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Vibrio ruber sp. nov., a red, facultatively anaerobic, marine bacterium isolated from sea water

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A red, heterotrophic, marine bacterium, designated strain VR1^T, was isolated from a sea-water sample collected in the shallow coastal region of Keelung, Taiwan. Cells of the novel strain were facultatively anaerobic, Gram-negative rods that were motile by means of a polar flagellum. The strain grew optimally at 25–30 °C and pH 6–7. Growth required the presence of NaCl, the optimal concentration being about 2 %. The red pigment produced by the cells was identified as prodigiosin. Strain VR1^T grew anaerobically by fermenting glucose and other carbohydrates and producing acids and gases. The strain did not require either vitamins or other organic growth factors for growth. It contained 2-OH-16:0 and 3-OH-14:0 as the major cellular fatty acids. The DNA G + C content was 45.8 mol%. Phenotypic and chemotaxonomic characterization indicated that strain VR1^T represents a novel species in the genus *Vibrio*. Strain VR1^T is phenotypically similar to *Vibrio gazogenes*. However, the reduction of nitrate to nitrite, the ability to utilize D-arabinose, melibiose and L-glycine as sole carbon sources, the inability to utilize sorbitol as a sole carbon source, resistance to O/129 and susceptibility to erythromycin and novobiocin allow differentiation between *V. gazogenes* and strain VR1^T. The name *Vibrio ruber* sp. nov. is proposed for the novel species, with strain VR1^T (=CCRC 17186^T =JCM 11486^T) as the type strain.

Marine, halophilic, facultatively anaerobic, Gram-negative, rod-shaped bacteria are currently placed in the genera *Vibrio* (Baumann *et al.*, 1984), *Photobacterium* (Baumann & Baumann, 1984), *Listonella* (MacDonell & Colwell, 1985) and *Moritella* (Urakawa *et al.*, 1998) of the family *Vibrionaceae*. They are ubiquitous in estuarine, coastal and oceanic waters and in marine sediments (Huq & Colwell, 1995; Simidu & Tsukamoto, 1985; Simidu *et al.*, 1982). They are closely associated with many kinds of marine organisms, from plankton to fish (Cerdà-Cuéllar *et al.*, 1997; MacDonald *et al.*, 1986; Nair *et al.*, 1988; Onarheim *et al.*, 1994; Simidu *et al.*, 1969, 1971; Sochard *et al.*, 1979). Some species are found as symbionts in specialized luminous organs of marine fish and invertebrates (Lee & Ruby, 1994; Leisman *et al.*, 1980; Reichelt *et al.*, 1977; Ruby & Asato, 1993; Ruby & Morin, 1978), whereas many other species are pathogens of humans or marine animals (Blake *et al.*, 1980; Borrego *et al.*, 1996; Egidius *et al.*, 1986; Hada *et al.*, 1984; Ishimaru *et al.*, 1996; Schiewe *et al.*, 1981). These halophilic, facultatively anaerobic, Gram-negative rods are known to include both nitrogen-fixers and denitrifiers

(Guerinot & Colwell, 1985; Guerinot *et al.*, 1982; Shieh & Lin, 1992, 1994; Shieh & Liu, 1996; Shieh & Yang, 1997; Shieh *et al.*, 1989), though the definitions given in *Bergey's Manual of Systematic Bacteriology* in 1984 indicated that these bacteria include neither nitrogen-fixers nor denitrifiers (Baumann & Baumann, 1984; Baumann & Schubert, 1984; Baumann *et al.*, 1984).

Vibrio aerogenes, a species capable of fermenting glucose and other carbohydrates, with the production of gas, was described in our recent report (Shieh *et al.*, 2000). Using an anaerobic enrichment culture method similar to that used in our earlier report, we have isolated more than 40 strains of gas-producing bacteria, belonging to the family *Vibrionaceae*, from sea-water samples collected in the shallow coastal regions of Keelung, Taiwan. Strain VR1^T was the only red bacterium among these isolates. Other strains isolated produced white to off-white colonies on agar plates. Strain VR1^T is of interest because the production of red pigment is a property found among only a few species of marine bacteria, such as *Pseudoalteromonas rubra* (Gauthier, 1976; Gauthier *et al.*, 1995) and *Vibrio gazogenes* (Baumann *et al.*, 1984; Harwood, 1978). Phenotypic and chemotaxonomic characterization data obtained in this study indicate that the red strain constitutes a novel species in the genus *Vibrio*, for which we propose the name *Vibrio ruber* sp. nov.

