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ORIGINAL PAPER

Vibrio tetraodonis subsp. *pristinus* subsp. nov., isolated from the coral *Acropora cytherea* at Palmyra Atoll, and creation and emended description of *Vibrio tetraodonis* subsp. *tetraodonis* subsp. nov

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Abstract Strain OCN044^T was isolated from the homogenised tissue and mucus of an apparently healthy *Acropora cytherea* coral fragment collected from the western reef terrace of Palmyra Atoll in the Northern Line Islands and was taxonomically evaluated with a polyphasic approach. The morphological and chemotaxonomic properties are consistent with characteristics of the genus *Vibrio*: Gram-stain-negative rods, oxidase- and catalase-positive, and motile by means of a polar flagellum. Strain OCN044^T can be differentiated as a novel subspecies based on 21 differences among chemotaxonomic features (e.g., fatty acids percentages for $C_{12:0}$ and $C_{18:1}$ ω7c), enzymatic activities (e.g., DNase and cystine arylamidase), and carbon sources utilized (e.g., L-xylose and D-melezitose) from its nearest genetic relative. Phylogenetic analysis and genomic comparisons show close evolutionary relatedness to *Vibrio tetraodonis* A511^T but the overall genomic relatedness indices identify strain OCN044^T as a distinct subspecies. Based on a polyphasic characterisation, differences in genomic and taxonomic data, strain OCN044^T represents a novel subspecies of *V. tetraodonis* A511^T, for which the name *Vibrio tetraodonis* subsp. *pristinus* subsp. nov. is proposed. The type strain is OCN044^T (=LMG 31895^T=DSM 111778^T).

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10482-022-01766-0) contains supplementary material, which is available to authorized users.

Repositories The strain collection identifiers for *Vibrio tetraodonis* subsp. *pristinus* subsp. nov. are LMG 31895 and DSM 111778. The 16S rRNA gene sequences for *Vibrio tetraodonis* subsp. *pristinus* subsp. nov. and *V. tetraodonis* A511^T are deposited at DDBJ/ENA/GenBank under the accession numbers MW872696 and ON808596, respectively, and the draft genome of *Vibrio tetraodonis* subsp. *pristinus* subsp. nov. was deposited at DDBJ/ENA/GenBank under the accession number WWEU00000000.

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Introduction

Members of the family *Vibrionaceae*, with over 190 accepted species, are Gram-stain-negative Gammaproteobacteria predominantly found in marine and estuarine environments (Pruzzo et al. 2005; Oliver et al. 2012; Jiang et al. 2021). A high abundance are detected in eutrophic aquatic ecosystems and colonised marine organisms (Thompson et al. 2004). *Vibrio* species are implicated as etiological agents of disease in coral, bivalves, shrimp, fish, and humans (Baker-Austin et al. 2018). Noteworthy examples of marine *Vibrio* pathogens and their hosts include *Vibrio pectenicida* and scallops (*Pecten maximus*) (Lambert et al. 1998), *Vibrio ostreicida* and Flat Oysters (*Ostrea edulis*) (Prado et al. 2005, 2014), *Vibrio tasmaeniensis* and Pacific oysters (*Crassostrea gigas*) (Gay et al. 2004), and *Vibrio coralliilyticus* and various coral, urchins, and bivalves (Ben-Haim et al. 2003; Sussman et al. 2008; Ushijima et al. 2014, 2016; Vezzulli et al. 2010; Estes et al. 2004; Balbi et al. 2019; Kesarcodi-Watson et al. 2009; Li et al. 2020; Nguyen et al. 2019; Richards et al. 2015). In contrast, some *Vibrio* species are considered mutualistic or commensal symbionts, like the bioluminescent *Aliivibrio fischeri* (Ruby 1996; McFall-Ngai et al. 2012). *A. fisheri* can colonise a specialised organ of the squid *Euprymna scolopes*, which facilitates counter-luminescent camouflage during the night. Genomic analysis of the recently described species *Vibrio tetraodonis* A511^T, isolated from the marine pufferfish *Sphoeroides spengleri*, identified gene families and clusters within the strain that may confer advantages to the pufferfish host (Azevedo et al. 2021). Additional *Vibrio* strains have been pursued as potential probiotics; for example, growth of the microalga *Chaetoceros muelleri* is improved when co-cultured with *Vibrio alginolyticus* C7b (Gomez-Gil et al. 2002; Verschuere et al. 2000; Sawabe et al. 2003; Riquelme et al. 2001). Researchers have also begun to assess the roles and interactions of mutualistic/commensal *Vibrio* in the coral holobiont (Koenig et al. 2011, Arboleda and Reichardt, 2009, Kvennefors et al. 2010). Studies on mutualistic *Vibrio* are less frequent than pathogenesis research, due in part to stigmatisation of this genus as being commonly associated with disease, which highlights the need for and novelty of additional work on non-pathogenic *Vibrio* species.

Strain OCN044^T was isolated from an apparently healthy *Acropora cytherea* colony and, based on partial 16S rRNA gene sequencing alone, strain OCN044^T was originally and incorrectly designated *V. nereis* strain OCN044 (Ushijima et al. 2016). This work assessed the virulence of various *V. coralliilyticus* strains and used strain OCN044^T as a negative control bacterium during infection trials, in which it did not induce obvious disease signs in apparently healthy *Acropora cytherea* or *Montipora capitata* coral fragments. Based on its isolation and inability to infect its host in laboratory trials, strain OCN044^T may be a member of the coral microflora. Much of the research on *Vibrio* species related to coral focuses on disease and pays little attention to members of the non-diseased microbial community. The continued study of this and other non-pathogenic *Vibrio* species may provide insight into interspecies interactions within the coral holobiont. The results of a polyphasic approach to characterize strain OCN044^T support the classification of the isolate as a novel subspecies of *V. tetraodonis* A511^T, for which the name *Vibrio tetraodonis* subsp. *pristinus* subsp. nov. is proposed.

