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Photobacterium rosenbergii sp. nov. and *Enterovibrio coralii* sp. nov., vibrios associated with coral bleaching

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Six new *Vibrio*-like isolates originating from different species of bleached and healthy corals around Magnetic Island (Australia) were investigated using a polyphasic approach. Phylogenetic analyses based on 16S rRNA, *recA* and *rpoA* gene sequences split the isolates in two new groups. Strains LMG 22223^T, LMG 22224, LMG 22225, LMG 22226 and LMG 22227 were phylogenetic neighbours of *Photobacterium leiognathi* LMG 4228^T (95·6 % 16S rRNA gene sequence similarity), whereas strain LMG 22228^T was related to *Enterovibrio norvegicus* LMG 19839^T (95·5 % 16S rRNA gene sequence similarity). The two new groups can be distinguished from closely related species on the basis of several phenotypic features, including fermentation of D-mannitol, melibiose and sucrose, and utilization of different compounds as carbon sources, arginine dihydrolase activity, nitrate reduction, resistance to the vibriostatic agent O/129 and the presence of fatty acids 15 : 0 iso and 17 : 0 iso. The names *Photobacterium rosenbergii* sp. nov. (type strain LMG 22223^T = CBMAI 622^T = CC1^T) and *Enterovibrio coralii* sp. nov. (type strain LMG 22228^T = CBMAI 623^T = CC17^T) are proposed to accommodate these new isolates. The G + C contents of the DNA of the two type strains are respectively 47·6 and 48·2 mol%.

Coral reefs are very important ecosystems for the marine environment as they harbour a great diversity of organisms. They are also relevant for the economy of several countries via tourism and fishing. In addition, coral reefs provide protection to coastal areas and may be a source of new bioactive compounds. The bacterial biodiversity associated with corals is poorly known (but see Rosenberg & Loya, 2004; Rosenberg & Falkovitz, 2004; Rohwer & Kelley, 2004). Rohwer *et al.* (2001, 2002) examined the bacterial community associated with different species of apparently healthy corals from Panama and Bermuda by both culture-dependent and culture-independent techniques. These authors found a high diversity of bacteria, including representative species of *Bacillus*, *Clostridium*, cyanobacteria, the *Cytophaga–Flavobacterium–Bacteroides* group and *Proteobacteria*. More than 80 % of the 1178 cloned 16S rRNA gene sequences originating from coral sources had not been allocated to recognized bacterial species as they had less than 93 % sequence similarity (Rohwer *et al.*, 2002).

Coral reefs have experienced a tremendous decline in recent decades (Hoegh-Guldberg, 2004). Global climate changes, sea-water pollution as a result of aquaculture, oil spills and urban sewage, coral bleaching and other infectious diseases are the main causes of this decline (Hoegh-Guldberg, 2004; Hughes *et al.*, 2003; Knowlton & Rohwer, 2003; Rosenberg & Loya, 2004; Rosenberg & Ben-Haim, 2002; Sutherland *et al.*, 2004). Kushmaro *et al.* (1996) suggested that bleaching is, in fact, the result of an infectious disease. Bacterial infections of corals caused by *Vibrio shilonii* (= *Vibrio mediterranei*) (Kushmaro *et al.*, 2001) and *Vibrio*

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA* and *rpoA* gene sequences determined in this study are shown in Table 1.

Neighbour-joining trees showing relationships among *Vibrio*-like species on the basis of *rpoA* and *recA* gene sequence analysis are available as supplementary material in IJSEM Online.

coralliilyticus (Ben-Haim *et al.*, 2003) were subsequently reproduced in the laboratory. *V. coralliilyticus* also causes disease in fish and shellfish (B. Austin, D. A. Austin, R. Sutherland, F. L. Thompson and J. Swings, unpublished results).

In the present study we analysed the taxonomic position of six new isolates originating from different species of bleached and healthy corals from Magnetic Island (Australia) in 2002. The taxa identified share the main phenotypic features of *Vibrio*, but 16S rRNA, *recA* and *rpoA* gene sequences clearly suggest that they represent two novel species for which we propose the names *Photobacterium rosenbergii* sp. nov. and *Enterovibrio coralii* sp. nov.

