C for 5 min and then placed on ice.

DNA homology experiments. DNA homology studies were performed by using the competition procedure of the membrane method (13). Competitor DNAs were sonicated at 50 W for two 15-s bursts. Membrane filters (type HAHY; Millipore Corp., Bedford, Mass.) containing reference DNA were placed in 5-ml screw-cap vials which contained the labelled, sheared, denatured DNA and the denatured, sheared competitor DNA. The ratio of the concentration of competitor DNA to the concentration of labelled DNA was at least 150:1. The final volume was adjusted to 140 μl, and the reaction mixture contained a final concentration of 2× SSC and 30% formamide solution. The hybridization temperature ranged between 56 and 59°C, which is below the upper limit allowed for the validity of the filter method (7). After hybridization, the filters were washed in 2× SSC at the optimum renaturation temperature. The radioactivity bound to the filters was measured with a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.), and the percentage of homology was calculated by the method of Johnson (13). All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Phenotypic characterization. Strains of *Vibrio* group P1 exhibited the typical features of the genus *Vibrio*. The major phenotypic characteristics that differentiate these strains from other reference *Vibrio* species are shown in Table 2. All named species differ widely in their phenotypic characteristics from *Vibrio* group P1 strain B1090. Routine identification of *Vibrio* group P1 is based on the following traits: indole production; growth at 4°C; inability to grow at 30°C and in 6% NaCl; arginine dehydrolase negative; β-galactosidase positive; ability

to hydrolyze gelatin, casein, lecithin, and starch; and inability to ferment sucrose, D-mannitol, or amygdalin.

On the basis of biochemical profiles, *Vibrio* group P1 was included previously in the *V. splendidus*-like group (2, 4). However, *Vibrio* group P1 is clearly distinguished from this group by the following tests: growth at 4 and 30°C; arginine dehydrolase, β-galactosidase, lecithinase, and indole tests; and fermentation of D-mannitol and amygdalin. In addition, unlike *Vibrio* group P1, *V. splendidus* can utilize aconitate, D-alanine, α-ketoglutarate, DL-lactate, maltose, D-mannitol, L-proline, pyruvate, and L-threonine.

Clustering analysis. Seventy-three tests were used for the cluster analysis of the strains studied. Figure 1 depicts a simplified dendrogram based on the simple matching similarity coefficient and the unweighted pair group method of association with arithmetic averages. All the presumptive *Vibrio* group P1 strains ($n = 24$) constituted a homogeneous phenon, clustering at a similarity level higher than 95% (Table 3).

The 15 other *Vibrio* species (12 type strains and 3 reference strains) did not cluster within the *Vibrio* group P1 phenon. None of them showed similarities higher than 70% with strains of the *Vibrio* group P1 phenon. *Vibrio* group P1 strain B1090^T was selected as the representative strain of the phenon.

DNA base composition. The G+C contents of the DNAs of nine representative strains of *Vibrio* group P1 ranged from 42.9 to 45.5 mol% (mean value ± standard deviation = 44.3 mol% ± 0.86 mol%) (Table 4). These values lie well within the accepted range of 38 to 51 mol% for members of the genus *Vibrio* (12).

Hybridization studies. The results of intraspecific and interspecific hybridizations between the type strain and other selected strains of *V. tapetis* and seven reference strains of several *Vibrio* species are summarized in Table 5. All the *V. tapetis* strains showed total homology (100%) with *V. tapetis* B1090^T. On the other hand, the percentage of homology of several *Vibrio* species with *V. tapetis* B1090^T ranged between 15 and 58% for *V. costicola* NCIMB 701 and *V. vulnificus* CECT 529, respectively. These results confirm that *V. tapetis* is genotypically a separate species from the other phenotypically related *Vibrio* species tested and are consistent with assignment of this species to the family *Vibrionaceae*.

On the basis of these results and taking into consideration the criteria recommended by Wayne et al. (32), the strains of the *Vibrio* group P1 phenon constitute a new taxonomic unit, for which we propose the new species name of *V. tapetis*.

FIG. 1. Dendrogram showing the result of cluster analysis of phenetic data using the simple matching similarity coefficient and unweighted pair group method with arithmetic averages for *V. tapetis* B1090^T and 15 other *Vibrio* species.

