NOTE

***Vibrio calviensis* sp. nov., a halophilic, facultatively oligotrophic 0.2 µm-filterable marine bacterium**

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A Gram-negative, facultatively anaerobic, straight to slightly curved rod-shaped bacterium (RE35F/12^T) sensitive to vibriostatic agent O/129 was previously isolated from sea water (Western Mediterranean Sea, Bay of Calvi, Corsica, France) by 0.2 µm-membrane filtration. Strain RE35F/12^T (= CIP 107077^T = DSM 14347^T) was facultatively oligotrophic, halophilic, required Na⁺ for growth and produced acid but no gas from D-glucose under anaerobic conditions. Comparative 16S rRNA gene-sequence analyses demonstrated that the bacterium is most closely related (94.3%) to *Vibrio scophthalmi*. Similarities to the sequences of all other established *Vibrio* species ranged from 93.6% (with *Vibrio aestuarianus*) to 90.7% (with *Vibrio rumoiensis*). Strain RE35F/12^T occupies a distinct phylogenetic position; this is similar to the case of *Vibrio hollisae*, because RE35F/12^T represents a relatively long subline of descent sharing a branching point with the outskirts species *V. hollisae*. The G+C content of the DNA was 49.5 mol%. Ubiquinone Q-8 was the main respiratory lipoquinone, and 16:1ω9cis, 16:0 and 18:1trans9, cis11 were the major cellular fatty acids, 16:1ω9cis being predominant. The polyamine pattern was characterized by the presence of the triamine sym-norspermidine. On the basis of the polyphasic information summarized above, a new *Vibrio* species is described for which the name *Vibrio calviensis* sp. nov. is proposed.

Keywords: 0.2 µm-filterable marine bacteria, starvation, polyphasic taxonomy, *Vibrio calviensis*

There is evidence that a significant part of the bacterial community in sea water could pass through membrane filters with a pore size of 0.45 µm, and even 0.2 µm. They represent a high proportion of the biomass in the oceans and are major agents in the biogeochemical cycling of elements (Cho & Azam, 1988, 1990; Kirchmann *et al.*, 1991). Filterable bacteria which respond to the addition of nutrients by increasing their size and undergoing cell division have been isolated from surface-marine waters as well as from deep-ocean water (Torella & Morita, 1981; Tabor *et al.*, 1981; MacDonell & Hood, 1982; Hood & McDonnell, 1987; Vybiral *et al.*, 1999). Some marine bacteria exhibit a nutrient-regulated life-cycle in which ultramicrocells develop as a result of exogenous nutrient limitation. Physiological and molecular studies of the effects of

starvation-induced differentiation programme on a number of different marine bacterial isolates revealed that this life-cycle includes pronounced morphological variations during growth, starvation and recovery, some of which are strain specific [for a review, see Kjelleberg (1993) and Morita (1997)].

In a recent investigation, we isolated, by 0.2 µm-membrane filtration, several marine proteobacteria from sea-water samples collected in the Bay of Calvi, Corsica, France (Vybiral *et al.*, 1999). One of these filterable bacteria, designated as (type) strain RE35F/12^T, was recognized as a representative of the family *Vibrionaceae* but no taxonomic consequences were drawn from the conclusions. Since strain RE35F/12^T is of special ecophysiological interest because of its ability to produce starvation forms (Fig. 1) and to reduce its cell size down to a volume of 0.1 µm³, we completed the taxonomic data on this bacterium by

Abbreviation: O/129, 2,4-diamino-6,7-diisopropylpteridine.