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Materials and methods

Isolation, cultivation, and phenotypic characterisation

Strain OCN044^T was isolated from a fragment of healthy *A. cytherea* coral harvested from the western reef terrace of Palmyra Atoll in the Northern Line Islands as previously described (Ushijima et al. 2016). Briefly, the fragment was crushed in autoclaved seawater and the resulting crushate was plated on glycerol seawater agar (4 g l^{-1} tryptone, 2 g l^{-1} yeast extract, and 2 g l^{-1} glycerol in a liter of seawater) as previously described (Ushijima et al. 2016). The plated crushate was incubated at 29 °C overnight and colonies were purified on the same medium.

Morphological, physiological, and culture-based characterisation of strain OCN044^T and the related species *V. tetraodonis* A511^T and *Vibrio aquimaris* DSM 109633^T were carried out as previously described with some modifications (Beurmann et al. 2017; Azevedo et al. 2021; Franco et al. 2020). *V. tetraodonis* A511^T and *Vibrio aquimaris* DSM 109633^T were provided by the Collection of Aquatic Microorganisms (CAIM) in Mazatlan, Sinaloa, Mexico, and the Belgian Co-ordinated Collection of Micro-Organisms (BCCM) LMG collection in Ghent, Belgium, respectively. Strain properties were determined using lysogeny broth (LB) medium (Sigma-Aldrich, St. Louis, MO), glycerol artificial seawater medium buffered with 50 mM Tris base to pH 8.3 (GASW-Tris) and solidified with 1.5% agar (Ushijima and Häse, 2018), and Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (HiMedia, West Chester, PA), which was prepared according to the manufacturer's instructions and supplemented with NaCl up to a 3% final concentration. Strains were routinely cultured at 28 °C in GASW-Tris, solidified with 1.5% agar as needed or shaken at 150 RPM overnight unless otherwise noted. Cell morphology was examined by transmission electron microscopy (TEM). Cells from an overnight culture of strain OCN044^T grown in GASW-Tris at 28 °C were deposited on Formvar-coated grids, fixed with 1% uranyl acetate, and imaged on a Hitachi HT770 TEM at 100 kV. Swimming and swarming motility were determined on GASW-Tris plates supplemented with 0.26% or 1.5% agar, respectively; plates were incubated at 28 °C for 48 h and cultures were evaluated for movement outward from the inoculation site. Swimming was additionally assessed via the hanging drop method. Anaerobic growth was assessed on GASW-Tris plates incubated at 28 °C for 48 h using the GasPak System according to the manufacturer's instructions (BD, Franklin Lakes, NJ). The pH range supporting growth was determined with GASW medium without TRIS base adjusted to pH 4.0–10.0 in increments of 0.5 using the following buffers: pH 4.0–6.0, citrate/Na_2HPO_4; pH 6.0–8.0, phosphate buffer; pH 9.0–10.0, glycine/NaOH (McCauley et al. 2015). The pH was adjusted prior to sterilisation, verified after sterilisation prior to inoculation, and verified again after incubation. The temperature range supporting growth was assessed on GASW-Tris medium incubated from 0 to 45 °C in 5 °C increments. Tolerance to NaCl was determined on LB medium buffered to pH 8.3 with 50 mM Tris base and supplemented with 0–10% NaCl in 0.5% increments from 0 to 2% and 1% increments from 3 to 10%.

Carbon source utilisation and enzyme activity tests were carried out using the API 50 CH, API ZYM, and API 20E kits according to the manufacturer's instructions (BioMérieux, Marcy-l'Étoile, France), the only modification was the final concentration of the suspension medium for these kits was adjusted to 3% NaCl. Utilisation of citrate was determined on Simmon's citrate medium (BD) supplemented with 3% NaCl according to the manufacturer's instructions. Catalase activity was assessed with the addition of 3% H_2O_2 to colonies grown on GASW medium, with the formation of bubbles interpreted as a positive result. Determination of oxidase activity was carried out with BD BBL DrySlide tests according to the manufacturer's instructions (BD). Nitrate and nitrite reduction tests were conducted as previously described [36], with the medium supplemented with 3% NaCl. SIM, MR-VP, and urease media (BD) were prepared with 3% NaCl and the tests were conducted according to the manufacturer's instructions after two days of incubation at 28 °C. Substrate degradation activity was assessed with GASW-Tris supplemented with 1% (w/v) Tweens 20, 40, 60, and 80, and clearance zones around colonies were determined after two days of incubation at 28 °C. Gelatinase activity was assessed with gelatinase medium (BD) supplemented with 3% NaCl after two days of incubation at 28 °C according to the manufacturer's instructions.

