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Refined identification of *Vibrio* bacterial flora from *Acanthaster planci* based on biochemical profiling and analysis of housekeeping genes

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ABSTRACT: We used a polyphasic approach for precise identification of bacterial flora (*Vibrionaceae*) isolated from crown-of-thorns starfish (COTS) from Lizard Island (Great Barrier Reef, Australia) and Guam (USA, Western Pacific Ocean). Previous 16S rRNA gene phylogenetic analysis was useful to allocate and identify isolates within the Photobacterium, Splendidus and Harveyi clades but failed in the identification of *Vibrio harveyi*-like isolates. Species of the *V. harveyi* group have almost indistinguishable phenotypes and genotypes, and thus, identification by standard biochemical tests and 16S rRNA gene analysis is commonly inaccurate. Biochemical profiling and sequence analysis of additional *topA* and *mreB* housekeeping genes were carried out for definitive identification of 19 bacterial isolates recovered from sick and wild COTS. For 8 isolates, biochemical profiles and *topA* and *mreB* gene sequence alignments with the closest relatives (GenBank) confirmed previous 16S rRNA-based identification: *V. fortis* and *Photobacterium eurosenbergii* species (from wild COTS), and *V. natriegens* (from diseased COTS). Further phylogenetic analysis based on *topA* and *mreB* concatenated sequences served to identify the remaining 11 *V. harveyi*-like isolates: *V. owensii* and *V. rotiferianus* (from wild COTS), and *V. owensii*, *V. rotiferianus*, and *V. harveyi* (from diseased COTS). This study further confirms the reliability of *topA–mreB* gene sequence analysis for identification of these close species, and it reveals a wider distribution range of the potentially pathogenic *V. harveyi* group.

KEY WORDS: *Vibrio* · Corallivores · Crown-of-thorns starfish · Harveyi clade · Splendidus clade

INTRODUCTION

Vibrios constitute an important part of the bacterial microflora of numerous marine animals (Harris 1993, Oxley et al. 2002). Some species are also recognized as important pathogens of marine and estuarine animals, causing substantial losses in commercial production systems and natural waters throughout the world (Kaysner & DePaola 2004). Particularly within the Echinodermata, several species not only integrate *Vibrio* spp. as part of their natural microflora, but also display susceptibility to this bacterium (Gilles & Pearse 1986, Morgan 2000, Becker et al. 2008). Moreover, remarkable similarities exist in the clinical signs, time to death, histology, scanning electron microscopy analysis, and identification of vibrios as etiological agents (Morgan 2000, Becker et al. 2004) with those described in experimentally-induced diseased crown-of-thorns starfish (COTS; Rivera-Posada et al. 2011).

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Resident bacteria constitute a critical barrier of resistance against colonization by exogenous microbes, preventing tissue invasion by pathogens (Guarner & Malagelada 2003). This function is accomplished through different mechanisms, including (1) secretion of antibacterial substances to reduce the number of viable pathogens, decrease bacterial metabolism, or inhibit toxin production; (2) competition for essential nutrients for pathogen survival; (3) supplementation with vitamins and short-chain fatty acids to the host tissues; (4) competition with pathogens preventing their adhesion to the intestine; (5) enhancement of the production of defensive molecules in the host; and (6) direct uptake or decomposition of organic matter or toxic material produced by pathogenic bacteria (Lievin et al. 2000, Guarner & Malagelada 2003, Macfarlane & Macfarlane 2003). Disrupting the bacterial ecological balance in healthy organisms allows overgrowth of pathogenic bacteria, and establishes a suitable environment for disease induction. Maladies and physiological manifestations such as intestinal inflammation, digestive ulcers, and multiple organ failure have been associated with microflora imbalances (Lemaire et al. 1997, Zhang & Chen 2010).

It is therefore important to compare bacterial communities in echinoderms and other related marine invertebrates in order to understand the role of bacterial balances in the health of these organisms. Bensoussan et al. (1984) analyzed bacterial isolates of digestive tracts in the asteroid *Solaster* sp. and the holothurian *Pseudostichopus villosus* in stable physicochemical conditions (constant environment), and reported that the most common bacteria isolated from these echinoderms were *Vibrio*-like Gram-negative rods, which constituted half of the total bacterial load; and that a clear separation existed between enteric bacterial communities and sediment microflora. Moreover, the authors suggested that microflora of echinoderms, and in particular of the Asteroidea, has a more specialized tendency to the catabolism of organic compounds; and they found a less diversified microflora in echinoderms in comparison with sediment microflora.