Fig. 1. Small coccoid cells of strain RE35F/12^T after 54 h nutrient limitation in MMS starvation medium (at 4 °C) containing the following (g l⁻¹): 26.0 NaCl, 5.6 MgCl₂·6H₂O, 7.6 MgSO₄·7H₂O, 1.54 CaCl₂·2H₂O, 0.1 Na₂HPO₄ and 0.5 mg l⁻¹ FeSO₄. The sample was examined by phase-contrast microscopy using a Leitz DRMB microscope (Leica) equipped with a Wild MPS 48/52 photoautomat. Bar, 2 µm.

using analysis of its genomic DNA G + C composition, electron microscopic examination of its cytological features, and detailed phylogenetic analysis. Sequence searches of the EMBL database using the program FASTA (Pearson & Lipman, 1988) revealed that the sequence of RE35F/12^T (EMBL/GenBank/DDBJ accession no. AF118021; Vybiral *et al.*, 1999) was most similar to species of the genus *Vibrio* (results not shown). Subsequently, nucleotide sequences of all validly described *Vibrio* species were retrieved from the EMBL database and aligned with the sequence of RE35F/12^T by using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually, and nucleotide positions of ambiguous alignment and gaps were omitted from the analysis; therefore, a stretch of 1331 nt was compared. Pairwise evolutionary similarities and distances (Jukes & Cantor, 1969) were calculated by using the program DNADIST implemented in the PHYLIP software package (version 3.57c; Felsenstein, 1995). A phylogenetic tree was constructed by using the neighbor-joining method (Saitou & Nei, 1987). The stability of groupings was estimated by bootstrap analysis (1000 replicates) using the programs SEQBOOT and CONSENSE from the same software package. The data analysis revealed that strain RE35F/12^T is most closely related (94.3%) to *Vibrio scophthalmi* CECT 4638^T (Cerdà-Cuellar *et al.*, 1997). Similarities to the sequences of all other established *Vibrio* species ranged from 93.6% (with *Vibrio aestuarianus* ATCC 35048^T) to 90.7% (with *Vibrio rumoiensis* FERM P-14531^T). Subsequently, a subset

Fig. 2. Unrooted phylogenetic dendrogram of strain RE35F/12^T and a subset of *Vibrio* species derived from 16S rDNA sequence data, using the neighbour-joining method for calculation. Numbers at nodes indicate the levels of bootstrap support based on data for 1000 replicates; only values greater than 60% are shown. Bar, 1 nucleotide substitution per 100 nucleotide positions.

of 13 sequences of the most closely related *Vibrio* species was used in a final phylogenetic analysis (Fig. 2). Restriction of the dataset allowed us to use a larger stretch of 1420 nt for comparison. The 16S rRNA sequence divergence values and treeing analysis showed that strain RE35F/12^T occupies a distinct position; this is similar to the case of *Vibrio hollisae*. Because RE35F/12^T represents a relatively long sub-line of descent sharing a branching point with the outskirts species *Vibrio hollisae* ATCC 33564^T, whereas the nearest phylogenetic relative of strain RE35F/12^T corresponds to *Vibrio scopthalmi* CECT 4638^T, a sequence divergence of 5.7% shows that this affinity is not particularly close but is comparable to that reported among existing species of the genus *Vibrio* (Dorsch *et al.*, 1992; Kita-Tsukamoto *et al.*, 1993; Aznar *et al.*, 1994).

It is recognized in this study that there is a body of knowledge concerning the heterogeneity of the genus *Vibrio* as it is currently defined (Dorsch *et al.*, 1992; Kita-Tsukamoto *et al.*, 1993). It is also recognized that strain RE35F/12^T is phylogenetically isolated on a side-branch of the genus *Vibrio*. A revision of the genus *Vibrio* was beyond the scope of this study, but the results of our phylogenetic analyses indicate that the genus designation of RE35F/12^T may be changed in the future. However, the chemotaxonomic data on RE35F/12^T supported its classification within the genus *Vibrio*. First of all, the major cellular fatty acids of the bacterium are 16:1 ω 9c (30.7%), 16:0 (19.0%) and 18:1 ω 7c (16.5%) (Vybiral *et al.*, 1999). This signature is characteristic of Gram-negative bacteria, and a ratio of 16:1 (higher than 16:0) is also typical for the family *Vibrionaceae* (Bøe & Gjerde, 1980; Lambert *et al.*, 1983). Moreover, Urdaci *et al.* (1990) and Bertone *et al.* (1996) reported that 16:1, 16:0 and 18:1 were the major fatty acids in the *Vibrio* species they tested, 16:1 being predominant. Cyclopropane acid (17:0 cyclo), which occurs, for instance, in *Photobacterium* species (Lambert *et al.*, 1983), was not detected in strain RE35F/12^T. Secondly, the cellular polyamine pattern of RE35F/12^T was characterized by the presence of the triamine *sym*-norspermidine (Vybiral *et al.*, 1999), a compound commonly found in *Vibrio* species (Yamamoto *et al.*, 1983; Hamana, 1997). Finally, the G+C content of the DNA (49.5 mol%), as determined by using the standard HPLC method (Cashion *et al.*, 1977; Mesbah *et al.*, 1989; Tamaoka & Kaneko, 1984; Visuvanathan *et al.*, 1989), in this study was within the range for *Vibrio* species (38–51 mol%).