DNase activity was determined with DNase test agar with methyl green (BD) supplemented with 3% NaCl after two days of incubation at 28 °C.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined by disc diffusion assays on Mueller-Hinton agar (BD) plates supplemented with 3% NaCl. After inoculation, plates were incubated at 28 °C for 18 h and zones of inhibition were recorded. Antibiotics were purchased from Thermo Fisher Scientific (Waltham, MA, USA), Hardy Diagnostics (Santa Maria, CA, USA), and Sigma-Aldrich and used in the following doses: ampicillin (Amp; 10 µg), penicillin G (Pn; 10 units), carbenicillin (Carb; 100 µg), vancomycin (Vanc; 30 µg), erythromycin (Ery; 15 µg), tetracycline (Tet; 30 µg), oxytetracycline (Oxy; 30 µg), streptomycin (Strep; 30 µg), spectinomycin (Spec; 100 µg), trimethoprim (Trim; 5 µg), gentamycin (Gm; 10 µg), chloramphenicol (Cm; 30 µg), kanamycin (Kan; 30 µg), neomycin (Neo; 30 µg), and vibriostatic agent 0129 (150 µg).

Chemotaxonomic characterisation

Cellular fatty acid analysis was carried out at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) using strain OCN044^T, *V. tetraodonis* A511^T, and *V. aquimaris* DSM 109633^T cells harvested during the exponential growth phase in GASW at 28 °C. Fatty acid methyl esters (FAMEs) were separated and identified according to the MIDI Sherlock Microbial Identification System, by Microbial ID (MIDI, Microbial ID, Newark, DE), and the published profiles of other related strains were used for comparison.

Phylogenetic analysis and genomic characterisation

Genomic DNA was isolated from strain OCN044^T and *V. tetraodonis* A511^T via phenol chloroform extraction and used in a PCR with the primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT −3') to amplify a fragment of the 16S rRNA gene (Aebischer et al. 2006; Sambrook 2001). The amplified product was purified with the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and sequenced by Sanger Sequencing in the Biotechnology Center at Southern Oregon University using the same primers. The 16S rRNA gene sequences of strain OCN044^T and 44 related strains were aligned using the MUSCLE algorithm, and a maximum likelihood phylogenetic tree was generated using the MEGAX software package (Kumar et al. 2018).

Multilocus sequence analysis (MLSA) was conducted as previously described (Jiang et al. 2021). Briefly, coding sequences of the *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* genes were retrieved from the genome of strain OCN044^T (Loughran et al. 2020) and from those of 44 related strains (Table S2), aligned and concatenated using MEGAX, and a maximum likelihood phylogenetic tree was generated.

A whole genome-based taxonomic analysis was performed as follows. Coding regions in all genomes were predicted and annotated with Prokka (version 1.14.5) set to default parameters (Seemann 2014). Phylogenetic analysis of the predicted amino acid sequences was conducted using PhyloPhlAN (version 3.0) with all parameters set to default except the "--diversity" parameter, which was set to "low" (Asnicar et al. 2020). A custom workflow was run in PhyloPhlAN using Diamond to map amino acid sequences and reference marker gene sets (Buchfink et al. 2015), MAFFT to align individual marker genes (Katoh and Standley, 2013), and trimAl to trim gaps and phylogenetically uninformative sites and to concatenate trimmed alignments (Capella-Gutiérrez et al. 2009). The concatenated alignment produced by the PhyloPhlAN pipeline was used to construct a maximum likelihood phylogeny of *Vibrio* species using IQ-Tree (version 2.0.3) (Minh et al. 2020). A total of 380 core conserved marker genes shared between 43 *Vibrio* genomes and 1 outgroup were identified by PhyloPhlAN, and the final concatenated alignment of trimmed alignments consisted of 90,492 characters. Model testing of the aligned and concatenated amino acid sequences was first performed using IQ-Tree with the "-m TESTONLY" option and the best-fit model "LG + F + G4" was used for phylogenetic inference. A maximum likelihood phylogenetic tree was constructed with IQ-Tree using the following parameters: −m LG + F + G4 -alrt 1000 -bb 1000 -T 24, with IQ-Tree run on the Pegasus high-performance computing cluster at The George Washington University. The resulting maximum likelihood phylogenomic

tree was drawn using the ETE3 toolkit and a custom Python script (version 3.1.1), and edited in Inkscape and Adobe Illustrator (Huerta-Cepas et al. 2016).

DNA-DNA Hybridisation (isDDH) was conducted *in silico* using GGDC 2.1 with the default parameters (Meier-Kolthoff et al. 2013). ANIb and FastANI tools were run with default parameters to calculate the pairwise average nucleotide identities (ANI) between eight closely related *Vibrio* genomes used for phenotypic comparison.

Results and discussion

Isolation and ecology

In the summer of 2011, our group embarked on a research trip to Palmyra Atoll in the Northern Line Islands focused on identifying pathogens involved in a tissue loss disease affecting Acroporids. It was necessary to isolate bacteria from healthy coral to use as controls in infection trials on island because we were quite limited in our bacterial importation abilities. The vast majority of colonies isolated from apparently healthy *Acropora cytherea* fragments demonstrated a fast-growing swarming phenotype and were incapable of growing on TCBS agar; this morphology was absent from both diseased *Acropora cytherea* fragments and seawater. One such isolate was utilized as the bacterial control in infection trials. Due to restrictions on the number of bacterial isolates we were allowed to export from Palmyra Atoll, we returned with only pathogenic isolates and the strain utilized as the bacterial control in infection trials, which is denoted strain OCN044^T (Ushijima et al. 2016). A comparison of the 16S sequence of strain OCN044^T with a subset of metagenomic sequencing projects conducted on members of the *Acropora* genus and other coral and uploaded to NCBI, indicated at least eight instances of 100% sequence identity of 16S sequences derived from coral with the strain OCN044^T 16S (Table S1). No such sequences with 100% identity to the strain OCN044^T 16S were present in seawater or other collected material nearby in sequencing projects where the metadata was sufficient to determine the nature of individual sets of reads. These data are consistent with the potential of strain OCN044^T associating with coral as a component of the holobiont.