Bacterial cultures were maintained on peptone/yeast extract (PY) slabs and were cultivated in PY broth, peptone/yeast

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Abbreviation: MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

The GenBank accession number for the 16S rDNA sequence of *Vibrio ruber* VR1^T is AF462458.

extract/glucose (PYG) broth or glucose/mineral (GM) medium for growth and other studies. PY broth contained the following ingredients (l^{-1} deionized water): 6 g Bacto peptone (Difco), 2 g Bacto yeast extract (Difco), 25 g NaCl, 3 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $CaCl_2$. The medium was adjusted to pH 7.0. Bacto agar (Difco) was added to this medium at 5 and 15 g l^{-1} for the preparation of stab and plate media, respectively. PYG broth was prepared in two parts. The first contained 6 g Bacto peptone, 2 g Bacto yeast extract, 25 g NaCl, 3 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $CaCl_2$ dissolved in 900 ml deionized water and was adjusted to pH 7.0. The second contained 5 g glucose dissolved in 100 ml deionized water. The two parts were autoclaved separately and mixed at room temperature. GM medium was also made up in two parts. Part 1 contained 0.54 g NH_4Cl , 25 g NaCl, 2 g $MgCl_2 \cdot 6H_2O$, 3 g K_2SO_4 , 0.2 g K_2HPO_4 , 0.01 g $CaCl_2$, 0.005 g $FeCl_3 \cdot 6H_2O$ and 5.4 g (approx. 25 mmol) MOPSO (Sigma) dissolved in 900 ml deionized water and adjusted to pH 7.0, while part 2 contained 5 g glucose dissolved in 100 ml deionized water. The two parts were also autoclaved separately and mixed at room temperature. Cultures were routinely grown at 25 °C in the dark unless otherwise stated. Growth studies were performed essentially as described in our previous report (Shieh *et al.*, 2000).

Phenotypic characteristics of strain VR1^T were determined according to methods described in our previous report (Shieh *et al.*, 2000). Susceptibility of strains VR1^T and *V. gazogenes* ATCC 29988^T to the following antibiotics was tested (amount per disc in parentheses): ampicillin (10 µg), carbenicillin (100 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), colistin (10 µg), erythromycin (15 µg), kanamycin (30 µg), lincomycin (5 µg), nalidixic acid (30 µg), neomycin (30 µg), novobiocin (30 µg), oxacillin (1 µg), penicillin G (10 U), polymyxin B (300 U), streptomycin (10 µg) and vancomycin (30 µg). The tests were done by spreading broth cultures (0.1 ml) of the strains on PY agar plates and then placing standard 6 mm antibiotics discs (Difco) on the plates. Growth-inhibition zones around the discs were noted after incubation of the plates aerobically at 25 °C for 24–30 h.

The red pigments produced by strains VR1^T and *V. gazogenes* ATCC 29988^T, grown aerobically in PYG broth at 25 °C, were extracted with acetone/methanol (7:2). The clear supernatant was taken after centrifugation and a Shimadzu UV-160A spectrophotometer was used to determine the absorption spectrum from 300 to 800 nm. For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS; Welham *et al.*, 1998), 0.5 µl of a 1:2 mixture (v/v) of the supernatant and α -cyano-4-hydroxycinnamic acid was dropped on the mass spectrometer sample probe and dried under ambient conditions. The MALDI-TOFMS analyses were performed on an HP G2025A mass spectrometer (Hewlett-Packard) using 337 nm radiation from a nitrogen laser. The mass spectrometer was operated in linear mode at an accelerating voltage

of 30 kV, giving an ion flight path of 1.7 m. The laser-beam energy was set within a range between 0.80 and 0.85 µJ.

Fatty acids of cells grown aerobically in PYG medium at 25 °C were extracted, saponified and esterified according to the method of Suutari *et al.* (1990). GLC analysis of the fatty acid methyl esters was performed on a gas chromatograph (GC-14A; Shimadzu) equipped with a flame ionization detector and a fused silica capillary column (Shieh & Jean, 1998).

