**Vibrio owensii** sp. nov., isolated from cultured crustaceans in Australia

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Keywords

Vibrio owensii, Vibrio harveyi, Vibrio campbellii; MLSA; crustacean; pathogen.

Abstract

Two bacterial strains (DY05T and 47666-1) were isolated in Queensland, Australia, from diseased cultured crustaceans *Panulirus ornatus* and *Penaeus monodon*, respectively. On the basis of 16S rRNA gene sequence identity, the strains were shown to belong to the Harveyi clade of the genus *Vibrio*. Multilocus sequence analysis using five housekeeping genes (*rpoA*, *pyrH*, *topA*, *ftsZ* and *mreB*) showed that the strains form a monophyletic group with 94.4% concatenated sequence identity to the closest species. DNA–DNA hybridization experiments showed that strains DY05T and 47666-1 had 76% DNA similarity to each other, but <70% to their closest neighbours *Vibrio harveyi* LMG 4044T (≤55%), *Vibrio campbellii* LMG 11216T (≤52%) and *Vibrio rotiferianus* LMG 21460T (≤46%). Strains DY05T and 47666-1 could be differentiated from their relatives on the basis of several phenotypic characteristics. The major fatty acids were C15:0 iso 2-OH and/or C16:1 α7, C16:0, C18:1 α7 and C14:0. Based on the polyphasic evidence presented here, it can be concluded that strains DY05T and 47666-1 belong to the same novel species of the genus *Vibrio*, for which the name *Vibrio owensii* sp. nov. is proposed. The type strain is DY05T (= JCM 16517T = ACM 5300T).

**Introduction**

Recently, the number of bacterial species of the genus *Vibrio* (Farmer et al., 2005) has increased noticeably. Currently, the Harveyi clade (Sawabe et al., 2007) includes eight species: *Vibrio harveyi*, *Vibrio campbellii*, *Vibrio rotiferianus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio mytii*, *Vibrio natriegens* and the newly described *Vibrio azureus* (Yoshizawa et al., 2009). Among this group, *V. harveyi* has been recognized as the most significant pathogen of marine-reared fish and crustaceans (Karunasagar et al., 1994; Zhang & Austin, 2000), and several studies have reported infections by this species in molluscs and corals (Nishimori et al., 1998; Sutherland et al., 2004). More recently, however, molecular analyses revealed that some disease causing strains of *V. campbellii* have been misidentified as *V. harveyi*, underestimating the significance of the former species as an aquaculture pathogen (Gómez-Gil et al., 2004). Here, we describe the physiological, chemotaxonomic and phylogenetic characteristics of two bacterial strains pathogenic to cultured crustaceans sharing the highest 16S rRNA gene sequence identities with *V. harveyi*, *V. campbellii* and *V. rotiferianus*.

**Materials and methods**

**Isolation and bacterial strains**

The strain 47666-1 was isolated from diseased *Penaeus monodon* larvae in a commercial prawn hatchery in North Queensland, Australia, and subsequently shown to be highly virulent to prawn larvae (Harris, 1993; Pizzuto & Hirst, 1995). Similarly, strain DY05T was isolated from diseased larvae of the ornate spiny lobster *Panulirus ornatus* in the Tropical Aquaculture Facility of the Australian Institute of Marine Science (AIMS), North Queensland, Australia, and subsequently shown to be highly virulent to lobster larvae (unpublished data). Bacteria (strains DY05T and 47666-1, *V. harveyi* LMG 4044T, *V. campbellii* LMG 11216T, *V. rotiferianus* LMG 21460T and *V. rotiferianus* CAIM 994) were cultured on thiosulphate–citrate–bile–salts–sucrose (TCBS) agar and marine agar (MA) at 28 °C. Stock cultures were
maintained frozen at $-80\,^{\circ}\mathrm{C}$ in either marine broth (MB) with 30% v/v glycerol or in Microbank™ cryovials (Pro-Lab Diagnostics).