Morphology, physiology, and biochemical analyses

Strain OCN044^T exhibited phenotypic and chemotaxonomic characteristics consistent with the genus *Vibrio*: Gram-stain-negative, rod-shaped cells, each with a single polar flagellum (Fig. 1), capable of swimming and swarming motility, anaerobic growth, and presence of the major fatty acids $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{18:1}\omega7c$, and/or $C_{18:1}\omega6c$, which are consistent with the genus *Vibrio* (Lambert et al. 1983). Growth of cream coloured, smooth, raised, opaque colonies with entire margins occurred from 10 to 37 °C, 1–6% NaCl, and at pH 6.5-9.0. Optimal growth was observed from 25 to 30 °C, 1–3% NaCl, and pH 7.5–8.5. Prolonged growth on GASW plates can result in colonies changing to a light-yellow colour but no diffusible pigment is observed, and strain OCN044^T does not grow on TCBS agar even with NaCl supplementation (1–3% NaCl final concentration). The inability to grow on TCBS agar is shared with the related strain *V. pectenicida* CAIM 594^T but is in contrast to the production of green colonies by *V. tetraodonis* A511^T and

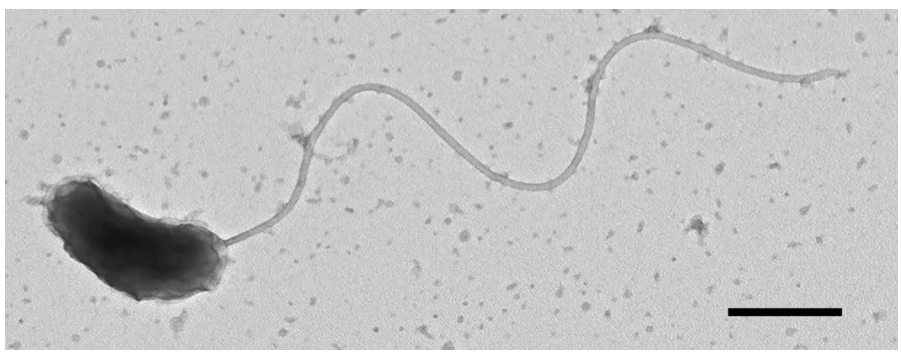

Fig. 1 Transmission electron micrograph of a uranyl acetate fixed cell of strain OCN044^T showing a polar flagellum. Bar, 500 nm

Vibrio aquimaris DSM 109633^T on the medium (Lambert et al. 1998). Strain OCN044^T was also unable to grow in 0.5 or 8% NaCl, which differentiates it from related species including *V. tetraodonis* A511^T, *Vibrio aquimaris* DSM 109633^T, *V. pectenicida* DSM 19584^T, and *Vibrio caribbeanicus* ATCC BAA-2122^T (Hoffmann et al. 2012). Further, only strain OCN044^T, *V. pectenicida* DSM 19584^T, and *V. ostreicida* DSM 21433^T (Prado et al. 2014) swarm while other related type strains do not display this motility. Notable characteristics that differentiate strain OCN044^T from closely related *Vibrio* species are shown in Table 1.

In biochemical assessments, strain OCN044^T is positive for oxidase, catalase, gelatinease, DNase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alpha-chymotrypsin, acid phosphatase, napthol ASBI phosphohydrolase, alpha glucosidase, and N-acetyl-beta-glucosamindase activites, ferments glucose, hydrolyses tween 40, 60, and 80, utilises glycerol, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, D-maltose, D-trehalose, amidon (starch), and glycogen as carbon sources, is negative for both nitrate and nitrite reduction, urease activity, and is not observed to produce indole or acetoin (Table 1 and Table S2). Only strain OCN044^T demonstrated cystine arylamidase activity among the type strains tested and used for comparison. Complete carbon source utilisation and enzyme activity profiles are presented in Tables S3&S4 in the Supplementary Material. Strain OCN044^T is susceptible to erythromycin, tetracycline, spectinomycin, trimethoprim, chloramphenicol, and vibriostatic agent 0129.

Chemotaxonomic characteristics

The predominant cellular fatty acids of strain OCN044^T are typical for the genus *Vibrio* (including $C_{12:0}$ (3.6%), $C_{14:0}$ (6.4%), $C_{16:0}$ (17.6%), summed feature 3 [$C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$] (41.9%), $C_{18:1}\omega7c$ (14.0%), and/or $C_{18:1}\omega6c$ (3.0%)) (Table S5) (Lambert et al. 1983). The FAME profiles of closely related species are presented in Table S6 in the Supplementary Material, and notable differences in fatty acid content percentages are observed with $C_{12:0}$, $C_{12:0}$ 3OH, $C_{16:0}$, $C_{18:1}\omega7c$, and $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$.

