Vibrio superstes sp. nov., isolated from the gut of Australian abalones *Haliotis laevigata* and *Haliotis rubra*

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Five alginolytic, facultatively anaerobic, non-motile bacteria were isolated from the gut of abalones *Haliotis laevigata* and *Haliotis rubra*. Phylogenetic analyses based on 16S rDNA data indicated that these strains are related closely to *Vibrio halioticoli* (98 % 16S rDNA sequence similarity). DNA–DNA hybridization and fluorescent amplified fragment length polymorphism fingerprinting demonstrated that the five strains constituted a single species that was different from all currently known vibrios. The name *Vibrio superstes* sp. nov. (type strain, LMG 21323^T = IAM 15009^T = G3-29^T; DNA G+C content, 48·0–48·9 mol%) is proposed to encompass this novel taxon. Several phenotypic features were disclosed that discriminate *V. superstes* from other *Vibrio* species: *V. superstes* sp. nov. and *V. halioticoli* can be differentiated on the basis of 17 traits (indole production, β-galactosidase test and assimilation of 15 carbon compounds).

Vibrio halioticoli and genetically related species, which are alginolytic, non-motile, fermentative marine bacteria, are abundant in the gut of *Haliotis* abalones in Japan and South Africa (Sawabe *et al.*, 1995, 2002). Hypothesized roles of *V. halioticoli* include its contribution to the digestion of alginate, which is a major polysaccharide in Japanese kelps ingested by these animals, and its conversion into volatile short-chained fatty acids via fermentation (Sawabe *et al.*, 2003). Nearly 80 species of abalone are known; they appear in offshore areas worldwide, but little is known about the presence of *V. halioticoli*-like bacteria in the gut of these molluscs. We recently isolated a set of five strains that were most similar phenotypically to *V. halioticoli* from the gut of Australian abalones (*Haliotis laevigata* and *Haliotis rubra*). DNA–DNA hybridization experiments, phenotypic characterization and phylogenetic and genetic analyses demonstrated that these strains represent a so far unknown species of the genus *Vibrio*.

Five strains of *V. superstes* sp. nov. [LMG 21319 (=IAM 15007=B1-5), LMG 21320 (=IAM 15008=B2-3), LMG 21321 (=G3-11), LMG 21322 (=G3-15) and LMG 21323^T (=IAM 15009^T=G3-29^T)] were isolated from the gut of the Australian abalones *H. laevigata* and *H. rubra*. These were collected at the coastal area of Clifton Springs, Victoria, Australia, by scuba-diving in December 2000. Strains were cultured on ZoBell 2216E agar (Oppenheimer & ZoBell, 1952) and stored at −80 °C in 10 % glycerol.

The sequence of a 1400 bp fragment of the 16S rDNA gene of strains LMG 21319, LMG 21320, LMG21321, LMG 21322 and LMG 21323^T was determined according to Sawabe *et al.* (1998) by using six sequencing primers (24F, 530F, 1100F, 520R, 920R and 1540R). The 16S rDNA sequences of *V. superstes* and related species were selected from a database of more than 60 000 already aligned bacterial 16S rDNA sequences. Selection of sequences was done according to previous phylogenetic analyses of the entire database

Abbreviations: FAFLP, fluorescent amplified fragment length polymorphism; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Vibrio superstes* are AF519806 (LMG 21319=B1-5), AY155582 (LMG 21320=B2-3), AY155583 (LMG 21321=G3-11), AY155584 (LMG 21322=G3-15) and AY155585 (LMG 21323^T=G3-29^T).

Figures showing a full phylogenetic tree and a transmission electron micrograph of *Vibrio superstes* LMG 21323^T are available as supplementary material in IJSEM Online.