**Phenotypic characterization**

For morphology and physiology studies, cells were grown for 24–48 h at $28\,^{\circ}\mathrm{C}$ on MA or in MB. Gram staining was performed using a Gram stain kit (Becton Dickinson, BD) according to the manufacturer’s instructions. Cell morphology, size and motility were determined by light microscopy (CX31, Olympus). Luminescence was observed in the dark and measured using a 1420 Wallac Multilabel Counter (Perkin Elmer) at 4-h intervals. Phenotypic analyses using API 20E, API 20NE and API ZYM commercial kits (bioMérieux) were performed according to the manufacturer’s instructions, except that a 2% NaCl (w/v) solution was used to prepare the inocula and the strips were incubated at $28\,^{\circ}\mathrm{C}$ for 48 h. The API 20E and 20NE were performed in triplicate, with *V. harveyi* LMG 4044$^{T}$ and *V. campbellii* LMG 11216$^{T}$ included as references. Salt tolerance was determined in PY broth [0.3% w/v neutralized peptone (Oxoid) and 0.1% w/v yeast extract (BD)] supplemented with NaCl concentrations between 0% and 10% (w/v) for 72 h at $28\,^{\circ}\mathrm{C}$ with shaking. Growth responses to temperatures between 4 and $45\,^{\circ}\mathrm{C}$ were tested in PY broth with 2% w/v NaCl for 72 h with shaking. Antibiotic sensitivity was determined using the disk susceptibility assay as described by Lane (1991) and sequenced using the BLASTN program (Altschul et al., 1990). Subsequently, sequences of our two strains, close relatives and type strains of related vibrios were aligned by ARR (Ludwig et al. 2004) or CLUSTAL_X (Thompson et al., 1997) for 16S rRNA and protein-coding genes, respectively. For ARR alignments, manual corrections were performed, where necessary, based on 16S rRNA gene secondary structure. Phylogenetic analyses were performed with PAUP v4.0B10 (Swofford, 2003). Distance matrices were generated according to the Kimura two-parameter correction (Kimura, 1980), and phylogenies were constructed by neighbor-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1973) methods. The stability of groupings was estimated by bootstrap analyses (1000 replications).

**DNA–DNA hybridization and DNA base composition**

DNA–DNA hybridization values between DY05$^{T}$ and 47666-1 and between these strains and type strains of *V. harveyi* (LMG 4044$^{T}$), *V. campbellii* (LMG 11216$^{T}$) and *V. rotiferianus* (LMG 21460$^{T}$) were determined. Genomic DNA was prepared according to a modification of the method described by Ezaki et al. (1989). The DNA mol% G+C content was determined by HPLC according to the method of Mesbah et al. (1989).

**Results and discussion**

**Phenotypic characteristics**

Phenotypically, strains DY05$^{T}$ and 47666-1 can be clearly assigned to the genus *Vibrio* (Alsina & Blanch, 1994). Characteristics distinguishing DY05$^{T}$ and 47666-1 from other strains in the Harveyi clade are presented in Table 1. The strains can be distinguished from most other arginine dihydrolase (ADH)-negative, ornithine and lysine decarboxylase (ODC and LDC)-positive vibrios by their inability to utilize citrate and their ability to produce acid from amygdalin. The latter characteristics are shared with
Table 1. Differential phenotypic characters between Vibrio owensii sp. nov. and species of the Harveyi clade

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Data of related species from Carson et al. (2006) unless otherwise indicated.

*Test used to differentiate ADH*, *LDC*, *ODC* *Vibrio* species (Alsina & Bianch, 1994).


+ positive; – negative; v, variable between strains; w, weak reaction; ND, no data.

V. rotiferianus and V. azureus, but DY05T and 47666-1 can be distinguished from these species by several tests including LDC (both species) and acid production from arabinoose (V. rotiferianus), sucrose and mannitol (V. azureus). It should be noted that 15 out of 62 previously classified *V. harveyi* strains were reported to be positive for amygdalin (Carson et al., 2006), and further genotypic analyses would be useful to determine the relatedness between these strains and the newly described species. Strains DY05T and 47666-1 showed similar biochemical profiles, except for the α-nitrophenyl-β-D-galactopyranosidase (ONPG) test, which was positive only for 47666-1.