Details of sources of strains are given in Table 1. Strains were grown on tryptone soy agar (TSA; Oxoid) supplemented with 2 % NaCl (v/v) at 28 °C for 24 h unless stated otherwise. Colony morphology was examined on cultures grown on thiosulphate/citrate/bile salts/sucrose (TCBS; Difco) agar by using a stereoscopic microscope. Cell morphology was examined on wet mounts via a phase-contrast microscope.

Gene sequences of 16S rRNA, *recA* and *rpoA* were generated on an ABI Prism 3100 DNA sequencer (Applied Biosystems) and analysed as described by Thompson *et al.* (2001, 2004a), but with minor modifications. The sequences of *recA* primers used for amplification and sequencing were recA-01-F (5′-TGARAARCARTTYGGT-AAAGG; position 222), recA-02-R (5′-TCRCCNTTRTA-GCTRTACC; 1040), recA-03-F (5′-TYGGBGTGATGTTY-GGTAACC; 767) and recA-04-R (5′-GGGTTACCRAAC-ATCACVCC; 769). These primers were designed using 15 *recA* gene sequences from *Vibrio cholerae* n16961_o1, *Vibrio parahaemolyticus* o3k6_rimd2210633, *Vibrio vulnificus* cmcp6, *Escherichia coli* (cft073, o157h7_edl933, o157h7_rimd0509952 and k12_mg1655), *Shigella flexneri* (2a_2457t and 2a_301), *Salmonella enterica* (ct18 and ty2_typhi 3), *Salmonella typhimurum* (lt2sgsc1412_atcc700720), *Yersinia pestis* (co92 and kim) and *Shewanella oneidensis* (mr1). The primers recA-01-F and recA-02-R are useful to amplify *recA* fragments of all recognized strains of *Vibrio*-like species. Sequence accession numbers are given in Table 1.

Overall, consensus sequences were obtained by at least two reads of the same region of the gene. The consensus sequences were transferred into BIONUMERICS 3.5 software (Applied Maths) and phylogenetic trees were constructed based on the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods. The G + C content of the DNA was determined by HPLC (Tamaoka & Komagata, 1984).

Phenotypic characterization of the isolates was performed using API 20E, API ZYM (bioMérieux) and Biolog GN2 metabolic fingerprinting (Biolog) following the manufacturers' instructions. Standard phenotypic tests were performed as described by Baumann *et al.* (1984), Farmer & Hickman-Brenner (1992) and Vandamme *et al.* (1998). Antibiograms were carried out using the disc diffusion methodology of Acar & Goldstein (1996) using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured for strains grown on Iso-sensitest agar (Oxoid) supplemented with 1·5 % (w/v) NaCl for 24 h at 28 °C. Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994).

The 16S rRNA gene sequences of strains LMG 22223^T (1505 nt) and LMG 22227 (1505 nt) were nearly identical, having more than 99·5 % similarity (Fig. 1). LMG 22223^T and LMG 22228^T were most closely related to *Photobacterium leiognathi* LMG 4228^T (95·6 %) and *Vibrio calviensis* LMG 21294^T (95·8 %), respectively. LMG 22228^T and *Enterovibrio norvegicus* LMG 19839^T had 95·5 % 16S rRNA gene sequence similarity. Clearly, *V. calviensis* should be transferred to *Enterovibrio*, but this remains to be done in future studies.

Table 1. Strains included in this study

Strain	Source	Accession numbers		
		16S rRNA gene	*recA*	*rpoA*
***Enterovibrio coralii* sp. nov.**				
LMG 22228^T (=CBMAI 623^T=CC17^T)	Water extract of bleached *Merulina ampliata*	AJ842343	AJ842347	AJ842530
***Photobacterium rosenbergii* sp. nov.**				
LMG 22223^T (=CBMAI 622^T=CC1^T)	Tissue extract of bleached *Pachyseris speciosa*	AJ842344	AJ842358	AJ842542
LMG 22224 (=R-21412=CC6)	Water extract of bleached *M. ampliata*	ND	AJ842359	AJ842543
LMG 22225 (=R-21417=CC11)	Water extract of apparently healthy *P. speciosa*	AJ842345	AJ842360	AJ842544
LMG 22226 (=R-21428=CC22)	Water extract of bleached *Barabattoia amicorum*	ND	AJ842361	AJ842545
LMG 22227 (=R-21429=CC23)	Tissue extract of bleached *B. amicorum*	AJ842346	ND	AJ842546

ND, Not determined.

