NOTE

***Vibrio agarivorans* sp. nov., a novel agarolytic marine bacterium**

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It is proposed that the new *Vibrio* species *Vibrio agarivorans* accommodates two agarolytic, halophilic, fermentative bacterial strains isolated from Mediterranean sea water. The cells were Gram-negative, oxidase-positive, polarly flagellated bacilli that fermented glucose without gas production and that produced no decarboxylases. They used a wide range of compounds as sole carbon and energy sources. The DNA G+C content was 44.8 mol%. Phylogenetic analysis based on complete 16S and 23S rDNA sequences revealed that the strains belong to the γ -Proteobacteria, and are specifically related to *Vibrio* species. Their nearest relatives were species of the *Vibrio fischeri* group, sharing 16S rDNA sequence similarities below 97% with the agarolytic strains. The type strain is 289^T (= CECT 5085^T = DSM 13756^T).

Keywords: γ -Proteobacteria, marine bacteria, *Vibrio agarivorans* sp. nov., agarolytic activity, 16S rDNA phylogeny

The ability to hydrolyse agar, a complex polysaccharide commonly used as a solidifying agent in microbiological media, is widespread among marine bacteria. This ability is very apparent, as colonies of agarolytic bacteria grown on solid media form a pit, more or less pronounced, which sometimes progresses to a big hole. Pigmented members of the *Bacteroides*–*Flavobacterium*–*Cytophaga* branch (*Cytophaga lytica*, *Cytophaga fermentans*, *Cytophaga latercula*, *Cytophaga uliginosa*, *Microscilla furvensens*, *Marinilabilia agarovorans*, *Persicobacter diffuens* and *Flameovirga aprica*) and strictly aerobic proteobacteria of the genera *Prosthecomicrobium* (*Prosthecomicrobium litorale*), *Pseudoalteromonas* (*Pseudoalteromonas atlantica*) and *Pseudomonas* (*Pseudomonas elongata*, *Pseudomonas gelidicola*, both *incertae sedis*) account for all of the currently described species showing this activity (Akawaga–Matsushita *et al.*, 1992; Bauld *et al.*, 1983; Nakawaga & Yamasato, 1996; Nakawaga *et al.*, 1997; Palleroni, 1984; Reichenbach, 1989). Recently, a thermophilic, facultatively anaerobic Gram-negative coccus, *Alerococcus agarolyticus*, has been described as an agarase producer (Shieh & Jean, 1998).

During an annual survey of *Vibrio* species from marine water and bivalves on the Spanish Mediterranean coast (Arias *et al.*, 1999), the occurrence of several large, agarolytic colonies on thiosulphate–citrate–bile–sucrose (TCBS) agar plates was noted. The strains recovered from these colonies behaved as typical *Vibrio* species: they showed good growth on this selective medium, they were Gram-negative rods with the typical motility of polarly flagellated cells, they required NaCl for growth, they were fermentative in the oxidation/fermentation test, and they were oxidase-positive and produced unpigmented, agarolytic colonies on marine agar. The only reference, in the literature, to agarolytic vibrios within the genus *Vibrio sensu stricto*, is strain Bar, an unnamed vibrio isolated and characterized along with the strains used to describe *Vibrio diazotrophicus* (Guerinot & Patriquin, 1981; Guerinot *et al.*, 1982). The strain was included in at least two numerical taxonomic studies and could not be placed in any of the defined groups (West *et al.*, 1985, 1986). To the best of our knowledge, this strain has not been deposited in any culture collection. Furthermore, none of the currently described *Vibrio* or *Photobacterium* spp. are recognized as agarolytic. We have investigated the taxonomic position of two of our agarolytic strains by phenotypic and phylogenetic characterization and we have concluded that they constitute a new species within the genus *Vibrio*, for which we propose the name *Vibrio*

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA sequences of *Vibrio agarivorans* CECT 5085^T and CECT 5084 are AJ310647 and AJ310648, respectively. The accession numbers for the respective 23S rRNA sequences are AJ310649 and AJ310650.

Fig. 1. Phylogenetic tree derived from maximum-parsimony analysis of the 16S rRNA gene sequences of *Vibrio agarivorans* strains CECT 5085^T (= DSM 13756^T) and CECT 5084 and other related species of the family *Vibrionaceae*. Bar, 5% estimated sequence divergence. Numbers near to the specific names correspond to the strain numbers and the accession numbers for the 16S rDNA gene sequences.

agarivorans, and strain 289^T (= CECT 5085^T = DSM 13756^T) as the type strain.