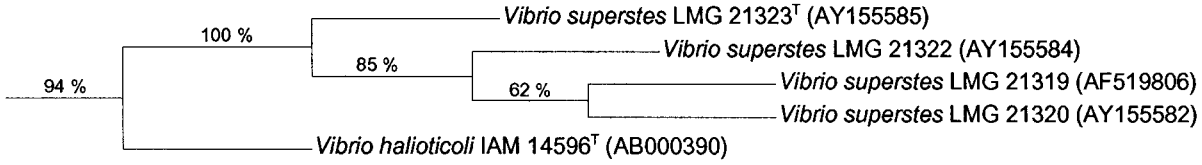

Fig. 1. Unrooted phylogenetic tree based on 16S rDNA sequence data. This figure combines the results of three analyses, i.e. NJ, MP and ML. The topology shown was obtained by using NJ and 1000 bootstrap replications (shown above branches).

and BLAST searches against the latest EBI release. Phylogenetic trees were constructed by using three different methods [BIONJ, maximum-likelihood (ML) and maximum-parsimony (MP)]. For neighbour-joining (NJ) analysis, distance matrices were calculated by using the Kimura two-parameter correction. BIONJ was done according to Gascuel (1997), ML and MP were from PHYLIP (Phylogeny Inference Package, version 3.573c; distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). Because of close relationships, no evident homoplasy was detected and almost-entire sequences that corresponded to positions 47–1364 of the sequence of the type strain of *V. superstes* were used for the analysis (a short insertion in the *Salinivibrio costicola* sequence between positions 159 and 160 of *V. superstes* was excluded from the analysis). Phylogenetic trees were drawn by using NJPLOT (Perrière & Gouy, 1996). The topology shown (Fig. 1) is that of the bootstrap tree, as it has been demonstrated that this topology is often better than that of a simple NJ or MP analysis (Berry & Gascuel, 1996). There is no distance bar in Fig. 1 as it would be meaningless because: (i) distances are corrected (see above) and (ii) this is a bootstrap tree.

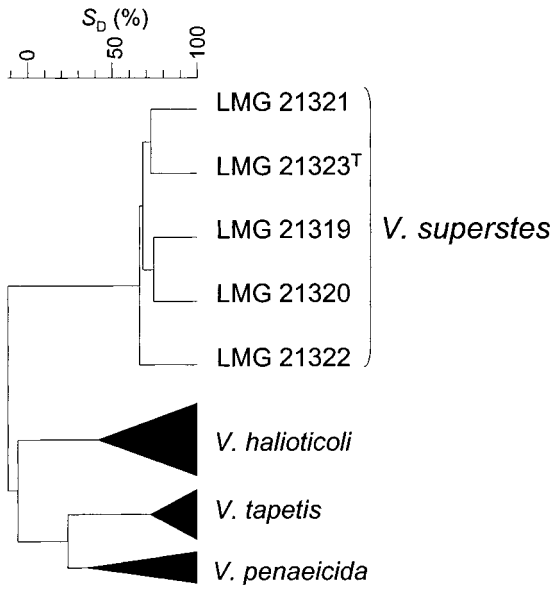

Fig. 2. Dendrogram of FAFLP patterns of the five novel *Vibrio* isolates from Australian abalone. *V. halioticoli*, *Vibrio tapetis* and *Vibrio penaeicida* were included as outgroups.

Fluorescent amplified fragment length polymorphism (FAFLP) analysis was performed as described previously (Thompson *et al.*, 2001; Sawabe *et al.*, 2002). Clustering of the patterns was done by using the Dice coefficient (S_D) and the Ward algorithm (Sneath & Sokal, 1973).

DNA of bacterial strains was prepared by the procedure of Marmur (1961). DNA G+C contents were determined by HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization experiments were performed in microdilution wells by using a fluorometric direct-binding method described by Ezaki *et al.* (1988, 1989).