In addition to the chemotaxonomic information, several key phenotypic traits of strain RE35F/12^T were congruent with the definition of the genus *Vibrio* (Baumann *et al.*, 1984). Also, strain RE35F/12^T is facultatively anaerobic, oxidase-positive, Gram-negative, halophilic (optimum growth occurs at 2.5–3.5% NaCl) and is a straight to slightly curved, rod-shaped, motile bacterium. Electron microscopic examinations showed that the cells of RE35F/12^T each have a single

Fig. 3. Transmission electron micrograph of strain RE35F/12^T showing the single polar flagellum. Cells were grown for 24 h in Bacto marine broth 2216 (Difco) at room temperature (22 ± 3 °C) and were harvested by centrifugation of the sample directly onto a collodion-coated 400-mesh nickel grid (ATHENE SIRA, diameter 3.05 mm; Smethurst High-light). The grid had been coated with a layer of carbon before use. The sample was examined with a Zeiss EM 902 transmission electron microscope. Bar, 0.47 µm.

polar flagellum (Fig. 3). These characteristics, together with several other phenotypic traits common to most of the *Vibrio* species [e.g. acid but no gas from D-glucose under anaerobic conditions, growth on thio-sulphate/citrate/bile salts/sucrose medium (TCBS agar; Oxoid), susceptibility to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), hydrolysis of gelatin, as well as the utilization of mannitol] define strain RE35F/12^T as a halophilic species of the genus *Vibrio*. A unique feature of RE35F/12^T was its ability to grow at concentrations of NaCl above 10%, which was only observed for a limited number of *Vibrio* species. Phenotypic characteristics distinguishing strain RE35F/12^T from other known halophilic *Vibrio* species able to grow at/or above 10% NaCl are summarized in Table 1. Strain RE35F/12^T and the phylogenetically closest species *V. scopthalmi* differ phenotypically in several respects. In contrast to *V. scopthalmi*, strain RE35F/12^T grows at NaCl concentrations up to 12%, does not possess an arginine dihydrolase, utilizes citrate and mannitol, hydrolyses

Table 1. Phenotypic characteristics distinguishing RE35F/12^T from other *Vibrio* species able to grow at/or above 10% NaCl

Data for strain RE35F/12^T were taken from Vybiral *et al.* (1999); data for other vibrios were compiled from Alsina & Blanch (1994), Baumann *et al.* (1984), Farmer & Hickman-Brenner (1991) and Pujalte *et al.* (1993). +, Positive for $\geq 90\%$; (+) positive for 75–89%; –, negative for $\geq 10\%$; (–), negative for 25–11%; v, variable for 26–74%; ND, no data. Percentages indicate positive results. 1, RE35F/12^T; 2, *Vibrio alginolyticus*; 3, *Vibrio cincinnatiensis*; 4, *Vibrio diazotrophicus*; 5, *Vibrio fluvialis*; 6, *Vibrio furnissii*; 7, *Vibrio gazogenes*; 8, *Vibrio hollisae*; 9, *Vibrio metschnikovi*; 10, *Vibrio mytili*; 11, *Vibrio nereis*; 12, *Vibrio proteolyticus*.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Oxidase	+	+	+	+	+	+	–	+	–	+	+	+
Gas from D-glucose	–	–	–	–	–	+	+	–	–	+	–	–
Voges–Proskauer reaction	–	+	–	–	–	–	–	–	+	–	–	+
ONPG	+	–	+	+	(+)	+	ND	–	(+)	ND	–	–
Nitrate reduction	+	+	+	+	+	+	–	+	–	+	+	+
Indole production	–	+	–	–	+	(+)	+	+	+	–	v	+
Utilization of:												
Citrate	+	+	+	+	+	+	+	–	v	v	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	–	+
Hydrolysis of starch	–	+	ND	+	+	+	+	ND	+	–	–	+
Liquefaction of gelatin	+	+	–	–	+	(+)	+	–	+	–	v	(+)
Susceptibility to 10 µg O/129*	+	+	+	v	+	+	–	ND	–	ND	(–)	+
Growth in the presence of:												
10% NaCl	+	+	v	v	(+)	+	+	(–)	v	+	(–)	+
12% NaCl	+	+	–	–	–	(–)	v	–	–	–	–	+
Growth at 40 °C	–	+	ND	v	+	+	+	ND	+	–	v	+

