Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica

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The aetiological agent of bleaching of the coral *Oculina patagonica* was characterized as a new *Vibrio* species on the basis of 16S rDNA sequence, DNA–DNA hybridization data and phenotypic properties, including the cellular fatty acid profile. Based on its 16S rDNA and DNA–DNA hybridization, the new *Vibrio* species is closely related to *Vibrio mediterranei*. The name *Vibrio shiloi* sp. nov. is proposed for the new coral-bleaching species, the type strain being AK1^T (= ATCC BAA-91^T = DSM 13774^T).

Keywords: coral bleaching, *Vibrio*, *Vibrio shiloi*

INTRODUCTION

Coral bleaching is the disruption of symbioses between coral hosts and photosynthetic microalgal endosymbionts, referred to as zooxanthellae (Brown *et al.*, 1995). The loss of the pigmented zooxanthellae causes the coral to lose colour (bleaching) and eventually die, since a major portion of the coral's nutrition comes from the photosynthetic products of the algae. Coral-bleaching events of unprecedented frequency and global extent were reported in the 1980s and 1990s (Glynn, 1991a, b; Goreau, 1990, 1994; Hoegh-Guldberg, 1999). Coral bleaching may be induced by a variety of environmental stimuli, including increased seawater temperature (Jokiel & Coles, 1990). There has been speculation that large-scale bleaching episodes are linked to global warming (Hoegh-Guldberg, 1999).

Recently, we reported that bleaching of the coral *Oculina patagonica*, present in the Mediterranean Sea, is the result of a bacterial infection (Kushmaro *et al.*, 1996, 1997). The causative agent, referred to as *Vibrio* sp. AK1, was present in all bleached *O. patagonica* and absent from all healthy (unbleached) corals examined. Pure cultures of AK1^T caused bleaching when inoculated onto unbleached corals in controlled aquarium experiments. The first step in the infection process is the adhesion of cells of AK1^T to the coral through a β -D-galactopyranoside receptor (Toren *et al.*, 1998). Interestingly, the binding occurs only when the bacteria are grown at elevated seawater temperatures, which may explain how increased temperature causes

coral bleaching (Kushmaro *et al.*, 1998). Following adhesion, AK1^T penetrates into the coral tissue, multiplies intracellularly (Banin *et al.*, 2000) and produces toxins that inhibit photosynthesis and bleach and lyse the intracellular zooxanthellae (Ben-Haim *et al.*, 1999). Although members of the genus *Vibrio* are common pathogens in the marine environment, AK1^T is the first vibrio that attacks zooxanthellae and has been proven to be responsible for the coral-bleaching disease. The present study was carried out in order to characterize the coral-bleaching pathogen further. The data suggest that strain AK1^T is a new marine species, for which the name *Vibrio shiloi* sp. nov. is proposed.

METHODS

Micro-organisms, media and growth conditions. *V. shiloi* AK1^T (= DSM 13774^T) was isolated from a bleached coral sample, as described previously (Kushmaro *et al.*, 1996, 1997). Using the same techniques, an additional isolate (*V. shiloi* AK2) was obtained from bleached corals at a different site in the Mediterranean Sea, off the coast of Israel. For comparative studies, *Vibrio mediterranei* ATCC 43341^T (Pujalte & Garay, 1986) was used. The *Vibrio* strains were routinely cultivated in MB medium (0.9 % NaCl plus 1.8 % Marine broth; Difco) or on MB agar (MB medium solidified with 1.8 % Difco Bacto agar).

Phenotypic characterization. Biochemical tests were performed by using the API-20 NE system (micromethod tests for the identification of Gram-negative rods; bioMérieux). The standard API-20 NE method was used except that the media were adjusted to 3 % NaCl. Growth and acid production from sucrose were measured on TCBS agar (Difco) after incubation at 30 °C for 48 h. Salt tolerance was determined in nutrient broth (Difco) containing varying concentrations of NaCl. Sensitivity to the vibriostatic compound O129 (2,4-diamino-6,7-diisopropylpteridine;

The GenBank/EMBL accession number for the 16S rDNA sequence of *Vibrio shiloi* AK1^T is AF007115.