Two agarolytic sea-water strains, 289^T (CECT 5085^T = DSM 13756^T) and 351 (CECT 5084), previously recovered during an annual study of the Spanish Mediterranean coast near Valencia, were isolated from TCBS agar (Oxoid) plates and repeatedly streaked on marine agar 2216 (Difco) plates until pure cultures were obtained. They were maintained on semi-solid marine agar stabs [marine broth 2216 (Difco) plus 0.2% agar] at room temperature and as suspensions in marine broth + 10% glycerol at -80 °C. They were routinely grown at 25 °C, and commercial media (except for marine agar and marine broth) were supplemented with NaCl up to a concentration of 2% (w/v). The phenotypic profiles of both strains were

determined. Most of the phenotypic tests used have been described previously (Macián *et al.*, 2001). The nutritional spectrum of sole-carbon-source utilization was analysed in liquid media, by using Biotype^R strips (bioMérieux) that allow the testing of 99 sole carbon sources and include a negative control. The strips were inoculated with 400 µl per tube from a dilution of 2 ml cell suspension (3.0 MacFarland scale) in 60 ml basal medium (Baumann & Baumann, 1981). Results were recorded after 2, 4 and 6 d incubation at 25 °C. Additional carbon sources not included on the Biotype^R strips were tested on basal medium supplemented with 0.1% (L-arginine, L-glutamate, pyruvate) or 0.2% (salicin) of the compound.

The DNA G+C content was determined from the midpoint value of the thermal denaturation profile

Fig. 2. Phylogenetic tree derived from distance-matrix analysis of the 23S rRNA gene sequences of *V. agarivorans* strains CECT 5085^T (= DSM 13756^T) and CECT 5084 and other related species of the genera *Vibrio*. Bar, 5% estimated sequence divergence. Numbers near to the specific names correspond to the strain numbers and the accession numbers for the 23S rRNA gene sequences.

obtained with a Gilford 2600 spectrophotometer (Mandel & Marmur, 1968). The G+C content was calculated by using the equation of De Ley (1970) and with *Escherichia coli* ATCC 11775^T DNA as the reference (G+C content 51 mol%).

Isolation of genomic DNA, amplification of almost full-length 16S and 23S rRNA gene fragments, and sequencing of rDNA using a LICOR automated sequencer (MWG Biotech) were performed (Macián *et al.*, 2001).

Sequences were added to the 16S and 23S rRNA sequence databases of the Technical University of Munich using the program package ARB (Ludwig & Strunk, 1997). The respective ARB tools were used for automated sequence alignment. The alignment was checked by eye and corrected manually using the sequence editor ARB_EDIT. Phylogenetic analyses were performed by applying the maximum-parsimony (with the full dataset of 20000 sequences, ARB, parsimony), distance-matrix (with all available members of the γ -*Proteobacteria* as well as selected references from other major phylogenetic groups, ARB; Felsenstein, 1982) and maximum-likelihood methods (with known selected references from the γ -*Proteobacteria*, fastDNAm1; Maidak *et al.*, 1996) on different datasets varying with respect to the inclusion of variable sequence positions (Ludwig *et al.*, 1998). The accession numbers for the 16S and 23S rRNA gene sequences used in the study appear in Figs 1 and 2, respectively.

The agarolytic Mediterranean sea-water bacteria are Gram-negative rods (approx. 2–4 μ m long $\times$ 0.4–0.6 μ m wide); each rod has one polar flagellum. They grew on marine agar plates as unpigmented, non-luminous colonies that produced a shallow pit on the medium (agarolytic), and they did not swarm. They

developed as agarolytic green colonies on TCBS agar (i.e. they were non-sucrose-fermenting). None of them was able to grow at 4 or 40 $^{\circ}$ C, and both grew in the range 20–37 $^{\circ}$ C. They were strictly halophilic, unable to grow in NaCl-free medium, and able to grow at NaCl concentrations of up to 6%. They were fermentative in the oxidation/fermentation test, fermenting glucose without gas production, and gave a negative Voges–Proskauer test result. Both strains reduced nitrate to nitrite and were sensitive to the vibriostatic agent O/129 (150 μ g). Test results for Møller's and Thornley's arginine dihydrolase, lysine and ornithine decarboxylases, indole and H₂S production were negative. The bacteria did not hydrolyse urea, gelatin, casein or starch. The nutritional profiles of both agarolytic strains [289^T (= CECT 5085^T = DSM 13756^T) and 351 (= CECT 5084)] were almost identical; a wide range of compounds, mainly sugars and organic acids, was utilized (see description). The strains were phenotypically different from other decarboxylase-negative *Vibrio* species not only because of the agarolytic ability but also on the basis of carbon-source use and the results of other biochemical tests (Table 1). Therefore, we sequenced their 16S and 23S rRNA genes in order to clarify their phylogenetic affiliations.