Phylogenetic analysis and genomic characteristics

Prior sequencing of the strain OCN044^T genome facilitated an assessment of the relevant features for existing within the coral holobiont (Loughran et al. 2020). Microbes on coral may quorum sense (QS) to maintain a competitive advantage during antagonistic interactions and nutrient source competition (Golberg et al. 2013). This strain potentially has the components of several QS systems (Table S7) but, like its close relative *V. tetraodonis* A511^T, lacks *luxI* and *luxM* homologs, which could indicate alternative systems that do not rely on AI-1 or AHLs. Iron is a critical component of *Vibrio* metabolism, as well as a major limiting factor in oligotrophic marine environments (e.g., coral reefs), and strain OCN044^T harbours genes for this purpose (Johnson 2013). Changes in seawater due to runoff and freshwater input can influence the osmoregulation of microbes, which can lead to changes in the microbial composition of the coral holobiont. Previous work indicates that, as salinity is modulated to alter osmotic conditions, coral communities can shift toward increasing levels of *Vibrios* (Röthig et al. 2016); strain OCN044^T contains genes that would aid in this manner of osmoregulation to maintain its place in the holobiont. Coral-associated microbes have been found to exhibit chemotaxis as a means of establishing and maintaining themselves as part of the holobiont (Tout et al. 2015), and strain OCN044^T has 34 proteins with potential MCP signal transduction domains as well as additional genes to facilitate chemotaxis. Together, these genomic components could promote strain OCN044^T to persist as a constituent of the coral holobiont.

Analysis of the 16S rRNA placed strain OCN044^T in the genus *Vibrio* and clusters with *V. aquimaris* DSM 109633^T and *V. tetraodonis* A511^T (Fig. S1 in the Supplementary Material). Comparison of the strain OCN044^T 16S rRNA sequence indicates 98.56% identity with *V. tetraodonis* A511^T, which is below the 98.65% same-species identity threshold (Kim et al. 2014). Within the complete genome of *V. aquimaris* DSM 109633^T, nine copies of the 16S rRNA sequence are present and comparison of the strain OCN044^T 16S rRNA sequence indicates

Table 1 Phenotypic characteristics that differentiate strain OCN044^T from related *Vibrio* type strains

Characteristic	1	2[a]	3[b]	4[c]	5[d]	6[e]	7[f]	8[g]
Pigmentation	Cream	Cream	Beige	Unpigmented	Cream	Unpigmented	Cream	Beige
Nitrate reduction	−	−	−	+	−	−	+	+
Indole production	−	−	−	−	+	−	−	−
Swarming	+	−	−	+	−	+	−	−
Growth on TCBS	−	Green	Green	−	Green	Green	Yellow	Yellow
Growth with/at:								
0.5% (w/v) NaCl	−	+	−	+	+	−	−	−
8.0% (w/v) NaCl	−	−	+	+	+	−	−	−
4 °C	−	−	−	+	−	−	−	−
37 °C	+	−	+	−	−	−	+	+
Enzyme activity:								
Lipase (C14)	+	+	variable	NA	−	+	+	+
Gelatinase	+	−	−	+	−	+	+	+
DNase	+	−	+	+	+	NA	NA	NA
Valine arylamidase	+	+	Variable	NA	−	+	−	+
Arginine dihydrolase	−	+	−	−	+	−	+	+
Trypsin	+	+	Variable	NA	−	+	−	+
Cystine arylamidase	+	−	−	NA	−	−	NA	−
Alpha-chymotrypsin	+	+	−	NA	−	+	NA	−
Alpha glucosidase	+	+	−	NA	−	−	NA	−
Beta galactosidase	−	+	−	NA	−	−	+	−
N-Acetyl-Beta- Glucosaminidase	+	−	+	NA	−	+	NA	+
Utilization of								
D-Glucose	+	+	−	−	−	+	+	+
D-Ribose	−	−	−	−	+	+	+	+
N-acetylglucosamine	+	+	−	−	+	+	+	+
Glycerol	+	+	−	+	−	variable	+	NA
Potassium 2-ketogluconate	−	−	+	NA	−	−	NA	NA
D-Mannitol	−	−	−	−	+	+	+	−
D-Sorbitol	−	−	+	−	−	−	NA	−
D-Sucrose	−	−	+	−	+	−	+	+
L-Xylose	−	+	−	NA	NA	−	NA	NA
Methyl-Ad-Mannopyranoside	−	+	−	NA	NA	−	NA	NA
Methyl-Ad-Glucopyranoside	−	+	−	NA	NA	−	NA	NA
Amygdalin	−	+	−	NA	−	−	−	−
Arbutin	−	+	−	NA	NA	−	NA	NA
Salicin	−	+	−	NA	NA	−	NA	NA
D-Lactose	−	+	−	NA	−	−	NA	−
Inulin	−	+	−	NA	NA	−	NA	NA
D-Melezitose	−	+	−	NA	NA	−	NA	NA
Gentiobiose	−	+	−	NA	NA	−	NA	−
D-Turanose	−	+	−	NA	NA	−	NA	−
DNA G+C content (mol%)	42.4	42.5	42.7	41.0	41.6	53.0	45.6	46.0

Strains: 1, strain OCN044^T; 2, *V. tetraodonis* A511 $^{T;}$ 3, *V. aquimaris* DSM 109633^T; 4, *V. pectenicida* DSM 19584^T; 5, *V. caribbeanicus* ATCC BAA-2122^T; 6, *V. ostreicida* DSM 21433^T; 7, *V. coralliilyticus* ATCC BAA-450^T; 8, *V. neptunius* LMG 20536^T

+ positive; − negative; NA, not available; strain data are [a]collected here and from Azevedo et al. (2021); [b]Franco et al. (2020) and verified here; [c]Lambert et al. (1998); [d]Hoffman et al. (2012); [e]Prado et al. (2014); [f]Ben-Haim et al. (2003); [g]Thompson et al. (2003)