* O/129, Vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine.

gelatin, and is able to grow at 4 °C but not at 37 °C. On the basis of both phylogenetic and phenotypic evidence, it is proposed that strain RE35F/12^T be classified as a new species of the genus *Vibrio*.

Description of *Vibrio calviensis* sp. nov.

Vibrio calviensis (cal.vi.en'sis. N.L. gen. n. *calviensis* of or pertaining to the Bay of Calvi, Corsica, from where the type strain was isolated).

The description is based on data compiled from Vybiral *et al.* (1999) and data generated in this study. The cells are straight to slightly curved and rod-shaped (0.25–1.0 × 0.75–2.5 µm), Gram-negative and motile by means of a single polar flagellum. Colonies on Bacto marine agar 2216 (Difco) as well as on sea-water agar are brownish, translucent, circular, smooth and convex with an entire edge. Bioluminescence was not observed. Growth occurs at room temperature, at 30 °C and within 10 d at 4 °C. No growth occurs at 37 °C. Grows on TCBS agar, producing green colonies. Sodium ions are essential for growth. Growth occurs between 1.5 and 12% NaCl. The optimal NaCl concentration for growth is 2.5–3.5% (w/v); there is no growth at 1.0% NaCl or when NaCl is absent. Susceptible to ampicillin, chloramphenicol, colistin sulphate, fusidic acid, kanamycin, penicillin G, polymyxin B, streptomycin, tetracycline and vibriostatic agent O/129. Resistant to lincomycin, oxacillin and

spectinomycin. Oxidase and catalase tests give positive results. Facultatively anaerobic. Acid but no gas is produced from glucose. Nitrate is reduced to nitrite, but not further to N₂. The Voges–Proskauer reaction is negative and the methyl red test is positive. Gelatin and Tween 80 are hydrolysed but casein, starch and agar are not. The following substrates are utilized as sole carbon and energy sources: β-gentiobiose, cell-lobiose, citrate, ribose, galactose, gluconate, mannitol, melibiose, maltose, D-mannose, D-turanose and saccharose. The following substrates are not utilized as sole carbon and energy sources: adipate, amidon, arbutine, L-arabinose, caprate, D-fructose, D-glucose, glycogen, inulin, methyl α-D-mannoside, N-acetylglucosamine, trehalose, D- and L-xylose. Alkaline phosphatase, acidic phosphatase, esterase (C₄), esterase (C₈), β-galactosidase, lipase (C₁₄), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and urease are produced. Arginine dihydrolase, α-chymotrypsin, cystine arylamidase, α-galactosidase, α-glucosidase, α-mannosidase, trypsin and valine arylamidase are not produced. The major cellular fatty acids are 16:1 *cis*, 16:0 and 18:1 *trans*9, *cis*11; in addition, hydroxylated fatty acids 12:0 3-OH and 14:0 3-OH are present in minor amounts. The cellular polyamine content is characterized by the presence of putrescine, spermidine, cadaverine, 1,3-diaminopropane and *sym*-norspermidine. The G+C content of the DNA is 49.5 mol%. The type strain is RE35F/12^T (= CIP

107077^T = DSM 14347^T); it was isolated from sea water by 0.2 µm membrane filtration.

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