In total, 78 phenotypic characteristics, including alginase

Table 1. DNA relatedness between *Vibrio superstes* and *Vibrio halioticoli*

Strain	G+C content (mol%)	Reassociation (%) with biotinylated DNA from:	
		V. superstes LMG 21323^T	*V. halioticoli* IAM 14596^T
V. superstes:			
LMG 21319	48·0	84·1	29·9
LMG 21320	48·9	75·3	28·5
LMG 21321	48·9	87·4	24·3
LMG 21322	48·3	94·4	25·7
LMG 21323^T	48·6	100·0	22·4
V. halioticoli IAM 14596^T	43·1	12·9	100·0

activity, were determined by standard methods (Leifson, 1963; Hidaka & Sakai, 1968; West *et al.*, 1977; Ostle & Holt, 1982; Baumann & Schubert, 1984; Holt *et al.*, 1994). These phenotypic characterizations were done at 20 °C.

The results of our phylogenetic analysis showed clearly that the strains belonged to the γ3 subgroup, phylum *Proteobacteria* (Garrity & Holt, 2001) (see Supplementary Fig. A in IJSEM Online). The closest phylogenetic neighbour of the five Australian abalone strains is *V. halioticoli* (Fig. 1). The five strains of *V. superstes* had high levels of 16S rDNA sequence similarity to each other, i.e. 99·7–99·9 %, and 98·3 % similarity to *V. halioticoli* IAM 14596^T. Similarity levels of <97·3 % were found with other *Vibrio* species.

The five strains had FAFLP patterns that consisted of 90 ± 10 fragments (minimum, 80; maximum, 108) and mutual similarity of at least 64·6 % (Fig. 2). *V. superstes* showed pattern similarities of <50 % to other *Vibrio* species, which shows clearly that this novel species is different from other vibrios (Thompson *et al.*, 2001). The FAFLP results are supported by our DNA–DNA hybridization experiments, which showed that the five strains of *V. superstes* (LMG 21319, LMG 21320, LMG 21321, LMG 21322 and LMG 21323^T) were conspecific strains that were clearly separated from *V. halioticoli* (Table 1).

The five Australian abalone strains have the main phenotypic features of the genus *Vibrio* (except for the absence of flagella). The strains are non-motile, Gram-negative and fermentative (Sawabe *et al.*, 1998). No flagellated cells were observed by transmission electron microsscopy, but short tubular projections were observed in *V. superstes* cells, similar to those around *Vibrio campbellii* cells [as reported by Allen & Baumann (1971)] (see Supplementary Fig. B in IJSEM Online). The function of these tubular projections has never been clarified (Allen & Baumann, 1971). These strains required salt for growth, did not accumulate poly-β-hydroxybutyrate and were oxidase-positive (Table 2). No peritrichous cells were observed when the strains were cultivated on solid media. Other phenotypic features of *V. superstes* are shown in Table 2. The five abalone isolates were most similar phenotypically to *V. halioticoli* IAM 14596^T, although the strains differed by 17 out of 78 traits tested (Table 2).

Despite the close phylogenetic relationship between *V. superstes* and *V. halioticoli*, *V. superstes* occurs in rather small populations, the size of which range from 0 to 20 % in the gut of Australian abalones (data not shown). Compared to the abundant populations of *V. halioticoli* and its speculated symbiotic contribution to the conversion of alginate to acetic acid in the gut of Japanese and South African abalones (Sawabe *et al.*, 2003), *V. superstes* may not be able to form such associations with Australian abalones because the major food of Australian abalones is red algae (Foale & Day, 1992). Differences in feeding behaviour of host abalones may affect the microflora of the gut microbial ecosystem and select for biochemical traits of these

Table 2. Phenotypic characteristics for distinguishing *Vibrio superstes* from previously described alginolytic *Vibrio* species