Description of *Vibrio tapetis* sp. nov. *Vibrio tapetis* (ta.pe'tis. L. gen. n. *tapetis*, of clams [the genus *Tapes*]) cells are gram-negative coccobacilli (1.0 to 1.5 by 0.5 μ m) motile by means of one polar flagellum. Grows on marine agar (Difco) and tryptone soya agar (Oxoid) containing 2% NaCl. Colonies develop within 48 h at $20 \pm 2^\circ\text{C}$ and are circular, regular in shape, translucent, and unpigmented. Grows on thiosulfate-citrate-bile-sucrose agar (green colonies). Swarming has not been detected in any of the media tested. Glucose metabolism is fermentative. Tests for catalase, oxidase, and nitrate reduction to nitrite are positive. Facultatively anaerobic. Chemoorganotrophic. No growth in the absence of NaCl in the culture medium. The salt requirement is specific for sodium ion.

Grows in media supplemented with 1 to 3% (wt/vol) NaCl but not in media with salinity higher than 5% NaCl. Susceptible to vibriostatic agent O/129 (150 μ g). Growth occurs between 4 and 22°C but not at higher temperatures. Optimal growth temperature is $18 \pm 2^\circ\text{C}$. Voges-Proskauer, H_2S production, arginine dehydrolase, and lysine and ornithine decarboxylase negative but indole production and β -galactosidase positive. The strains degrade casein, gelatin, starch, and Tween 80 but are not able to hydrolyze urea and alginate. Acid is produced from D-fructose, D-glucose, D-galactose, D-mannose, N-acetylglucosamine, and maltose but not from sucrose, L-arabinose, D-melibiose, L-rhamnose, D-sorbitol, myo-inositol, mannitol, or lactose.

V. tapetis B1090^T utilizes the following substrates as sole carbon and energy sources: D-fructose, α -D-glucose, N-acetylglucosamine, D-psicose, D-trehalose, Tween 40, succinate, and propionate. Other substrates specified in Table 2 are not utilized as sole carbon and energy sources.

TABLE 3. Phenotypic intraspecific variation in *V. tapetis* strains

Characteristic	No. of strains positive (%)	Differing strain(s) ^a	Reaction of type strain (B1090)
Hydrolysis of casein	23 (95.8)	IS-5	+
Utilization of:			
D-Mannose	23 (95.8)	IS-1	+
Succinate	23 (95.8)	2.3	+
N-Acetyl-D-glucosamine	22 (91.7)	IS-1, 9.5	+
D-Trehalose	20 (83.3)	IS-1, 9.5, 2.3, 11.1	+
Propionate	23 (95.8)	9.5	+
L-Arabinose	3 (12.5)	8.5, 9.5, 11.1	—
myo-Inositol	1 (4.2)	8.5	—
D-Galactose	1 (4.2)	8.5	—
DL-Lactate	1 (4.2)	8.5	—
D-Gluconate	1 (4.2)	8.5	—
Glycerol	1 (4.2)	8.5	—

^a Strains which differ from *V. tapetis* B1090^T phenotypic characteristics.

TABLE 4. Midpoint T_m s and DNA base compositions of the nine *V. tapetis* strains tested

Strain	T_m ($^\circ\text{C}$)	G+C content (mol%)
B1090 ^T	70.9	43.2
8.3	71.7	44.8
8.4	72.0	45.5
IS-1	71.2	43.8
IS-5	71.4	44.3
IS-7	71.3	44.1
11.1	71.6	44.7
9.4	70.7	42.9
2.3	72.0	45.5

TABLE 5. Intraspecific and interspecific DNA-DNA homology between *V. tapetis* B1090^T and other related strains of genus *Vibrio*

Strain ^a	% Homology with ³ H-labelled DNA from <i>V. tapetis</i> B1090 ^T
<i>V. tapetis</i>	
B1090 ^T	100
8.3.....	100
8.4.....	100
9.4.....	100
2.3.....	100
11.1.....	100
IS-1.....	100
IS-5.....	100
IS-7.....	100
<i>V. alginolyticus</i> CECT 521 ^T	53
<i>V. anguillarum</i> CECT 522 ^T	29
<i>V. costicola</i> NCIMB 1281 ^T	15
<i>V. fischeri</i> CECT 524 ^T	48
<i>V. pelagius</i> ATCC 25916 ^T	38
<i>V. splendidus</i> CECT 528 ^T	46
<i>V. vulnificus</i> CECT 529 ^T	58

^a CECT, Colección Española de Cultivos Tipos; NCIMB, National Collection of Industrial and Marine Bacteria (Aberdeen, United Kingdom); ATCC, American Type Culture Collection.

V. tapetis has been isolated exclusively from manila and fine clams (*T. philippinarum* and *T. decussatus*), in which it causes the brown ring disease.

The G+C content of strain B1090^T is 43.2 mol% (*T_m* method). The type strain of *V. tapetis* is strain B1090 (CECT 4600).

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