Species of the Harveyi clade have been described as major pathogens to aquatic animals, causing disease outbreaks responsible for severe economic losses in the aquaculture industry worldwide. Increasing infections in marine vertebrates and invertebrates and even in humans have been linked to increased marine temperatures with diffusion of pathogens to higher latitudes (Bossart 2007, Igbinosa & Okoh 2008). According to current molecular taxonomy studies, the Harveyi clade (Sawabe et al. 2007) includes 10 *Vibrio* species: *V. harveyi, V. campbellii, V. rotiferianus, V. alginolyticus, V. parahaemolyticus, V. mytili, V. natriegens*, the newly described species *V. azureus* (Yoshizawa et al. 2009), *V. owensii* (Cano-Gomez et al. 2010), and *V. sagamiensis* (Yoshizawa et al. 2010). Among this clade, the *V. harveyi*-related species belonging to the so-called *V. harveyi* group (*V. harveyi, V. campbellii, V. rotiferianus*, and *V. owensii*) are phenotypically and genetically indistinguishable, sharing almost identical biochemical profiles and 16S rRNA gene sequences (Gomez-Gil et al. 2004, Cano-Gomez et al. 2010). Therefore, conventional culture-based techniques, biochemical tests, and 16S rRNA gene analysis frequently lead to misidentification of *V. harveyi*-like isolates (Pedersen et al. 1998, Vandenberghe et al. 2003, Gomez-Gil et al. 2004). Previous analyses of 16S rRNA gene sequences allocated some *Vibrio* strains isolated from wild and sick COTS in Lizard Island (Great Barrier Reef [GBR], Australia) and Guam (USA) within the Harveyi clade, and some of these clustered within the *V. harveyi* group (Rivera-Posada et al. 2011). However, due to the low resolution of 16S rRNA, we were prompted to perform additional biochemical tests and sequence analysis of 2 additional housekeeping genes in order to precisely identify these isolates at the species level.

Multilocus sequence analysis (MLSA) is a recent approach that employs sequence analysis of several housekeeping genes in bacteria and subsequent phylogenetic analysis of their concatenated sequences to delineate species and infer genetic relationships (Gevers et al. 2005). For the Harveyi clade, this method has been described as an alternative to the expensive and labor-intensive DNA–DNA hybridization (Gomez Gil et al. 2003, Cano-Gomez et al. 2009), still considered the gold standard for bacterial species delineation (Stackebrandt et al. 2002). However, MLSA is costly, time consuming, and requires a considerable amount of experience to analyze, concatenate, and construct phylogenies with DNA sequences of multiple loci. Recent efforts have focused on the design of a fast, practical, but still accurate identification method for *Vibrio harveyi*-related species as an alternative to 16S rRNA gene analysis and DNA–DNA hybridization. Short-term epidemiology studies or environmental surveys, for example, involve a high number of isolates to be identified rapidly and efficiently. In these cases, and under the evidences pointing to the Harveyi clade, the number of genes sequenced could be minimized for a more practical identification (Thompson et al.

2007, Cano-Gomez et al. 2011). While more than 5 genes should be analyzed for taxonomic studies of cryptic bacterial species, the use of at least 2 independent housekeeping genes has been suggested for bacterial identification purposes (Martens et al. 2008).

A recent MLSA study focused on the identification of *Vibrio harveyi*-related species by analysis of protein-coding genes (*rpoA, pyrH, topA, ftsZ*, *mreB*; Cano-Gomez et al. 2011). Concatenation of only *topA* and *mreB* gene sequences offered similar resolution to that of full MLSA (5 genes) for identification of *V. harveyi*-related species. These authors suggested that initial allocation of *V. harveyi*-like isolates into the *V. harveyi* group (by biochemical or 16S rRNA gene analysis) and additional *topA–mreB* gene analysis offers a reliable identification of these close species, with resolution power comparable that that of a full MLSA analysis.

The purpose of this study was to analyze data from phenotypic characterization, concatenated sequences of *topA* and *mreB* genes, and previously obtained 16S rRNA gene-based phylogenies to: (1) identify bacterial strains isolated from COTS in the Pacific and discriminate between those naturally present from those inducing disease and death, (2) validate an identification approach for *Vibrio harveyi*-related strains based on sequence analysis of *topA* and *mreB* protein-coding genes; and (3) provide baseline data for better understanding of the gut microbe role in the physiological processes of COTS.