The predominant fatty acids of strains DY05T and 47666-1 were C15:0 iso 2-OH and/or C16:1ω7 36.6–37.5%, C16:0 16.6–16.7%, C18:1ω7 14.6–16.4% and C14:0 (6.0–6.3%). For other fatty acids, see the species description and Table S1. No clear differences from the closely related species *V. harveyi*, *V. campbellii* and *V. rotiferianus* grown under identical conditions (Gómez-Gil et al., 2003) were observed (Table S1). None of the strains showed luminescence in vitro. Strain 47666-1 was originally reported as luminescent (Harris, 1993), but we could not confirm this.

### Phylogenetic analyses

The 16S rRNA gene sequence analysis showed that strains DY05T and 47666-1 belong to the Harveyi clade. The strains shared 99.2–99.5% 16S rRNA gene sequence identities with other species of this clade, but the two strains formed a monophyletic group with 99% bootstrap support (Fig. 1) and 100% 16S rRNA gene sequence identity, supporting their close affiliation. The mean sequence identity for the concatenated five protein-coding loci was 98.8% between strains DY05T and 47666-1 and 94.4% between these strains and the relatives *V. harveyi*, *V. campbellii* and *V. rotiferianus*. Discrimination between these species on the basis of phenotypic and 16S rRNA gene analyses is difficult and additional molecular methods such as MLSA have become important tools for the correct species delineation and identification (Sawabe et al., 2007; Thompson et al., 2007). Phylogenetic trees generated for concatenated sequences of the five protein-coding loci using NJ, MP and ML methods confirmed the clustering of strains DY05T and 47666-1 (bootstrap values of 100%, 100% and 95%, respectively) and their distinction to close species (Fig. 2, Fig. S1a and b).

An extended phylogenetic analysis was performed to detect public database sequences that could potentially belong to the same species as strains DY05T and 47666-1. Using database sequences for the *pyrH*, *topA* and *mreB* loci, *Vibrio* sp. CAIM 994 clustered with DY05T and 47666-1 in single-gene phylogenetic analyses. Thus, we acquired this strain, isolated from snapper (*Lutjanus guttatus*) in the northwest coast of Mexico, and determined its 16S rRNA and *rpoA* gene sequences. Strain CAIM 994 was initially identified as *V. rotiferianus*, but described as a possible intermediate strain according to MLSA (Thompson et al., 2007). Phylogenies based on 16S rRNA gene sequences (Fig. 1) and concatenated sequences of five protein-coding loci (Fig. 2) confirmed that CAIM 994, 47666-1 and DY05T formed a monophyletic group with bootstrap support values of 99–100%. CAIM 994 shared 99.9% (16S rRNA gene) and 98.3% (five protein-coding loci) gene sequence identities with DY05T and 47666-1. These are greater than the identities shared between CAIM 994 and DY05T and 47666-1. These are greater than the identities shared between CAIM 994 and DY05T and 47666-1. Therefore, 16S rRNA gene and MLSA support the notion that CAIM 994 was previously misidentified. Further studies based on phenotypic and genotypic characterization would be required to clarify the relatedness of this and other strains clustering with the *Vibrio owensii* sp. nov. proposed here.
DNA--DNA hybridization and DNA base composition

Strains DY05\(^T\) and 47666-1 showed 76% DNA--DNA hybridization values with each other and 44–55% with *V. harveyi* LMG 4044\(^T\), *V. campbellii* LMG 11216\(^T\) and *V. rotiferianus* LMG 21460\(^T\) (Table S2). As a DNA--DNA hybridization value of 70% is generally accepted as the limit for species delineation (Wayne *et al*., 1987), it can be concluded that strains DY05\(^T\) and 47666-1 belong to a single novel species.

The DNA mol% G\(_1\)C content of DY05\(^T\) (45.3 mol%) and 47666-1 (45.9 mol%) support their affiliation with *Vibrio* (Baumann & Schubert, 1983).

It can be concluded that strains DY05\(^T\) and 47666-1 are closely related to *V. harveyi*, *V. campbellii* and *V. rotiferianus* in terms of 16S rRNA gene sequences and phenotypic profiles, but that they can be differentiated from all *Vibrio* species described previously by means of MLSA (rpoA, pyrH, topA, fsiZ and mreB genetic loci), DNA–DNA hybridizations and several biochemical characteristics. The strains can be identified by performing tests for LDC and ODC, citrate utilization and acid production from amygdalin, arabinose and sucrose (API 20E system). Based on these results, strains DY05\(^T\) and 47666-1 clearly represent a novel species of the genus *Vibrio*, for which the name *V. owensii* sp. nov. is proposed.