98.84–99.25% identity, which is slightly above the 98.65% same-species identity threshold. A multilocus sequence analysis (MLSA) was used to assess the relationship between strain OCN044^T and other *Vibrio* species (Ushijima et al. 2016; Jiang et al. 2021). This analysis placed strain OCN044^T next to *V. tetraodonis* A511^T and in a cluster with *V. aquimaris* DSM 109633^T and *V. pectenicida* CAIM 594^T (Fig. S2 in the Supplementary Material). Grouping with *V. pectenicida* CAIM 594^T in the MLSA indicates that strain OCN044^T belongs within the eponymous Pectenicida clade. The G + C content of the genome of strain OCN044^T is 42.40%, which is in line with reported values for related strains (Table 1) and falls within the 38–51 mol% typically observed for this genus (Farmer et al. 2005).

For a more robust analysis of phylogeny, a whole genome-based taxonomic analysis was performed. The resulting phylogenomic tree placed strain OCN044^T as sister to *V. tetraodonis* A511^T with a 100% bootstrap value (Fig. 2). This result contrasts with the 16S tree but is consistent with the MLSA tree where *V. tetraodonis* A511^T is a closer relative to strain OCN044^T than *V. aquimaris* DSM 109633^T,

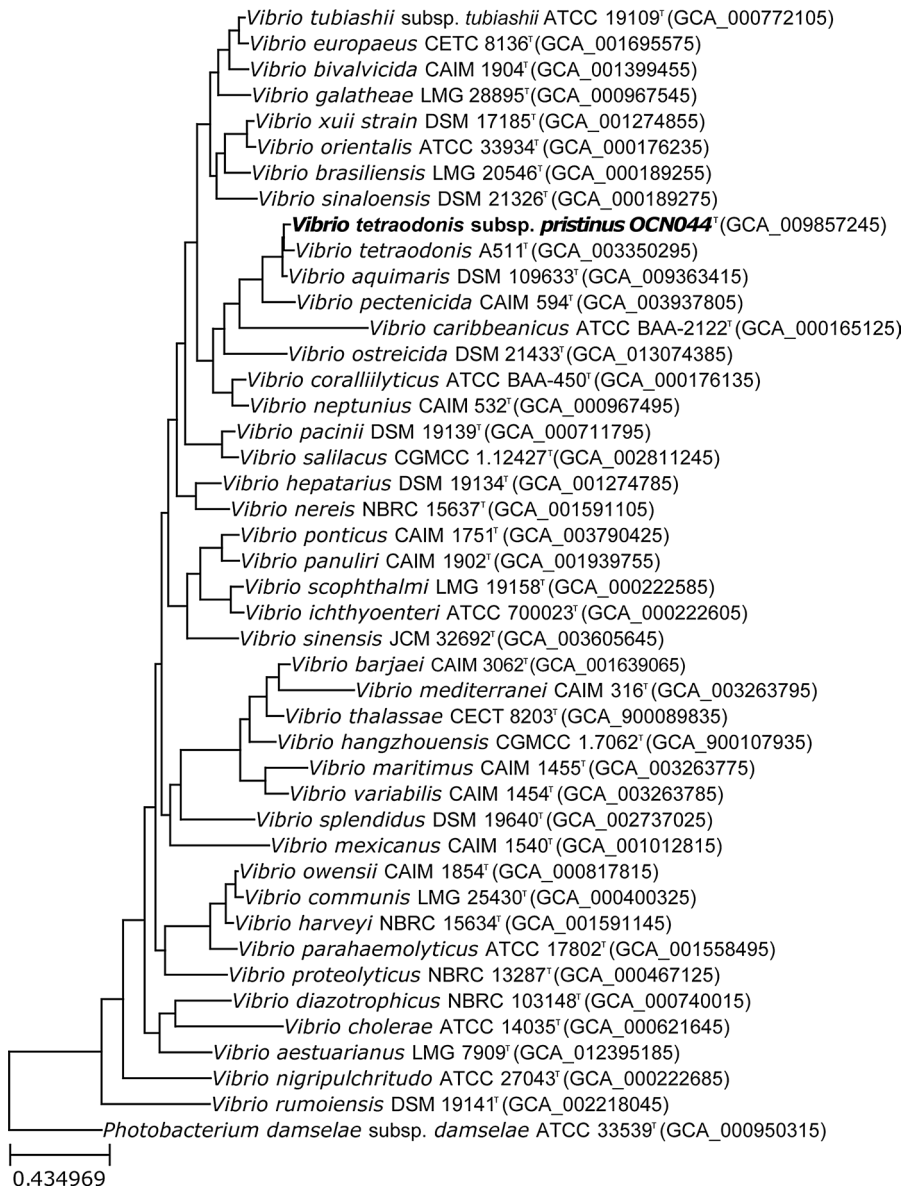

Fig. 2 A maximum likelihood phylogenomic tree using 381 conserved core genes (90,623 characters) shared between the 43 *Vibrio* species and the outgroup, *Photobacterium damselae* subsp. *damselae* strain ATCC 33539^T. Ultrafast bootstrap (UFBoot) values were determined and, though not shown, are 100% at each node (Hoang et al. 2018). Accession numbers of genomes are shown in parentheses. Bar, number of substitutions per nucleotide position

which is not unexpected given the higher reliability of species relatedness in MLSA analyses than 16S phylogenies in the family *Vibrionaceae*. The grouping of strains near strain OCN044^T in Fig. 2, further informed by the results of the MLSA in Figure S2, indicated that *V. tetraodonis* A511^T, *V. aquimaris* DSM 109633^T, *V. pectenicida* DSM 19584^T, *V. caribbeanicus* ATCC BAA-2122^T, *V. ostreicida* DSM 21433^T, *V. coralliilyticus* ATCC BAA-450^T, *Vibrio neptunius* LMG 20536^T represented the most closely related type strains and served as the basis of comparison throughout this study.