MATERIALS AND METHODS

Samples

Samples consisted of DNA from 19 strains recovered from individual adult sick or wild COTS across 2 sampled sites in the Pacific Ocean in 2009 (Rivera-Posada et al. 2011). Eleven of these strains were obtained at Lizard Island (GBR): 5 isolated from wild COTS and 6 sick COTS induced from thiosulfate-citrate-bile-sucrose agar (TCBS) injection (see Rivera-Posada et al. 2011). The other 8 strains were obtained from COTS at Guam (USA, Mariana Archipelago): 3 from wild COTS and 5 from diseased COTS after TCBS injection.

Biochemical tests

Phenotypic analysis for all 19 bacterial isolates was performed in triplicate by employing API 20NE commercial kits (bioMérieux) according to the manufacturer's instructions with the following modifications (1) 2% NaCl (w/v) solution was used to prepare the inocula, (2) the strips were incubated at 30°C for 48 h, and (3) several colonies of pure cultures of a single organism were used when bacterial colonies were tiny to ensure that all tubes and cupules had enough bacteria for growth.

PCR amplification and sequencing

PCR amplification and sequencing was carried out with DNA from all 19 COTS bacterial isolates. Partial regions of the housekeeping genes *topA* (topoisomerase I) and *mreB* (rod shaping protein MreB) were amplified and sequenced as described by Sawabe et al. (2007) (Table 1). All PCR amplifications were performed in a Perkin Elmer Applied Biosystems GENEAMP PCR System 9700 thermocycler. PCR reactions (20 µl) contained approximately 20 ng of genomic DNA, 1× PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7; Qiagen), 0.5 µM of each primer, 200 µM dNTPs, and 0.5 units of *Taq* DNA polymerase (Qiagen). Finally, PCR products were visually inspected in 1% agarose gels and subsequently sequenced by Macrogen Ltd, Korea, with appropriate primers.

Table 1. Amplification and sequencing primers

Gene (gene product), length	Primer name	Primer sequence (5′–3′)	Annealing temp (°C)	Source
16S rRNA (16S ribosomal RNA), 1421 nt	27F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	54	Lane (1991)
topA (topoisomerase I), 800 nt	VtopA400F VtopA1200R	GAGATCATCGGTGGTGATG GAAGGACGAATCGCTTCGTG	50	Sawabe et al. (2007)
mreB (rod shaping protein MreB), 1000 nt	VmreB12F VmreB999R	ACTTCGTGGCATGTTTTC CCGTGCATATCGATCATTTC	50	Sawabe et al. (2007)

Sequence and phylogenetic analysis

Electropherograms of *topA* and *mreB* sequences were assembled in Sequencher 4.9 (Gene Codes). Sequences were manually corrected and trimmed, and BLASTn searches were performed against public databases for preliminary identification and comparison with 16S rRNA-based identification. Subsequently, phylogenetic analysis was performed with those 11 isolates belonging to the *Vibrio harveyi* group as indicated by initial biochemical characterization and 16S rRNA gene sequence analysis. Protein-coding *mreB* and *topA* gene sequences were aligned employing Clustal_X (Thompson et al. 1997). These alignments also included publicly available sequences of 11 *Vibrio* species type strains and *Photobacterium phosphoreum* LMG 4233^T as an outgroup. Finally, individual *topA* and *mreB* sequences from all 22 strains were concatenated and used to construct a multilocus phylogenetic tree for more accurate and supported identification. Phylogenies were obtained by the neighbor-joining (NJ) method (Saitou & Nei 1987) in PAUP v.4.0B10 for Windows (Swofford 2003). Bootstrap (BT) support values were calculated on 1000 replicates. For the NJ analysis, uncorrected 'p' distances and the Kimura 2-parameter correction (Kimura 1980) were tested.

Accession numbers

The gene sequences were deposited in GenBank under accession numbers HQ540692–HQ540710 and HQ540711–HQ540729 for the *topA* and *mreB* genes, respectively (Table 2).