Sigma) was determined after incubation for 48 h at 30 °C on MB agar containing 30 µg of the compound on a disc.

Carbon utilization was analysed using BIOLOG GN microwell plates. Pure cultures were removed from MB plates and suspended in 20 ml seawater and the density of the suspension was adjusted so that the OD₅₉₀ was 0.130–0.145. The suspension was then distributed into BIOLOG GN microwell plates. Each plate contained 96 microwells with a different carbon source in each and tetrazolium violet as an indicator of metabolic activity. The plates were incubated for 48 h at 30 °C. Wells that changed to purple were marked as positive for metabolic activity.

The microbial fatty acid profile was analysed using the MIDI/Hewlett Packard microbial identification system (Analytical Services Inc.), which uses GC profiles of fatty acid methyl esters (Urdaci *et al.*, 1990). Bacteria were grown on MB agar at 30 °C for 48 h.

For electron microscopy, exponentially growing bacteria in MB medium, as well as bacteria inside coral cells, were centrifuged and resuspended in seawater. The samples were adhered to a carbon-coated grid and stained with 1% uranyl acetate and photographed under a JEM-1200EX electron microscope (JEOL).

DNA isolation, amplification, sequencing and sequence analysis. Total DNA was extracted from 10 ml bacterial cultures by using the SDS/proteinase/phenol/chloroform method described by Sambrook *et al.* (1989). 16S rDNA was amplified in a 50 µl reaction consisting of 5 µl 10× PCR buffer, 1 µl 2.5 mM total dNTP mixture, 5 µM of each primer, 10 ng template DNA and 2.5 units Ex Taq DNA polymerase (Takara Shuzo). The primers used were 5'-GAGTTTGATCCTGGCTCAG-3' (positions 9–27, *Escherichia coli* numbering system) and 5'-AGAAAGGAGGTGATCCAGCC-3' (positions 1525–1544) (Rudner *et al.*, 1998). Amplification conditions for the PCR (PTC-100; MJ Research Inc.) included an initial denaturation step at 95 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 5 min. Reaction products were checked for size and purity on 0.8% agarose gels and the DNA fragments were then recovered with a QIAquick gel extraction kit (Qiagen). DNA sequencing was performed using the chain-termination method in an ABI Prism (model 377, version 2.1.1) automatic sequencer. Primers used for the sequencing reaction were complementary to the conserved regions of the 16S rDNA. The novel sequence of the *V. shiloi* AK1^T 16S rDNA (1501 bp) was compared with homologous *Vibrio* 16S rDNA sequences taken from published databases (see Fig. 2). Calculation of similarity values and construction of the phylogenetic tree were performed as described by Kita-Tsukamoto *et al.* (1993).

DNA–DNA hybridization. DNA–DNA similarity experiments between *V. shiloi* AK1^T and *V. mediterranei* ATCC 43341^T were carried out by the renaturation method (Escara & Hutton, 1980; Huß *et al.*, 1983), using DNA samples isolated by the method of Cashion *et al.* (1997). Reassociation was performed in 2× SSC at 65 °C (optimal conditions). Similarity values were calculated according to Jahnke (1992).

Cell fixation, antibodies and immunofluorescence microscopy. Overnight cultures of *V. shiloi* AK1^T and *V. mediterranei* ATCC 43341^T grown in MB were fixed with 4% freshly prepared paraformaldehyde (final concentration) and incubated for 1–3 h at the growth temperature. The cells were washed five times in TBS (10 mM Tris base,