**Description of *V. owensii* sp. nov.**

*Vibrio owensii* (o.wens’i.i. N.L. gen. n. owensii, of Owens, named to honor L. Owens, an Australian microbiologist and specialist in the biology of *V. harveyi*-related species).

Cells are slightly curved Gram-negative rods, 1.0 \(\mu\)m wide \(\times\) 3.1 \(\mu\)m long, facultative anaerobic and motile by...
means of at least one flagellum. After growth for 48 h at 28\degree C, the strains form translucent (DY05\textsuperscript{T}) or opaque (47666-1), nonluminescent, nonswarming, smooth and round colonies (2–3 mm) on MA, and bright, yellow and round colonies (2–3 mm) on TCBS agar. Growth occurs in the presence of 1–8\% NaCl (w/v), but not at 0\% or 10\% NaCl. The minimum temperature for growth is 12–15\degree C, while the maximum temperature for growth is 35–37\degree C. No growth occurs at 4\degree C. Both strains are ADH-negative, LDC- and ODC-positive. Tests for citrate utilization, production of H\textsubscript{2}S, urease, Voges–Proskauer, assimilation of arabinose, and acid production from inositol, sorbitol, rhamnose, melibiose and arabinose are negative, while tests for nitrate reduction, indole production, tryptophan deaminase, gelatinase, oxidase, hydrolysis of esculin, assimilation of glucose, mannose, mannotol, potassium gluconate and malate and fermentation of glucose, mannitol, sucrose and amygdalin are positive. Enzyme activities detected by API ZYM tests are alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, \alpha-chymotrypsin, acid phosphatase and naphtol-AS-\beta-phosphohydrolase. A difference between strains was seen for the ONPG test, which was positive for 47666-1 and negative for DY05\textsuperscript{T}. Both strains were susceptible to chloramphenicol (30 \textmu g), gentamicin (10 \textmu g), sulphisoxazole (300 \textmu g), trimethoprim-sulphamethoxazole (1/19) (1.25–23.75 \textmu g) and tetracycline (30 \textmu g) and vibrio-static agent O/129 (10 and 150 \textmu g); intermediate to erythromycin (15 \textmu g) and kanamycin (30 \textmu g), and resistant to ampicillin (10 \textmu g). The major fatty acids (> 1\% for at least one strain) are summed feature 3 (C\textsubscript{16:1} \textit{ω}7c and/or C\textsubscript{15} iso 2-\textit{OH}), C\textsubscript{16:0}, C\textsubscript{18:1} \textit{ω}7c, C\textsubscript{14:0}, C\textsubscript{16:0} iso, C\textsubscript{12:0}, summed feature 2 (C\textsubscript{14:0} 3-\textit{OH} and/or C\textsubscript{16:1} iso 1), C\textsubscript{17:0} iso, C\textsubscript{17:1} \textit{ω}8c, C\textsubscript{17:0} 3-\textit{OH} and C\textsubscript{18:0}. The DNA G+C content is 45.3–45.9 mol\%. The type strain is DY05\textsuperscript{T} (= JCM 16517\textsuperscript{T} = ACM 5300\textsuperscript{T}), isolated from cultured larvae of the ornate spiny lobster \textit{P. ornatus} in Queensland, Australia.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Phylogenetic analysis based on the (a) MP and (b) ML methods, using concatenated sequences of *rpoA* (884 bp), *pyrH* (421 bp), *topA* (587 bp), *ftsZ* (443 bp) and *mreB* (507 bp) loci (total length, 2842 bp) from *Vibrio owensii* strains and other species of the Harveyi clade.

**Table S1.** Fatty acid composition of *Vibrio owensii* sp. nov. and related species as reported by Gómez-Gil *et al.* (2003).

**Table S2.** DNA–DNA hybridization values among *Vibrio owensii* sp. nov. and type strains of related species.

**Table S3.** List of strains and sequence accession numbers included in the MLSA.

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