Species/strains: 1, *V. superstes* LMG 21319–LMG 21323^T; 2, *V. halioticoli* IAM 14596^T; 3, *Vibrio pelagius* biovar 1 ATCC 25916^T. +, Positive; −, negative; V+, variable (type strain is positive); V−, variable (type strain is negative). All species are negative for the following characteristics: pigmentation, swarming, poly-β-hydroxybutyrate accumulation, luminescence, growth at 4 and 40 °C, amylase, gelatinase, chitinase, agarase, gas production from D-glucose, acetoin production, lysine decarboxylase, arginine dehydrolase, ornithine decarboxylase, acid from L-arabinose, inositol and L-rhamnose, requirement for organic growth factors, utilization of D-sorbitol, L-tyrosine, meso-erythritol, L-arabinose, citrate, DL-malate, δ-aminovalerate and aconitate. All species are positive for the following characteristics: Na^+ requirement, growth at 15 and 30 °C, oxidase, catalase, alginase, nitrate reduction, O/129 sensitivity, methyl red test, growth on TCBS (thiosulfate/citrate/bile salts/sucrose) agar, growth in 3 % NaCl, acid from D-glucose, D-mannitol and maltose, utilization of alginate, D-fructose, D-glucose, maltose, D-mannitol, D-glucosamine, *N*-acetylglucosamine, fumarate and succinate. All species are fermentative in the oxidation–fermentation test.

Characteristic	1 ($n=5$)	2	3
Motility	−	−	+
Growth at 37 °C	−	−	+
Production of:			
Lipase	−	−	+
Indole	−	+	−
ONPG test	−	+	+
Growth in:			
1 % NaCl broth	−	−	+
6 % NaCl broth	−	−	+
Acid from:			
Sucrose	−	−	+
D-Sorbitol	−	−	+
Utilization of:			
D-Mannose	+	−	+
Sucrose	+	−	+
D-Gluconate	+	−	+
Glycerol	−	+	+
2-Oxoglutarate	−	−	+
D-Galactose	+	−	+
Cellobiose	+	−	−
Melibiose	+	−	−
Lactose	+	−	−
D-Glucuronate	+	−	−
Trehalose	V+	−	+
Putrescine	V−	−	+
γ-Aminobutyrate	+	−	+
Acetate	+	−	+
Pyruvate	−	−	+
Propionate	V+	−	+
L-Glutamate	+	−	+
D-Xylose	+	−	−

symbiotic vibrios. Major phenotypic traits of *V. superstes* differed from those of *V. halioticoli* in that the former was positive for use of 14 carbon compounds (Table 2). *V. superstes* may have acquired the ability to assimilate multiple carbon compounds to survive in the gut of the Australian abalone.

In conclusion, our polyphasic study demonstrated clearly that the five abalone isolates represent a so far undescribed species of the genus *Vibrio*, for which we propose the name *Vibrio superstes* sp. nov. The name *V. superstes*, which means survivor, has been chosen in this respect. Global whole-genome analyses may clarify the evolutionary history of *V. superstes* and *V. halioticoli*. Studies on the ecology of *V. superstes* are under way in order to better understand its interactions in the gut of marine herbivores, particularly abalones.

Description of *Vibrio superstes* sp. nov.

Vibrio superstes (su.per′stes. L. masc. adj. *superstes* remaining alive after another's death, outliving, surviving).

Gram-negative, facultatively anaerobic, non-motile and non-flagellated. Cells in ZoBell 2216E broth are rod-shaped with rounded ends (0·6–0·8 × 1·2–1·3 μm). No endospores or capsules are formed. Flagellation is not observed when the organism is cultivated on solid and/or in liquid media. Colonies on ZoBell 2216E agar are beige, circular, smooth and convex with entire edges. Sodium ions are essential for growth. Mesophilic and neutrophilic chemo-organotroph: grows between 15 and 30 °C. No growth occurs at 40 °C. Positive for acid production from glucose, nitrate reduction, hydrolysis of alginate, oxidase and catalase and assimilation of D-mannose, sucrose, D-gluconate, D-galactose, cellobiose, melibiose, lactose, D-glucuronate, trehalose, γ-aminobutyrate, acetate, propionate, L-glutamate, D-xylose, D-fructose, maltose, D-glucosamine, *N*-acetylglucosamine, D-mannitol, fumarate, succinate, D-glucose and alginate. The following tests are negative: gas production from glucose, acetoin production, lysine decarboxylase, arginine dehydrolase, ornithine decarboxylase, indole production, β-galactosidase test, luminescence, pigmentation, requirement for organic growth factors, hydrolysis of starch, gelatin, chitin, Tween 80 and agar, accumulation of poly-β-hydroxybutyrate, assimilation of D-sorbitol, glycerol, citrate, meso-erythritol, DL-malate, 2-oxoglutarate, putrescine, δ-aminovalerate, pyruvate, L-tyrosine, aconitate and L-arabinose. DNA G+C content is 48·0–48·9 mol%.