The DNA G+C content of strain 289^T (= CECT 5085^T = DSM 13756^T) was 44.8 mol%, as determined by the thermal denaturation method, and thus was within the range for the genus *Vibrio*.

The almost complete sequences of the 16S rRNA and 23S rRNA genes were determined for strains 289^T (= CECT 5085^T = DSM 13756^T) and 351 (= CECT 5084) and were deposited in the EBI databases under the accession numbers AJ310647 and AJ310648, respectively. Phylogenetic analysis was performed by applying the three alternative treeing methods, and the results were congruent with respect to the positioning of the new isolates. The comparative analysis of the sequences confirmed the affiliation of the strains to the family *Vibrionaceae*. The sequences of both strains were identical, and showed similarity of less than 97% to other *Vibrio* and *Photobacterium* species. The closest phylogenetic neighbours of our isolates were *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wadonis*. The 16S rDNA sequence from *V. fischeri* was the most similar, having 96.5% identical bases. *V. logei* and *V. salmonicida* showed 95.2 and 94.8% similarity, respectively, whereas the *V. wadonis* 16S rDNA sequence similarity was 94.6%. According to the criteria of Stackebrandt & Goebel (1994), these 16S sequence similarities indicate that the strains represent a different species. Fig. 1 is a phylogenetic tree for *Vibrionaceae*, based on the maximum-parsimony method, which includes the 16S rDNA sequence data for the two strains. The phylogenetic position of these representatives is peripheral to the *V. fischeri*–*V. logei*–*V. salmonicida*–*V. wadonis* group. In several studies (Kita-Tsukamoto *et al.*, 1993; Lunder *et al.*, 2000; Ruimy *et al.*, 1994; Sawabe *et al.*, 1998; Shieh *et*

Table 1. Characteristics useful for distinguishing *Vibrio agarivorans* sp. nov. from its closest phylogenetic neighbours and other amino-acid-negative *Vibrio* spp.