150 mM NaCl). Polyclonal antibodies were obtained from the serum of a rabbit, immunized with fixed *V. shiloi* cells. Fixed cells (10⁶ cells ml⁻¹) were attached to microscope slides covered with poly-L-lysine (50–100 µg ml⁻¹) and incubated for 2 h at room temperature. The slides were then washed once in TBS and incubated for 12 h at 4 °C with the polyclonal antibodies (1:1000 dilution in TBS). The slides were washed five times in TBS and incubated with 5 µg Rhodamine Red-X-conjugated anti-rabbit IgG ml⁻¹ (Jackson ImmunoResearch). After incubation, the slides were washed five times in TBS and mounted with a solution of 90% glycerol containing 20 µg 4,6-diamidino-2-phenylindole (DAPI) ml⁻¹ and 1 mg *p*-phenylenediamine ml⁻¹. Coverslips were sealed and the slides were stored at –30 °C until examination. The slides were examined using a Leica microscope (model DMRBE) with set filters A for DAPI and M2 filter for rhodamine.

RESULTS

Exponentially growing *V. shiloi* AK1^T is a Gram-negative, motile, rod-shaped bacterium (2.4 × 1.6 µm) that has a single, polar, sheathed flagellum (Fig. 1a).

Fig. 1. Electron micrographs of negatively stained cells of *V. shiloi* AK1^T. (a) Exponentially growing cells. (b) Cells growing intracellularly. Bars, 0.5 µm.

Fig. 2. Phylogenetic tree based on 16S rDNA sequences, showing the relationships among 27 *Vibrio* reference strains in comparison to *V. shiloi* AK1^T (arrow). Sequence accession numbers are given. *Salmonella typhi* was used as the outgroup.

When growing intracellularly in coral tissue, the cells lack the flagellum and are smaller ($2.0 \times 1.0 \mu\text{m}$; Fig. 1b). These properties, together with its ability to form yellow colonies on TCBS agar and its sensitivity to the vibriostatic agent O129, suggest that AK1^T is a species of the genus *Vibrio* (Farmer & Hickman-Brenner, 1992). The sequence of the 16S rDNA of strain AK1^T was determined and compared with data for known *Vibrio* species (Fig. 2). The sequence of strain AK1^T is related most closely to *V. mediterranei*, with 1425 of 1438 of the nucleotides being identical, 9 being different (including a nucleotide gap) and 4 undefined (in *V. mediterranei*). Assuming that the undefined residues are identical, the overall 16S rDNA similarity is 99.4%. DNA–DNA hybridization results showed that strain AK1^T and *V. mediterranei* are 72% related at the total DNA level.

The cellular fatty acid composition of *V. shiloi* AK1^T is summarized in Table 1. Comparison of the fatty acid profile of strain AK1^T with bacteria in the database also placed it clearly in the genus *Vibrio*. However, as seen in Table 1, the fatty acid composition of *V. shiloi* AK1^T is very different from that of *V. mediterranei*.

Table 1. Fatty acid profiles of *V. shiloi* AK1^T and *V. mediterranei* ATCC 43341^T

Values are percentages of the total fatty acid. Fatty acids are listed as follows: numbers before the colon indicate the number of carbon atoms and numbers after the colon refer to the number of double bonds; 3-OH indicates a hydroxy group at carbon-3; $\omega 5c$, $\omega 7c$, $\omega 9t$ etc. indicate the double-bond position from the hydrocarbon end of the carbon chain; *c*, *cis* isomer; *t*, *trans* isomer. ND, Not detected.