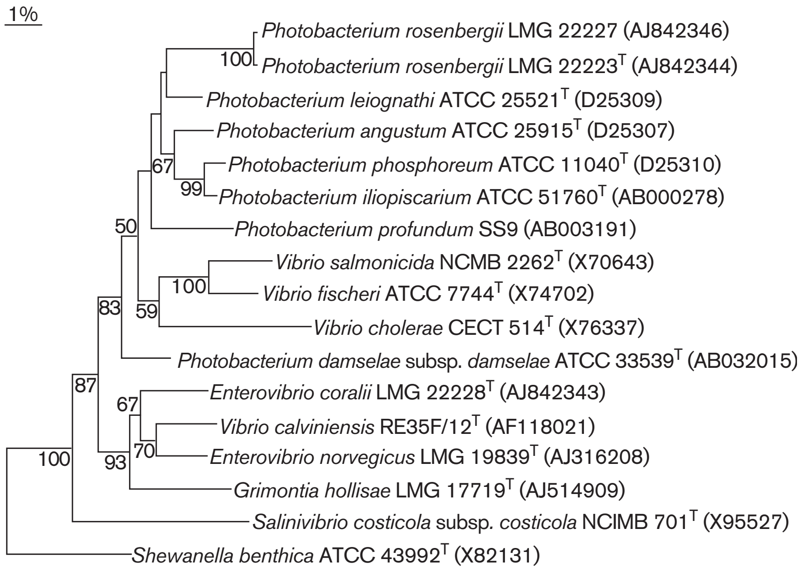

Fig. 1. Neighbour-joining tree showing the relationships among representative *Vibrio*-like species on the basis of 16S rRNA gene sequence analysis (1400 nt). Bootstrap values after 500 replications are shown. Bar, 1 % estimated sequence divergence.

The *rpoA* gene sequences (928 nt; corresponding to about 94 % of the coding region of this locus) of strains LMG 22223^T, LMG 22224, LMG 22225, LMG 22226 and LMG 22227 were nearly identical, having more than 99·6 % similarity (see Supplementary Fig. A in IJSEM Online). The closest phylogenetic neighbours of LMG 22223^T and LMG 22228^T were *Photobacterium phosphoreum* (91·2 %) and *Grimontia hollisae* (96·1 %), respectively. *Enterovibrio norvegicus* LMG 19839^T and the novel strain LMG 22223^T had 95·5 % *rpoA* sequence similarity. This low level of similarity suggests that LMG 22223^T and LMG 22228^T represent two novel phylogenetic branches within the current families *Photobacteriaceae* and *Enterovibrionaceae* (Thompson *et al.*, 2004b). The usefulness of the *rpoA* gene as an alternative phylogenetic marker for bacterial classification has been proposed recently (Zeigler, 2003; Gevers *et al.*, 2004; Lerat *et al.*, 2003). The *rpoA* gene is a single-copy and ubiquitous gene in the bacterial genomes sequenced to date. According to Zeigler (2003) this gene evolves as slowly as the 16S rRNA gene. It seems to be resistant to lateral gene transfer (Lerat *et al.*, 2003) and is indeed a chronometer for *Vibrio*-like species (unpublished results).

Strains LMG 22224, LMG 22225 and LMG 22226 had identical *recA* gene sequences (797 nt) (see Supplementary Fig. B in IJSEM Online). LMG 22223^T had 94·5 % *recA* gene sequence similarity to the other conspecific strains, but only 83·9 % to its closest phylogenetic neighbour, *Photobacterium leiognathi*. LMG 22228^T had 88·2 and 85 % *recA* gene sequence similarity to *Enterovibrio norvegicus* LMG 19839^T and *Grimontia hollisae* LMG 17719^T, respectively. Collectively, 16S rRNA, *recA* and *rpoA* gene sequence data indicate that the coral isolates represent two novel species for which we propose the names *Photobacterium rosenbergii* and *Enterovibrio coralii*.