RESULTS

All PCR primer sets specifically amplified the target sequences of all 19 isolates. Sequence lengths of *topA* and *mreB* sequenced regions were 626 nt and 834 nt, respectively. After BLASTn alignments with closest relatives in GenBank, isolates were initially identified as *Vibrio fortis, V. natriegens, V. harveyi*, *V. owensii*, *V. rotiferianus*, and *Photobacterium eurosenbergii* with sequences identities of 99 to 100% for both loci. However, for the 11 isolates belonging to the *V. harveyi* group (*V. harveyi, V. owensii*, and *V. rotiferianus*), BLASTn-based identification was not achieved since high sequence similarities (99 to 100%) with 2 or more *V. harveyi*-related species were obtained for each isolate. Phylogenetic reconstructions based on these 2 genes were consistent with previously obtained topologies based on the 16S rRNA gene (Rivera-Posada et al. 2011; Fig. 1), but this time clades were supported by higher BT values (Fig. 2). Isolates were allocated into 3 well supported clusters (100% BT support values), each containing the type strain of

Table 2. Accession numbers for the *topA* and *mreB* genes from 19 *Vibrio* isolates

Isolate	*topA*	*mreB*
G2	HQ540692	HQ540729
Y2	HQ540693	HQ540728
HAEM	HQ540694	HQ540715
NA	HQ540695	HQ540711
CREAM TCBS	HQ540696	HQ540714
GREEN AGRESS	HQ540697	HQ540712
GREEN+	HQ540698	HQ540716
CREAM 12-13	HQ540699	HQ540717
GU2Y	HQ540700	HQ540723
GU5YEN	HQ540701	HQ540724
GU5DARKEN	HQ540702	HQ540725
GU4YEN	HQ540703	HQ540721
GUIGEN	HQ540704	HQ540722
GU5PALEEN	HQ540705	HQ540720
GUMAB	HQ540706	HQ540719
GU1*	HQ540707	HQ540718
X1	HQ540708	HQ540726
X2	HQ540709	HQ540727
YG	HQ540710	HQ540713

Table 3. *Vibrio* spp. Sequence analysis and statistics of single-gene and 2-locus (*topA–mreB*) sequence alignments. Values shown are % similarities. Intra- and interspecies similarities were deduced from the number of nt substitutions per site between populations: Dxy (K2P). Gene sequence lengths (nt) are as follows: 16S rRNA, 1298; *topA*, 626; *mreB*, 834; 2-locus multilocus sequence (MLS; i.e. *topA* and *mreB* concatenated sequences), 1460

	16S rRNA	*topA*	*mreB*	2-locus MLS
Interspecies				
V. natriegens/V. fortis	98	73.6	86.5	81.1
V. natriegens/V. harveyi	98.8	80	90.8	86.4
V. natriegens/V. owensii	99.4	79.2	92.1	86.6
V. natriegens/V. rotiferianus	99.4	79	90	85.5
V. fortis/V. harveyi	97.3	75.1	87.1	82.2
V. fortis/V. owensii	98.3	74.3	86.6	81.4
V. fortis/V. rotiferianus	98.2	75.3	85.3	81.2
V. harveyi/V. owensii	99	90.1	93.9	92.3
V. harveyi/V. rotiferianus	99.1	88.6	93.2	90.7
V. owensii/V. rotiferianus	99.4	92.3	91.8	92.1
Intraspecies				
V. harveyi	99.9	99.3	99.6	99.5
V. owensii	99.9	99.3	98.1	98.6
V. rotiferianus	100	99.2	98.1	98.6