The type strain is LMG 21323^T = IAM 15009^T. The bacterium was isolated from the gut of the Australian abalones *Haliotis rubra* and *H. laevigata*.

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References

Allen, R. D. & Baumann, P. (1971). Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. *J Bacteriol* **107**, 295–302.

Baumann, P. & Schubert, R. H. W. (1984). *Vibrionaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 516–550. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Berry, V. & Gascuel, O. (1996). On the interpretation of bootstrap trees: appropriate threshold of clade selection and induced gain. *Mol Biol Evol* **13**, 999–1011.

Ezaki, T., Hashimoto, Y., Takeuchi, N., Yamamoto, H., Liu, S.-L., Miura, H., Matsui, K. & Yabuuchi, E. (1988). Simple genetic method to identify viridans group streptococci by colorimetric dot hybridization and fluorometric hybridization in microdilution wells. *J Clin Microbiol* **26**, 1708–1713.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Foale, S. & Day, R. W. (1992). Recognizability of algae ingested by abalone. *Aust J Mar Freshw Res* **43**, 1331–1338.

Garrity, G. M. & Holt, J. G. (2001). The road map to the *Manual*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 119–166. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer-Verlag.

Gascuel, O. (1997). BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* **14**, 685–695.

Hidaka, T. & Sakai, M. (1968). Comparative observation of inorganic salt requirement of the marine and terrestrial bacteria. *Bull Misaki Mar Biol Inst Kyoto Univ* **12**, 125–149.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). Facultatively anaerobic Gram-negative rods. Subgroup 2: family *Vibrionaceae*. In *Bergey's Manual of Determinative Microbiology*, 9th edn, pp. 190–253. Baltimore: Williams & Wilkins.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Oppenheimer, C. H. & ZoBell, C. E. (1952). The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. *J Mar Res* **11**, 10–18.

Ostle, A. G. & Holt, J. G. (1982). Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. *Appl Environ Microbiol* **44**, 238–241.

Perrière, G. & Gouy, M. (1996). WWW-Query: an on-line retrieval system for biological sequence banks. *Biochimie* **78**, 364–369.

Sawabe, T., Oda, Y., Shiomi, Y. & Ezura, Y. (1995). Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microb Ecol* **30**, 193–202.

Sawabe, T., Sugimura, I., Ohtsuka, M., Nakano, K., Tajima, K., Ezura, Y. & Christen, R. (1998). *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone *Haliotis discus hannai*. *Int J Syst Bacteriol* **48**, 573–580.

Sawabe, T., Thompson, F. L., Heyrman, J. & 7 other authors (2002). Fluorescent amplified fragment length polymorphism and repetitive

extragenic palindrome-PCR fingerprinting reveal host-specific genetic diversity of *Vibrio halioticoli*-like strains isolated from the gut of Japanese abalone. *Appl Environ Microbiol* **68**, 4140–4144.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M. & Ezura, Y. (2003). Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone–*V. halioticoli* association. *Aquaculture* **219**, 671–679.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy*. San Francisco: W. H. Freeman.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thompson, F. L., Hoste, B., Vandemeulebroecke, K. & Swings, J. (2001). Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Syst Appl Microbiol* **24**, 520–538.

West, M., Burdash, N. M. & Freimuth, F. (1977). Simplified silver-plating stain for flagella. *J Clin Microbiol* **6**, 414–419.