To assess genomic similarity, *in silico* DNA-DNA hybridisation (isDDH) was conducted and ANI values compared. The isDDH values ranged from 18.1 to 63.8% between strain OCN044^T and the other related type strains used for comparison, which are below the 70% cutoff for members of different species (Table 2) (Goris et al. 2007). The ANI values between the eight closely related *Vibrio* genomes named above (Table 3) were used for genomic comparison (Jain et al. 2018; Richter et al. 2015). When compared to strain OCN044^T, an average ANI value of 95.3% was recorded for *V. tetraodonis* A511^T, which falls within the threshold range of 95–96% commonly used to delineate species (Varghese et al. 2015; Jain et al. 2018; Chun et al. 2018; Ciufo et al. 2018; Kim et al. 2014).

On the basis of 16S rRNA sequence comparison alone, strain OCN044^T and *V. aquimaris* DSM 109633^T would be considered more related. However, the advent of genome sequencing has resulted in a transition from 16S rRNA-based phylogenies to whole-genome comparisons as the basis for taxonomy due to greater resolution from longer inputs (Parks et al. 2018; Murray et al. 2020; Hugenholtz et al. 2021). It has also been noted that the 16S rRNA gene is more conserved than the whole

Table 2 isDDH values (%) measured via pairwise comparison between related *Vibrio* species

	1	2	3	4	5	6	7	8
1		63.8	58.9	21.8	19.4	18.1	18.2	19.4
2			60.3	21.9	19.6	18.1	18.2	19.3
3				22.1	19.7	18.4	18.7	19.8
4					19.7	18.4	18.6	19.5
5						19.0	19.0	34.6
6							18.0	18.9
7								18.4
8								

Strains: 1, strain OCN044^T; 2, *V. tetraodonis* A511 ^{T}; 3, *V. aquimaris* DSM 109633^T; 4, *V. pectenicida* DSM 19584^T; 5, *V. neptunius* LMG 20536^T; 6, *V. ostreicida* DSM 21433^T; 7, *V. caribbeanicus* ATCC BAA-2122^T; 8, *V. coralliilyticus* ATCC BAA-450^T

Table 3 ANI values (%) measured via pairwise comparison between related *Vibrio* species with ANIb values above the midline and FastANI values below

	1	2	3	4	5	6	7	8
1		95.2	94.4	79.1	73.9	71.9	70.6	74.2
2	95.4		94.8	79.1	74.2	72.0	70.5	74.2
3	94.6	95.0		79.3	74.2	72.3	70.8	74.5
4	80.8	80.8	80.9		74.6	72.4	70.9	74.7
5	77.6	77.7	77.7	77.6		73.8	70.8	87.1
6	76.5	76.7	76.7	77.0	78.0		70.0	74.0
7	76.0	75.8	75.8	75.8	76.0	75.8		70.9
8	77.5	77.5	77.6	77.5	88.4	77.8	75.9	

Strains: 1, strain OCN044^T; 2, *V. tetraodonis* A511 ^{T}; 3, *V. aquimaris* DSM 109633^T; 4, *V. pectenicida* DSM 19584^T; 5, *V. neptunius* LMG 20536^T; 6, *V. ostreicida* DSM 21433^T; 7, *V. caribbeanicus* ATCC BAA-2122^T; 8, *V. coralliilyticus* ATCC BAA-450^T

genome and does not provide sufficient resolution among species, whereas ANI and other whole-genome comparisons facilitate finer resolution among species (Varghese et al. 2015; Jain et al. 2018). Strain OCN044^T and *V. tetraodonis* A511^T display a 95.3% average ANI for the concordant pair and isDDH of 63.8%, whereas strain OCN044^T and *V. aquimaris* DSM 109633^T display a 94.5% average ANI for the concordant pair and isDDH of 58.9%. Based on these data, it is clear that strain OCN044^T is more closely related to *V. tetraodonis* A511^T than *V. aquimaris* DSM 109633^T. While the ANI cutoff for different species is 95–96% and the isDDH cutoff is 70%, previous work has indicated that the isDDH cutoff for subspecies classification is 79–80% (Meier-Kolthoff et al. 2014). Additional work has indicated that conspecific genomes have ANI values of ≥ 97% (Van Rossum et al. 2020). Our data demonstrate that strain OCN044^T is below the species and subspecies cutoff values for isDDH when compared to *V. tetraodonis* A511^T, but is also not conspecific with *V. tetraodonis* A511^T. Because 95.3% ANI is slightly higher than the 95% lower bound for species demarcation, we define OCN044^T as a subspecies rather than a novel species to remain within accepted genomic parameters. When considered with the other results, these data fulfil the currently accepted genomic criteria to indicate that strain OCN044^T is sufficiently genetically divergent to be considered to represent a novel subspecies.