Enterovibrio coralii sp. nov. can be differentiated from *Enterovibrio norvegicus* on the basis of various phenotypic features. *Enterovibrio coralii* utilizes cellobiose, melibiose and L-histidine and reduces nitrate, but *Enterovibrio norvegicus* does not. *Enterovibrio norvegicus* produces indole, but *Enterovibrio coralii* does not. *Enterovibrio coralii* produces alcohol dehydrogenase and ferments D-mannitol, whereas *Enterovibrio norvegicus*, *G. hollisae* and *V. calviensis* do not. *Enterovibrio coralii* utilizes glycogen, D-fructose and D-trehalose, but *V. calviensis* does not. *Enterovibrio coralii* is resistant to O/129 (150 µg per disc), fusidic acid (10 µg per disc) and streptomycin (10 µg per disc), but *V. calviensis* is not. *Photobacterium rosenbergii* sp. nov. ferments D-mannitol and melibiose, whereas other *Photobacterium* species do not. *Photobacterium rosenbergii* utilizes citrate, cellobiose, melibiose, lactose, formate, propionate, D-raffinose, aconitate, D-alanine, L-alanine and L-histidine, whereas other *Photobacterium* species do not. *Photobacterium* species produce acetoin, but *Enterovibrio coralii* does not. None of the *Photobacterium* species produces the fatty acids 15 : 0 iso and 17 : 0 iso that are found in *Photobacterium rosenbergii*.

Description of *Photobacterium rosenbergii* sp. nov.

Photobacterium rosenbergii (ro.sen.ber′gi.i. N.L. gen. n. *rosenbergii* of Rosenberg, after the Israeli microbiologist Eugene Rosenberg).

Cells are Gram-negative, motile and oxidase-positive. Strains grow on the selective medium TCBS. Cells are 2–4 µm long and 1–2 µm wide after 1 day at 28 °C in TSA. Colonies are convex, round (1 cm in diameter), beige and opaque with entire and smooth margins after 2 days at 28 °C on TSA. Forms yellow colonies with raised margins on TCBS. Prolific growth occurs between 20 and 30 °C and at NaCl concentrations (w/v) of 2–6 %. No growth is observed at 4 or 40 °C or in 0 or 8 % NaCl. Utilizes the following carbon compounds as sole energy sources: dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, methyl β-D-glucoside, psicose, sucrose, D-trehalose, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-ornithine, L-serine, L-threonine, inosine, uridine, thymidine, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Does not utilize adonitol, L-arabinose, D-arabitol, α-D-lactose lactulose, xylitol, D-galactonic acid, γ-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, malonic acid, D-saccharic acid, sebacic acid, hydroxy-L-proline, L-leucine, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, 2-aminoethanol or 2,3-butanediol. Ferments glucose, D-mannitol, sucrose, melibiose and amygdalin. Does not ferment sorbitol or arabinose and is negative for acetoin and indole production. Reduces nitrate and is positive for arginine dihydrolase, β-galactosidase, alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Lysine and ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. The following features are variable within the species (the reaction for the type strain is indicated in parentheses): utilization of α-cyclodextrin (+), *N*-acetyl-D-galactosamine (−), i-erythritol (−), L-fucose (−), gentiobiose (−), myo-inositol (+), α-lactose (−), D-raffinose (−), L-rhamnose (+), D-sorbitol (−), turanose (+), D-galacturonic acid (−), D-gluconic acid (−), D-glucosaminic acid (−), D-glucuronic acid (+), α-hydroxybutyric acid (+), β-hydroxybutyric acid (−), quinic acid (−), succinamic acid (+), glucuronamide (−), L-phenylalanine (−), L-proline (+), L-pyroglutamic acid (+) and putrescine (−) as sole carbon sources. Fermentation of L-rhamnose and inositol, as well as lipase, α-galactosidase and α-glucosidase activity are variable, but positive for the type strain. Resistant to O/129 (150 μg per disc), fusidic acid (10 μg), streptomycin (10 μg), intermediately resistant to trimethoprim (1·25 μg), but susceptible to polymyxin B (300 U), oxolinic acid (2 μg), oxytetracycline (30 μg), penicillin G (10 μg) and chloramphenicol (30 μg). The most abundant fatty acids are summed feature 3 (41–44 %; comprising 16 : 1ω7*c* and/or 15 iso 2-OH), 18 : 1ω7*c* (17–19 %), 16 : 0 (10–15 %), 17 : 0 iso (3–6 %), 14 : 0 (3–4 %), 15 : 0 iso (2–4 %), summed feature 2 (2–3 %; comprising 14 : 0 3-OH, 16 : 1 iso I, an unidentified fatty acid with equivalent chain-length of 10·928 and/or 12 : 0 ALDE), 12 : 0 3-OH (2–3 %), 12 : 0 (2 %), 17 : 1ω9*c* iso (1–2 %), 15 : 0 (1–2 %), 13 : 0 iso (1 %), 17 : 0 (1 %), 17 : 1ω8*c* (1 %), 15 : 0 iso 3-OH (1 %) and 16 : 1ω7*c* alcohol (1 %). The G+C content of the DNA ranges from 47·6 to 47·9 mol%.