DNA base composition was determined as described previously by Shieh & Liu (1996).

Total genomic DNA was extracted from bacterial cells by using a Puregene DNA purification kit (Gentra Systems) following the instructions of the manufacturer. Two primer pairs were used for the PCR amplification: 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-CGGTTACCT-TGTTAGGACTTCACC-3' (positions 8–27 and 1488–1511, *Escherichia coli* numbering system) (Bennasar *et al.*, 1998) and 5'-GGCAGGCCTAACACATGCAAGT-3' and 5'-GGGCTACCTTGTTACGACTTCACC-3' (positions 41–62 and 1488–1511). The latter primer pair was designed by us according to the 16S rDNA sequences of species in the family *Vibrionaceae* that are available in the GenBank database. The PCR products were checked for size and purity on agarose gels and the DNA fragments were then purified and recovered with a QIAquick PCR purification kit (Qiagen). Sequencing of the 16S rDNA was carried out using an Applied Biosystems ABI 377 DNA sequencer.

The 16S rDNA sequences of strains VR1^T, ATCC 43942 and ATCC 43943 determined in this study were aligned using the PILEUP program (Genetics Computer Group, Wisconsin package) and adjusted with GeneDoc version 2.4 and CLUSTAL X version 1.8 (Thompson *et al.*, 1997). The aligned sequences were analysed by distance and parsimony methods embodied in the software PHYLIP version 3.6a2.1. Distance matrices for the aligned sequences were calculated with the two-parameter model of Kimura (1980). The distance matrices were then used to reconstruct a phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap confidence values (Felsenstein, 1985) were obtained with 1000 resamplings with an option of stepwise addition.

DNA relatedness was determined between strain VR1^T and *V. gazogenes* ATCC 29988^T and some reference bacteria. Unlabelled DNA was also isolated and extracted using the Puregene DNA-purification kit. The DNA was transferred to a Hybond-N⁺ membrane (Amersham Pharmacia Biotech) and dot-blot hybridization was done as described by Fesefeldt *et al.* (1998). Total DNA of strain VR1^T labelled with a DIG DNA labelling kit (Roche Diagnostics) was used as a probe. Hybridization was done at 68 °C and hybrid detection was performed by enzyme immunoassay and enzyme-catalysed colour reaction using a DIG nucleic acid detection kit (Boehringer Mannheim).

Strain VR1^T grew at pH values in the range 5–9, with optimal growth at about pH 7. The strain grew significantly at temperatures in the range 20–40 °C, and grew most rapidly at 25–30 °C. Growth was not observed at 4 or 45 °C. Strain VR1^T grew aerobically under air and anaerobically under argon in GM medium (Fig. 1), indicating that the strain is a facultative anaerobe without a requirement for vitamins or other organic growth factors. Anaerobic growth was accompanied by a remarkable decrease in the pH of the medium during the exponential phase of growth (Fig. 1), regardless of the larger buffer content (25 mM MOPSO). This indicated that the strain achieved anaerobic growth in the medium by fermenting glucose, with the production of considerable amounts of organic acids. Similar acidification occurring in the aerobic GM cultures could be due to oxidative and/or fermentative degradation of glucose; fermentative degradation of glucose would possibly have occurred because of the creation of an oxygen-depleted microenvironment in such static cultures. Fig. 2 shows the effect of NaCl on growth. Strain VR1^T grew aerobically in PY broth containing 1–10 % NaCl (approx. 0.17–1.7 M); growth was most rapid at 2 % NaCl (approx. 0.34 M) and there was no growth in the absence of NaCl. Substitution of KCl (2–5 %) for NaCl did not support growth (not shown).

Acetone/methanol extracts of the red pigments produced by VR1^T and *V. gazogenes* ATCC 29988^T both showed maximal absorption at about 535 nm. Infrared spectrophotometry showed that the red pigment produced by *V. gazogenes* ATCC 29988^T is prodigiosin (Harwood, 1978). The MALDI-TOFMS analyses performed in this study confirmed that the composition of the major cellular pigment extracted by acetone/methanol from VR1^T was also prodigiosin, similar to that of *V. gazogenes* ATCC 29988^T (data not shown).