Fatty acid	<i>V. shiloi</i> AK1 ^T	<i>V. mediterranei</i> ATCC 43341 ^T
12:0 iso	0.1	ND
12:0	4.7	5.0
13:1 iso	ND	1.1
13:0 iso	0.4	2.0
12:0 iso 3-OH	0.1	ND
12:0 3-OH	2.0	2.2
14:0 iso	4.0	1.3
14:1	ND	1.0
14:0	5.9	8.0
15:0 iso	0.5	2.3
15:0 anteiso	0.5	ND
15:1	ND	2.0
15:0	0.4	0.3
14:0 iso 3-OH	1.1	ND
14:0 3-OH	ND	2.6
16:1 iso or 14:0 3-OH	3.1	ND
16:0	10.0	2.5
16:1	ND	30.0
16:1 $\omega 7c$ or 15:0 iso 2-OH	32.5	ND
16:1 $\omega 5c$	0.1	ND
16:0	12.1	16.0
17:0 iso	0.2	1.6
17:0 anteiso	0.3	ND
17:1	ND	2.7
17:1 $\omega 8c$	0.6	ND
17:0	0.2	0.3
18:1 iso H	0.5	ND
18:0 iso	1.6	ND
18:1	ND	19.0
18:1 $\omega 7c$ or $\omega 9t$ or $\omega 12t$	18.4	ND
18:0	0.2	0.3
Unknown or 19:0 cyclo $\omega 10c$	0.1	ND

Furthermore, it could not be placed in any of the 40 *Vibrio* species in the database. The closest fits were to *Vibrio cholerae* Inaba, *Vibrio fluvialis* and *Vibrio furnissii*, but strain AK1^T differed from each of them by more than these species differed from one another. A further indication that the cell surface of strain AK1^T is very different from that of *V. mediterranei* was that antibodies prepared against strain AK1^T did not react with *V. mediterranei* ATCC 43341^T.

Table 2. Phenotypic differences between *V. shiloi* AK1^T and *V. mediterranei* ATCC 43341^T

Character	<i>V. shiloi</i> AK1 ^T	<i>V. mediterranei</i> ATCC 43341 ^T
Bleaching of <i>Oculina patagonica</i> *	+	—
Fatty acid content (% of total):		
16:1	ND	30.0
16:1 ω 7c or 15:0 iso 2-OH	32.6	ND
17:1	ND	2.5
18:1	ND	19.0
18:1 ω 7c or ω 9t or ω 12t	18.4	ND
Reaction to anti- <i>Vibrio</i> sp. AK1 antibodies	+	—
Growth on 6% NaCl	—	+
Utilization of:		
N-Acetyl D-galactosamine	—	+
m-Inositol	—	+
Monomethyl succinate	—	+
β -Hydroxybutyric acid	+	—
Methyl β -D-glucoside	+	—
D-Lactose	+	—
L-Rhamnose	+	—
Glucuronamide	+	—

* Bleaching experiments were conducted by spotting 10⁶ bacterial cells on corals and incubating them for 3 weeks in a 2 l aerated aquarium at 25 °C.

ND, Not detected.

Biochemical tests and carbon compound utilization data of the two independently isolated coral pathogens *V. shiloi* AK1^T and *V. shiloi* AK2 were identical. For comparative purposes, all of the tests were performed on *V. mediterranei* ATCC 43341^T under the same conditions. The phenotypic differences between *V. shiloi* AK1^T and *V. mediterranei* are summarized in Table 2.

DISCUSSION

The causative agent of bleaching of the coral *O. patagonica* (Kushmaro *et al.*, 1996, 1997) is a *Vibrio* species, based on all genotypic and phenotypic tests performed. The question of whether to classify it as a new species, closely related to *V. mediterranei*, or as a subspecies of *V. mediterranei* is arbitrary. The 16S rDNA sequence similarity (99.4%) and total DNA–DNA hybridization data (72%) are both borderline values for what would currently be defined as a new species. For example, *V. cholerae* (classic Ogawa) and *Vibrio mimicus* show 99.6% sequence identity in their 16S rDNAs and are considered different species. When one takes additional data into consideration (Table 2), it would appear that the definition of a new species is justified. The property that led to the isolation of *V. shiloi* AK1^T and AK2 is its ability to infect and bleach its host coral. This virulence property is not only of considerable ecological significance, but also leads to a unique lifestyle. *V. shiloi* AK1^T adheres specifically to its host coral, penetrates into the coral tissue, multiplies intracellularly and subsequently kills the coral

zooxanthellae, leading to coral bleaching (Banin *et al.*, 2000; Ben-Haim *et al.*, 1999). *V. mediterranei* is not a coral pathogen and has never been shown to grow intracellularly.