The type strain is strain LMG 22223^T (=CBMAI 622^T= CC1^T).

Description of *Enterovibrio coralii* sp. nov.

Enterovibrio coralii (co.ra′li.i. L. gen. sing. n. *coralii* of coral).

Gram-negative, motile, oxidase-positive. Strains grow on the selective medium TCBS. Cells are 1 μm in diameter after 1 day at 28 °C in TSA. Colonies are umbonate, round (5 mm in diameter), beige and transparent with entire and smooth margins after 2 days at 28 °C on TSA. Forms small, green colonies (2 mm in diameter) with raised margins on TCBS. Prolific growth occurs between 20 and 30 °C and at NaCl concentrations (w/v) of 2–6 %. No growth is observed at 4 or 40 °C or in 0 or 8 % NaCl. Utilizes the following compounds as sole carbon sources: dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, α-D-glucose, α-lactose, α-D-lactose lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β-D-glucoside, psicose, D-raffinose, D-sorbitol, sucrose, D-trehalose, turanose, acetic acid, *cis*-aconitic acid, D-gluconic acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, inosine, uridine and thymidine. Does not utilize Tweens 40 or 80, adonitol, L-arabinose, D-arabitol, i-erythritol, formic acid, D-galactonic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, malonic acid, D-saccharic acid, sebacic acid, glucuronamide, L-leucine, L-phenylalanine, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, γ-DL-glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. Ferments glucose and D-mannitol and is positive for arginine dihydrolase, α- and β-galactosidases, alkaline phosphatase, esterase, esterase lipase, leucine and valine arylamidases, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase and α-glucosidase. Does not ferment inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. Activity of lysine and ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, lipase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase is not detected. Resistant to O/129 (150 μg per disc), fusidic acid (10 μg), streptomycin (10 μg), trimethoprim (1·25 μg), but susceptible to polymyxin B (300 U), oxolinic acid (2 μg), oxytetracycline (30 μg), penicillin G (10 μg) and chloramphenicol (30 μg). The most abundant fatty acids are summed feature 3 (32 %; comprising 16 : 1ω7*c* and/or 15 iso 2-OH), 18 : 1ω7*c* (23 %), 16 : 0 (16 %), 18 : 1ω9*c* (5 %), 16 : 1ω9*c* (4 %), 14 : 0 (4 %), 12 : 0 (3 %), summed feature 2 (3 %; comprising 14 : 0 3-OH, 16 : 1 iso I, an unidentified fatty acid with equivalent chain-length of

10·928 and/or 12 : 0 ALDE), 12 : 0 3-OH (2 %), 18 : 0 (2 %), 16 : 0 iso (1 %) and 17 : 1ω8*c* (1 %).

The type strain is LMG 22228^T (=CBMAI 623^T=CC17^T). The G+C content of the DNA of LMG 22228^T is 48·2 mol%.

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