Both VR1^T and *V. gazogenes* ATCC 29988^T contained 2-OH-16:0 and 3-OH-14:0 as the major cellular fatty acids. However, VR1^T had a far higher 2-OH-16:0 content and a significantly lower 3-OH-14:0 content than did *V. gazogenes* ATCC 29988^T (58.0 vs 36.7 mol% for 2-OH-16:0

Fig. 1. Changes in OD₆₀₀ (circles) and pH (squares) during aerobic (filled symbols) and anaerobic (open symbols) growth of strain VR1^T in GM medium.

Fig. 2. Influence of NaCl concentration on maximal growth (●) and specific growth rate (▲) of strain VR1^T in PY broth under aerobic conditions.

and 20.0 vs 37.0 mol% for 3-OH-14:0). The other cellular fatty acids present at levels greater than 1 mol% included 3-OH-12:0 (4.1 mol%), iso-16:0 (9.8 mol%) and 18:1 ω 9 (4.3 mol%) for VR1^T and 3-OH-12:0 (4.5 mol%), iso-16:0 (14.8 mol%), 18:2 ω 9,12 (2.4 mol%) and 18:1 ω 9 (5.0 mol%) for *V. gazogenes* ATCC 29988^T.

Strain VR1^T was a mesophilic, Gram-negative bacterium that produced flat, circular, red colonies on PY and PYG plates. The strain was a halophile, unable to grow in the absence of NaCl. Cells grown in PY and PYG broth cultures were curved rods (approx. 1.8–2.4 μ m long and 0.7–0.8 μ m wide) and each cell normally possessed a single, sheathed, polar flagellum as viewed by electron microscopy (Fig. 3). Strain VR1^T was a facultative anaerobe able to ferment glucose and other carbohydrates with the production of acids and gases. The test for catalase was positive but that for oxidase was negative. The strain reduced nitrate to nitrite

Fig. 3. Electron micrograph of strain VR1^T, showing a sheathed, polar flagellum. The flagellum has lost the distal part of its sheath, exposing the inner core. Bar, 0.5 μ m.

and was not sensitive to the vibriostatic agent O/129 at 10–150 µg per disc. These data support the inclusion of strain VR1^T in the genus *Vibrio* of the family *Vibrionaceae*. Additional phenotypic characteristics are given below in the species description.

The diameters (mm) of the inhibition zones of strain VR1^T in response to various antibiotics were: ampicillin, 20; carbenicillin, 25; cephalothin, 10.5; chloramphenicol, 26.5; clindamycin, 5; colistin, 17; erythromycin, 17; kanamycin, 16; lincomycin, 5; nalidixic acid, 23.5; neomycin, 21.5; novobiocin, 15; oxacillin, 5; penicillin G, 12; polymyxin B, 18; streptomycin, 14; and vancomycin, 5. With reference to the interpretive standards (National Committee for Clinical Laboratory Standards, 1990), strain VR1^T was susceptible to ampicillin, carbenicillin, chloramphenicol, colistin, erythromycin, kanamycin, nalidixic acid, neomycin, novobiocin, penicillin G, polymyxin B and streptomycin and resistant to cephalothin, clindamycin, lincomycin, oxacillin and vancomycin. *V. gazogenes* ATCC 29988^T exhibited similar reaction patterns for the test antibiotics, except that the strain was resistant to erythromycin and novobiocin.

Strain VR1^T had a G+C content of 45.8 mol%, falling within the range of G+C contents for the genus *Vibrio* (38–51 mol%).

An almost-complete 16S rDNA sequence (approx. 94.7%; estimated by comparison with the *E. coli* sequence, J01859) of strain VR1^T was determined. Comparative analysis confirmed the affiliation of the newly isolated strain to the genus *Vibrio*. However, the levels of similarity between the 16S rDNA sequence of strain VR1^T and those of all known *Vibrio* species were never greater than 96%; the highest similarity level was to *V. gazogenes* ATCC 29988^T (95.8%). As shown in Fig. 4, strain VR1^T formed a cluster with *V. gazogenes* strains ATCC 43492 and ATCC 29988^T within the radiation of the *Vibrio* species. The results of the 16S rDNA-based phylogenetic analysis revealed that strain VR1^T represents a novel species within the genus *Vibrio* (Stackebrandt & Goebel, 1994). Strains ATCC 29988^T, ATCC 43942 and ATCC 43943 were categorized as belonging to various subgroups of *V. gazogenes* (Farmer *et al.*, 1988). Our analysis, however, indicated that the three strains could be differentiated at the species level.