The data shown are from the following references: Baumann *et al.* (1984), Benediktsdóttir *et al.* (2000), Borrego *et al.* (1996), Fidopiastis *et al.* (1999), Guerinot & Patriquin (1981), Guerinot *et al.* (1982), Ishimaru *et al.* (1995, 1996), Macián *et al.* (2001) and Sawabe *et al.* (1998). The numbers represent the following species/strains of the genus *Vibrio*: 1, *V. agarivorans*; 2, *V. diazotrophicus*; 3, strain Bar; 4, *V. fischeri*; 5, *V. logei*; 6, *V. salmonicida*; 7, *V. wodanis*; 8, *V. natrieensis*; 9, *V. pelagius* biotype 1; 10, *V. pelagius* biotype 2; 11, *V. ordalii*; 12, *V. gazogenes*; 13, *V. ichtyoenteri*; 14, *V. splendidus* biotype 2; 15, *V. nigripulchritudo*; 16, *V. penaeicida*; 17, *V. navarrensis*; 18, *V. tapetis*; 19, *V. halioticoli*; 20, *V. lentus*. Test results: +, positive; –, negative; v, variable response; ND, no data available.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Luminescence	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pigment	–	–	–	Yellow	Yellow	–	Yellow	–	–	–	–	Red	–	–	Black	–	–	–	–	–
Thornley's arginine dihydrolase	–	v	–	–	–	–	ND	–	–	–	–	–	–	–	–	ND	–	–	–	+
Lysine decarboxylase	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Indole	–	+	–	–	–	–	v	–	–	+	–	–	–	+	+	v	+	+	+	v
Acid from sucrose	–	+	+	–	–	–	v	+	+	+	+	+	v	+	–	–	+	–	–	–
Growth at 4 °C	–	+	+	–	+	+	+	+	v	v	–	–	–	–	–	–	–	+	–	v
Growth at 40 °C	–	v	+	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	–	–
Hydrolysis of																				
Agar	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch	v	+	+	–	–	ND	+	v	–	–	–	+	–	+	+	v	+	+	–	+
Use of:																				
D-Ribose	–	+	ND	+	v	v	ND	+	+	+	+	+	ND	+	–	ND	+	ND	ND	v
D-Xylose	+	+	ND	–	–	–	ND	–	–	–	–	+	–	–	–	–	–	ND	+	–
L-Arabinose	–	+	ND	–	–	–	ND	+	–	–	–	+	ND	–	–	ND	–	–	–	–
Mannose	–	–	+	+	v	v	+	v	v	+	–	+	ND	–	+	ND	ND	+	v	+
Cellobiose	+	+	+	+	+	–	ND	v	–	–	–	+	–	v	+	+	v	–	v	+
Sucrose	–	+	+	–	–	–	ND	+	+	–	+	+	v	–	–	–	+	–	–	–
Lactose	+	+	+	–	–	–	ND	–	v	v	–	–	ND	–	+	ND	–	–	–	–
Melibiose	+	–	ND	–	–	–	ND	v	–	–	–	ND	ND	–	+	ND	–	–	–	–
D-Gluconate	v	+	ND	–	+	+	ND	+	+	+	–	–	v	–	v	v	+	–	–	v
D-Glucuronate	–	+	ND	–	–	ND	ND	v	–	–	–	–	–	+	–	–	–	–	–	v
Sorbitol	–	–	–	–	–	–	ND	–	–	–	–	+	ND	–	+	ND	–	–	–	–
2-Ketoglutarate	–	+	ND	–	–	v	ND	+	–	–	–	+	ND	+	+	ND	v	–	–	v
Putrescine	–	+	ND	–	–	ND	ND	+	+	+	–	–	–	–	–	–	–	–	–	–
L-Arginine	–	+	+	–	–	ND	ND	+	+	+	ND	–	ND	–	–	ND	ND	ND	ND	–

* From Fidopiastis *et al.* (1999).

al., 2000; Yumoto *et al.*, 1999), this group remains clearly isolated from the groups containing the *Photobacterium* spp., the *Vibrio anguillarum*–*Vibrio ordalii*–*Vibrio diazotrophicus*–*Vibrio vulnificus*–*Vibrio navarrensis* clade, and the so-called 'core' of marine *Vibrio* species (*Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio campbellii*, *Vibrio natrieensis*).

The almost complete sequence of the 23S rRNA gene was also determined for both strains; these sequences were deposited at the EBI databases under the accession numbers AJ310649 and AJ310650. The distance-matrix analysis yielded the dendrogram shown in Fig. 2. The currently available number of complete 23S rDNA sequences of *Vibrio* spp. (seven out of 44 validly described species) is still very low. The 23S rDNA sequences of *Vibrio lentus*, *Vibrio mediterranei*, *V. navarrensis*, *Vibrio pelagius* and *Vibrio splendidus* were determined by our group in a previous study (Macián *et al.*, 2001). The relevance of using 23S rDNA sequences lies in the higher number of informative positions they possess because of the larger length of the molecule. Moreover, 23S rRNA sequences, as well as 16S rRNA sequences, can be used as evolutionary markers, and confirm the results of the

phylogenetic studies based on the small-subunit RNA. In the present study, the phylogenetic organization of the available *Vibrio* spp. 23S rDNA sequences (Fig. 2) is congruent with the one based on 16S rDNA sequences (Fig. 1). The value for 23S rDNA sequence similarity between strains 289^T (= CECT 5085^T = DSM 13756^T) and 351 (= CECT 5084) was 99.6%, whereas the levels of similarity between the sequences of these two strains and those of other *Vibrio* species varied between 92.9 and 96.8% (with *V. navarrensis* and *V. mediterranei*, respectively).

Strain Bar (isolated from sea urchin; Guerinot & Patriquin, 1981), the only strain within the genus *Vibrio* described as agarolytic, was not available in any culture collection and therefore could not be included in the sequence study. This strain was reported to be able to grow at 4 and 42 °C as well as on L-arginine and salicin (but not succinate) as sole carbon sources, whereas the responses of our agarolytic sea-water strains for these five tests were just the opposite. Phenotypic characterization showed that strains 289^T (= CECT 5085^T = DSM 13756^T) and 351 (= CECT 5084) share properties common in members of the genus *Vibrio*, according to Baumann *et al.* (1984), but at the same time they can be easily differentiated from

other decarboxylase-negative or phylogenetically related *Vibrio* species (Table 1).

