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, 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; Borreto 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) stabs and were cultivated in PY broth, peptone/yeast...
extract/glucose (PYG) broth or glucose/mineral (GM) medium for growth and other studies. PYG 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 MgSO4.7H2O and 0.01 g CaCl2. 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 MgSO4.7H2O and 0.01 g CaCl2 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 NH4Cl, 25 g NaCl, 2 g MgCl2.6H2O, 3 g K2SO4, 0.2 g K2HPO4, 0.01 g CaCl2, 0.005 g FeCl3.6H2O and 5.4 g K2HPO4 (approx. 25 mmol) MOPS (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 VR1T were determined according to methods described in our previous report (Shieh et al., 2000). Susceptibility of strains VR1T and V. gazogenes ATCC 29988T 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 (50 μg), lincomycin (5 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), oxacillin (1 μg), penicillin G (10 μg), polymyxin B (300 μg), 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 VR1T and V. gazogenes ATCC 29988T, 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’-AGAGTTTGATCMTGCGTCAAG-3’ and 5’-CCTACACCTGTTAGGACTTCACC-3’ (positions 8–27 and 1488–1511, Escherichia coli numbering system) (Bennasar et al., 1998) and 5’-GCGAGGCTAACATGCAAGT-3’ and 5’-GCGCTACCTTGAGGACTTCACC-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 VR1T, 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. 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 VR1T and V. gazogenes ATCC 29988T 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 VR1T 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 immunoadsorpt and enzyme-catalysed colour reaction using a DIG nucleic acid detection kit (Boehringer Mannheim).
Strain VR1T 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 VR1T 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 VR1T 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 VR1T and V. gazogenes ATCC 29988T both showed maximal absorption at about 535 nm. Infrared spectrophotometry showed that the red pigment produced by V. gazogenes ATCC 29988T 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 VR1T was also prodigiosin, similar to that of V. gazogenes ATCC 29988T (data not shown).

Both VR1T and V. gazogenes ATCC 29988T contained 2-OH-16:0 and 3-OH-14:0 as the major cellular fatty acids. However, VR1T had a far higher 2-OH-16:0 content and a significantly lower 3-OH-14:0 content than did V. gazogenes ATCC 29988T (58.0 vs 36.7 mol% for 2-OH-16:0

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 18:1ω9 (4.3 mol%) for VR1T and 3-OH-12:0 (4.5 mol%), iso-16:0 (14.8 mol%), 18:2ω9,12 (2.4 mol%) and 18:1ω9 (5.0 mol%) for V. gazogenes ATCC 29988T.

Strain VR1T 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 μm long and 0.7–0.8 μm wide) and each cell normally possessed a single, sheathed, polar flagellum as viewed by electron microscopy (Fig. 3). Strain VR1T 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.

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Fig. 1. Changes in OD600 (circles) and pH (squares) during aerobic (filled symbols) and anaerobic (open symbols) growth of strain VR1T in GM medium.

Fig. 2. Influence of NaCl concentration on maximal growth (●) and specific growth rate (▲) of strain VR1T in PY broth under aerobic conditions.

Fig. 3. Electron micrograph of strain VR1T, showing a sheathed, polar flagellum. The flagellum has lost the distal part of its sheath, exposing the inner core. Bar, 0.5 μm.
and was not sensitive to the vibriostatic agent O/129 at 10–150 μg per disc. These data support the inclusion of strain VR1T 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 VR1T 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 interpretative standards (National Committee for Clinical Laboratory Standards, 1990), strain VR1T 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 29988T exhibited similar reaction patterns for the test antibiotics, except that the strain was resistant to erythromycin and novobiocin.

Strain VR1T 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 VR1T 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 VR1T and those of all known Vibrio species were never greater than 96%; the highest similarity level was to V. gazogenes ATCC 29988T (95.8%). As shown in Fig. 4, strain VR1T formed a cluster with V. gazogenes strains ATCC 43492 and ATCC 29988T within the radiation of the Vibrio species. The results of the 16S rDNA-based phylogenetic analysis revealed that strain VR1T represents a novel species within the genus Vibrio (Stackebrandt & Goebel, 1994). Strains ATCC 29988T, 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 VR1T 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 VR1T gave relatedness values of 55.9, 52.1, 32.1, 25.1 and 15.5% with V. gazogenes strains ATCC 29988T, ATCC 43942 and ATCC 43943, V. aegyptiacus ATCC 700797T and Vibrio vulnificus ATCC 27562T, respectively. Strain VR1T, 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 VR1T 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 VR1T 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, argarase, 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 VR1T (=CCRC 11486T = CCRC 17186T), which has a DNA G+C content of 45.8 mol%.

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**References**