Strain OCN044^T may be distinguished from phylogenetically related strains by its cystine arylamidase activity and its inability to grow at 8% NaCl. Strain OCN044^T differs from the most closely related strain, *V. tetraodonis* A511^T, in growth on TCBS agar in 0.5% NaCl, or at 37 °C; swarming motility; in gelatinase, DNase, arginine dihydrolase, beta galactosidase, and N-acetyl-beta-glucosaminidase activities; and in the utilization of L-xylose, amygdalin, arbutin, salicin, D-lactose, inulin, D-melezitose, gentiobiose, and D-turanose. Additionally, while *V. tetraodonis* A511^T was isolated from a pufferfish and is thought to act as a potentially beneficial component of its microbiome (Azevedo et al. 2021), strain OCN044^T was isolated from coral and identical 16S sequences are found in metagenomic projects assessing the coral holobiont, which indicates the potential for these two strains to associate with different hosts. The preceding summary of characteristics and genomic analyses support the inclusion of strain OCN044^T in the genus *Vibrio* and demonstrate that it exists as a distinct and novel subspecies, for which the name *Vibrio tetraodonis* subsp. *pristinus* is proposed.

Description of *Vibrio tetraodonis* subsp. *pristinus* subsp. nov.

Vibrio tetraodonis subsp. *pristinus* (pris.tin'us L. nom. n. pristinus, meaning pristine; referring to the pristine nature of the reefs surrounding Palmyra Atoll, the location from which the strain was isolated).

The cells are Gram-stain-negative, non-spore-forming rods, motile by a single polar flagellum with swimming and swarming activity. Colonies are cream-colored and opaque on GASW-Tris agar, with growth at 10–37 °C, 1–6% NaCl, and at pH 5.5–10; they do not grow on TCBS agar. Luminescence is not observed. Facultative anaerobe that ferments glucose but not mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose. Positive for oxidase and catalase activity, does not reduce nitrate or nitrite, does not utilise citrate, and is positive for gelatinase activity. Does not produce acetoin, indole, hydrogen sulfide, and does not demonstrate β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, or tryptophan deaminase activity. Tween 40, 60, and 80 are hydrolysed but Tween 20 is not. Positive for gelatinase, DNase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alpha-chymotrypsin, acid phosphatase, napthol ASBI phosphohydrolase, alpha glucosidase, and N-acetyl-beta-glucosamindase; and is negative for alpha and beta galactosidase, beta glucuronidase, beta glucosidase, alpha mannosidase, and alpha fucosidase. Utilised carbon sources include glycerol, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, D-maltose, D-trehalose, amidon (starch), and glycogen; but not erythritol, D- or L-arabinose, D-ribose, D- or L-xylose, D-adonitol, Methyl-BD-Xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, Methyl-ad-Mannopyranoside, Methyl-ad-Glucopyranoside, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-lactose, D-meliboise, D-saccharose (sucrose), inulin, D-melezitose, D-raffinose, xylitol,

gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fuccose, L-fuccose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate.

Dominant fatty acids are $C_{16:0}$ (17.6%), $C_{18:1}$ ω7c (14.0%), and $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c (41.9%). Susceptible to erythromycin, tetracycline, spectinomycin, trimethoprim, chloramphenicol, and vibriostatic agent 0129, but resistant to ampicillin, penicillin G, carbenicillin, vancomycin, oxytetracycline, streptomycin, gentamycin, kanamycin, and neomycin.

The type strain, OCN044^T (=LMG 31895^T=DSM 111778^T), was isolated from the tissues of a fragment of apparently healthy *A. cytherea* coral off the western reef terrace of Palmyra Atoll in the Northern Line Islands. The DNA G+C content of the type strain is 42.40%.

Emended description of *Vibrio tetraodonis* subsp. *tetraodonis* subsp. nov.

Characteristics are as those given for the species description by Azevedo et al. (2021) with the following additions. Growth occurs at pH 6.0–10.0 with optimum growth between pH 7.0–9.0. Swimming but not swarming motility. Ferments glucose and Tween 40, 60, and 80 are hydrolysed but Tween 20 is not. Does not produce hydrogen sulfide, reduce nitrate or nitrite, or utilise citrate. Negative for DNase, gelatinase, urease, cystine arylamidase, alpha galactosidase, beta glucuronidase, beta glucosidase, N-acetyl-beta-glucosamindase, alpha mannosidase, and alpha fucosidase. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, trypsin, alpha-chymotrypsin, acid phosphatase, napthol ASBI phosphohydrolase, beta galactosidase, and alpha glucosidase. Utilised carbon sources include glycerol, D-xylose, Methyl-BD-Xylopyranoside, D-glucose, D-fructose, D-mannose, Methyl-ad-Mannopyranoside, Methyl-ad-Glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, D-maltose, D-lactose, D-trehalose, inulin, D-melezitose, amidon (starch), glycogen, gentiobiose, and D-turanose; but not erythritol, D- or L-arabinose, D-ribose, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, esculin, D-cellobiose, D-meliboise, D-saccharose (sucrose), D-raffinose, xylitol, D-lyxose, D-tagatose, D-fuccose, L-fuccose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate.

Dominant fatty acids are $C_{16:0}$ (18.7%), $C_{18:1}$ ω7c (10.4%), and $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c (42.4%). Susceptible to erythromycin, gentamycin, kanamycin, trimethoprim, chloramphenicol, vancomycin, and vibriostatic agent 0129, but resistant to ampicillin, penicillin G, carbenicillin, tetracycline, oxytetracycline, streptomycin, spectinomycin, and neomycin. The type strain is A511^T (=CBAS 712^T=A511^T).

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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