The large differences in cellular fatty acids between *V. shiloi* AK1^T and *V. mediterranei* should be considered as an additional valuable tool in evaluating taxonomic differences between the two strains. The major fatty acids in *V. mediterranei* are hexadecane (30%) and octadecane (19%), both of which are not present in *V. shiloi* AK1^T. Also, the major fatty acids in *V. shiloi* AK1^T are not present in *V. mediterranei* (Table 2). It is also relevant that polyclonal antibodies generated against *V. shiloi* AK1^T reacted strongly with *V. shiloi* AK2, but reacted very weakly, if at all, with *V. mediterranei*. It may be that the very different fatty acids and cell-surface antigens reflect a different style of growth. In this regard, we have never been able to isolate *V. shiloi* from seawater, only from bleached corals.

Another significant difference between *V. shiloi* and *V. mediterranei* is their salt tolerance. All *V. mediterranei* strains grow in the presence of 6% NaCl, whereas *V. shiloi* AK1^T failed to grow in media containing more than 4% NaCl. Comparison of carbon compound utilization data of *V. shiloi* AK1^T and *V. mediterranei* showed eight differences (Table 2). The ability of *V. shiloi* AK1^T to utilize β -hydroxybutyrate is not common amongst members of the genus *Vibrio*. The literature indicates that all *V. mediterranei* strains fail to utilize methyl β -D-glucoside, D-lactose and

L-rhamnose (Farmer & Hickman-Brenner, 1992), whereas *V. shiloi* AK1^T utilized these compounds.

Description of *Vibrio shiloi* sp. nov.

Vibrio shiloi (shi'lo.i. N.L. gen. n. *shiloi* of Shilo, named after the late microbiologist Moshe Shilo).

Vibrio shiloi conforms to the descriptions of the family *Vibrionaceae* and of the genus *Vibrio* (Farmer & Hickman-Brenner, 1992). Cells are Gram-negative, non-spore-forming rods (2.4 × 1.6 µm) that are motile by a single, polar, sheathed flagellum when grown on solid or liquid medium. Colonies are pale yellow on Marine agar after 48 h incubation at 30 °C, have slightly serrated edges and do not luminesce. When grown on 0.4% agar, the cells swarm. Oxidase- and catalase-positive. Produces acid, but not gas, aerobically and anaerobically from D-glucose and sucrose. Growth in Marine broth occurs at 16–37 °C but not at 42 °C. Grows in Nutrient broth containing 2–4% NaCl, but no growth in 1 or 6% NaCl. Reduces nitrate to nitrite and produces indole. Shows positive reactions for lysine decarboxylase, β-galactosidase, gelatinase and β-glucosidase and negative reactions for ornithine decarboxylase, urease and arginine dihydrolyase. Cells utilize dextrin, glycogen, Tweens 40 and 80, N-acetyl D-glucosamine, cellobiose, D-fructose, L-fructose, D-mannitol, D-mannose, methyl β-D-glucoside, D-psicose, L-rhamnose, D-sorbitol, D-trehalose, turanose, methyl pyruvate, *cis*-aconitate, citric acid, D-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, β-hydroxybutyric acid, DL-lactic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartate, glycyl L-glutamate, L-histidine, L-ornithine, L-proline, L-serine, L-threonine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, putrescine, glycerol, glucose 1-phosphate and glucose 6-phosphate. Susceptible to 2,4-diamino-6,7-diisopropylpteridine (O129 vibriostatic agent), penicillin, ampicillin, tetracycline, oxytetracycline, streptomycin and kanamycin.

Isolated from and pathogenic for the Mediterranean coral *Oculina patagonica*, causing death of the endosymbiotic zooxanthellae and bleaching of the coral at temperatures of 20 to 32 °C. The type strain is strain AK1^T (= ATCC BAA-91^T = DSM 13774^T).

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