On the basis of phenotypic characterization and rRNA sequence analysis it can be concluded that the two agarolytic strains studied are different from any *Vibrio* and *Photobacterium* species described so far. Although there is a high probability that both strains do pertain to the same species, in view of the nearly identical phenotype and 16S and 23S rRNA sequences, further DNA–DNA reassociation experiments will definitively confirm the ascription of strain 351 (= CECT 5084) to the proposed new species, according to the criteria established for genospecies recognition (Wayne *et al.*, 1987). The name *V. agarivorans* sp. nov. is proposed for this species, and strain 289^T (= CECT 5085^T = DSM 13756^T) is proposed as the type strain.

Further studies involving genetic and phylogenetic characterization of the rest of agarolytic strains recovered in the original study are in progress (Arias *et al.*, 1999).

Description of *Vibrio agarivorans* sp. nov.

Vibrio agarivorans (a.ga.ri.vo'rans. Malayan n. agar agar; N.L. n. *agarum*, -i agar; M.L. v. *vorare* to devour; N.L. part. adj. *agarivorans* agar-eating).

Gram-negative rods, approximately 2–4 µm long × 0.4–0.6 µm wide, facultatively anaerobic, motile by one polar flagellum. Colonies are regular in shape and unpigmented. On TCBS agar, they grow as green colonies. Glucose metabolism is fermentative without gas production. Tests for catalase, oxidase and nitrate reduction to nitrite are positive. No growth occurs without the addition of NaCl to the culture medium. Growth occurs between 20 and 37 °C. Not luminescent. Indole, H₂S production from thiosulphate, Møller's and Thornley's arginine dihydrolase, and ornithine and lysine decarboxylase test results are negative. Hydrolyses agar. Utilizes the following substrates as sole carbon and energy sources: D-glucose, D-fructose, D-galactose, D-melibiose, maltotriose, maltose, α-lactose, 1-O-methyl β-galactopyranoside, 1-O-methyl α-galactopyranoside, D-cellobiose, D-xylose, D-mannitol, mucate, L-malate, 2-keto-D-gluconate, N-acetylglucosamine, caprate, succinate, fumarate, DL-glycerate, D-glucosamine, L-glutamate, L-serine and L-aspartate. None of the strains utilizes the following substrates: D-trehalose, D-mannose, L-sorbose, sucrose, D-raffinose, lactulose, β-gentibiose, 1-O-methyl β-D-glucopyranoside, D-ribose, L-arabinose, palatinose, L-rhamnose, L-fucose, D-melezitose, D-arabitol, L-arabitol, xylitol, dulcitol, D-tagatose, glycerol, myo-inositol, maltitol, D-turanose, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, D-lyxose, i-erythritol, 1-O-methyl α-D-glucopyranoside, 3-O-methyl D-glucopyranose, D-saccharate, L-tartrate, D-tartrate, m-tartrate, D-malate, *cis*-aconitate, *trans*-aconitate, tricarballoylate, citrate, D-glucuronate, D-galacturonate, 5-keto-D-gluconate, L-tryptophan,

phenylacetate, protocatechuate, *p*-hydroxybenzoate, quinate, gentisate, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, betain, putrescine, DL-α-aminobutyrate, histamine, DL-lactate, caprylate, L-histidine, glutarate, DL-α-aminovalerate, ethanolamine, tryptamine, itaconate, DL-β-hydroxybutyrate, D-alanine, malonate, 2-oxoglutarate, L-arginine and salicin. Isolated from sea water at the Mediterranean coast of Valencia (Spain). Type strain 289^T has been deposited at the Colección Española de Cultivos Tipo (Valencia, Spain) as strain CECT 5085^T, and at the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 13756^T. The G+C content of DNA of the type strain is 44.8 mol %. Strain 351 has been also deposited at both collections as CECT 5084 and DSM 13755, respectively.

Characteristics of the type strain of *Vibrio agarivorans* sp. nov. The type strain of *V. agarivorans* is 289^T, isolated in Spain from Mediterranean sea water. In addition to the characteristics that define the species, it has the following features: it utilizes aesculin and L-proline as sole carbon and energy sources, but not D-gluconate, L-alanine, L-tyrosine or pyruvate.

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