Fig. 4. Unrooted phylogenetic tree derived from neighbour-joining analysis of the 16S rDNA sequences of strain VR1^T and other related species of the genus *Vibrio*. GenBank accession numbers are given. Numbers above nodes represent bootstrap confidence values obtained with 1000 resamplings; values below 500 are not shown. Bar, 5 nucleotide substitutions per 100 nucleotides.

DNA–DNA hybridization experiments showed that strain VR1^T gave relatedness values of 55.9, 52.1, 32.1, 25.1 and 15.5 % with *V. gazogenes* strains ATCC 29988^T, ATCC 43942 and ATCC 43943, *V. aerogenes* ATCC 700797^T and *Vibrio vulnificus* ATCC 27562^T, respectively. Strain VR1^T, therefore, appeared to represent a novel genospecies within the genus *Vibrio*, for which the name *Vibrio ruber* sp. nov. is proposed.

Although the phenotypic characteristics of *V. ruber* VR1^T are rather similar to those of *V. gazogenes*, the reduction of nitrate to nitrite, the ability to utilize D-arabinose, melibiose and L-glycine as sole carbon sources, the inability to utilize sorbitol as a sole carbon source, resistance to O/129 and susceptibility to erythromycin and novobiocin allow differentiation of the proposed novel species from *V. gazogenes*. Differences in contents of major cellular fatty acids, as described above, also make the two species distinguishable. The following combination of characteristics distinguishes *V. ruber* VR1^T from other *Vibrio* species: positive for red pigment, gas production from glucose, growth in 10 % NaCl, utilization of D-arabinose, lactose, melibiose, xylose, acetate and L-glycine; negative for oxidase, arginine dihydrolase, utilization of trehalose and O/129 sensitivity.

To date, *V. ruber* has been found only in coastal sea water. Whether it is also distributed in marine sediment or other habitats awaits future investigation.

Description of *Vibrio ruber* sp. nov.

Vibrio ruber (ru'ber. L. masc. adj. *ruber* red).

Cells are Gram-negative, curved rods (approx. 1.8–2.4 µm long and 0.7–0.8 µm wide) that are motile by means of a single, polar, sheathed flagellum when grown in liquid media. Colonies produced on agar media are red, non-luminescent and circular with an entire margin. Swarming is not detected. Facultative anaerobe capable of both aerobic and anaerobic fermentative growth. Acid and gas are produced from fermentation of glucose. Other carbohydrates such as cellobiose, galactose, lactose, mannose, sucrose, xylose, mannitol and salicin are also fermented. Trehalose, dulcitol and inositol are not fermented. Catalase, amylase, gelatinase and lipase tests are positive while oxidase, agarase, caseinase, arginine dihydrolase and lysine and ornithine decarboxylases tests are negative. Indole is not produced. Nitrate is reduced to nitrite but not further to N₂O or N₂. Optimal growth occurs at 25–30 °C, pH 7 and about 2 % NaCl. Growth occurs between 20 and 40 °C but not at 4 or 45 °C. Growth occurs in 1–10 % NaCl levels, while no growth occurs in the absence of NaCl. Grows in a mineral medium containing glucose and NH₄Cl. Resistant to the vibriostatic agent O/129 (10 or 150 µg per disc). The major cellular fatty acids are 2-OH-16:0 and 3-OH-14:0. D-Arabinose, cellobiose, galactose, glucose, lactose, mannose, melibiose, sucrose, xylose, mannitol, acetate, citrate, fumarate, pyruvate, L-aspartate, L-glutamate and L-glycine can be utilized as sole carbon and energy sources. Trehalose,

adonitol, dulcitol, inositol, sorbitol, DL-malate, malonate, tartrate, L-alanine, L-arginine, L-lysine, L-ornithine and L-threonine are not utilized as sole sources of carbon and energy. Susceptible to ampicillin, carbenicillin, chloramphenicol, colistin, erythromycin, kanamycin, nalidixic acid, neomycin, novobiocin, penicillin G, polymyxin B and streptomycin. The type strain is strain VR1^T (=JCM 11486^T=CCRC 17186^T), which has a DNA G+C content of 45.8 mol%.

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