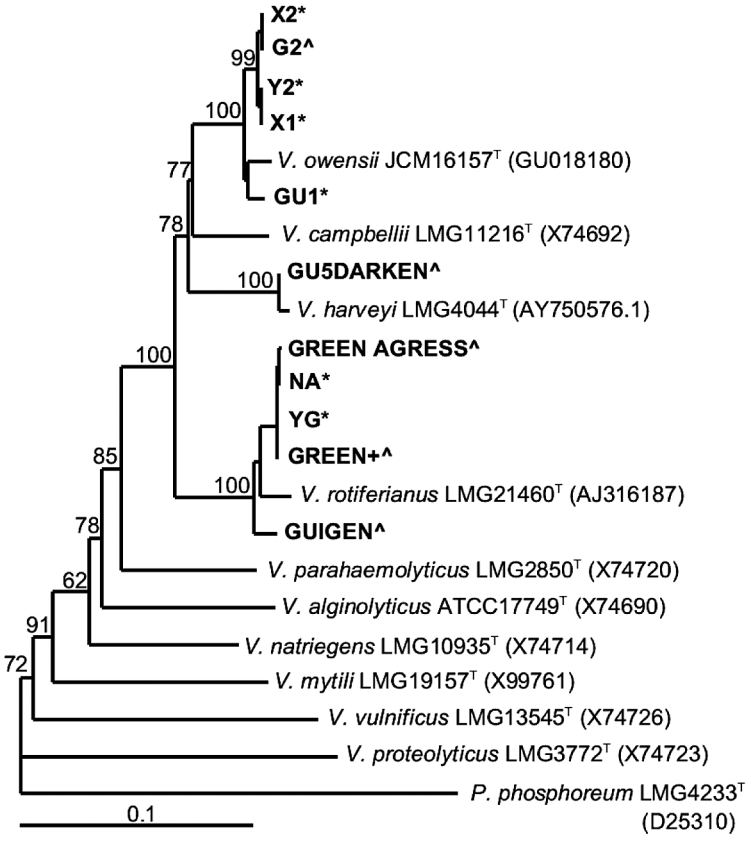

Fig. 1. *Vibrio* spp. Neighbor-joining phylogenetic analysis of *Vibrio* isolates from *Acanthasther planci* based on partial 16S rRNA gene sequences (1302 nt). GenBank accession numbers are provided in parentheses. *Photobacterium phosphoreum* LMG 4233^T was used as an outgroup. Bootstrap support values after 1000 simulations are shown. Bar shows 1% sequence divergence. GU: strains isolated from Guam (USA), *: strains isolated from wild *A. planci*. ^: strains isolated from TCBS-injected moribund *A. planci*

the identified species (*V. harveyi*, *V. owensii*, or *V. rotiferianus*). One *V. harveyi* strain was identified from sick COTS at Guam (GU5DARKEN), while *V. owensii* was found at both locations: 3 strains from wild (X1, X2, and Y2) and 1 from infected COTS (G2) in Lizard Island, and 1 strain from wild COTS at Guam (GU1*). Finally, 4 strains of *V. rotiferianus* were found in both wild (NA and YG) and sick COTS (GREEN AGRESS and GREEN+) at Lizard Island and 1 strain from diseased specimens at Guam (GUIGEN). Distance matrix analysis of concatenated *topA–mreB* gene sequences revealed intraspecies similarities of 98.6 to 99.5% for *V. harveyi*, *V. rotiferianus*, and *V. owensii* clusters and interspecies similarities of only 90.7 to 92.3% (Table 3). These 3 species showed only 81.2 to 86.6% sequence similarity with the less related species identified, *V. natriegens* and *V. fortis*.

Results from the biochemical characterization are presented in Table 4. From the 21 tests contained in each API 20NE strip, *Vibrio* isolates were positive for potassium nitrate, L-tryptophane, D-glucose, esculin, gelatin, β-galactosidase, malate, glucose, and oxidase and negative to L-arginine, urea, arabinose, adipate, and caprate. N-acetylglucosamine was 50% positive and the other 50% showed weak results. The potassium gluconate test was negative only for a *Photobacterium eurosenbergii* strain. The remaining tests showed weak or variable results from *V. harveyi*-related species, and only citrate utilization was found as a discriminatory character between isolates identified as *V. harveyi* and *V. owensii*.

DISCUSSION

In this study, biochemical characterization, analysis of *topA* and *mreB* protein-coding sequences, and comparison with previous 16S rRNA gene-based phylogenies were used for identification of 19 *Vibrio* isolated from *Acanthaster planci* in the Pacific. Isolates were identified as *Photobacterium eurosenbergii*, *V. natriegens*, and *V. fortis*, showing 100% *topA* and *mreB* sequence similarities by BLASTn alignments with type strains of these species. This identification was consistent with previous 16S rRNA based-phylogenies showing high BT support values (85 to 100%) for these clusters (Fig. 1). For the other 11 isolates belonging to the *V. harveyi* group, 16S rRNA gene (Rivera-Posada et al. 2011) and biochemical analysis profiles reported here failed in the identification at the species level. In contrast, the phylogenetic analysis of additional genetic markers (*topA* and *mreB*) offered a more definitive and discriminative identification of these closely related isolates.

The 16S rRNA gene is still necessary for species delineation, but its low resolution power for certain bacterial taxa, such as the *Vibrio harveyi* group (Gomez Gil et al. 2004, Thompson et al. 2005, Cano-Gomez et al. 2010), and the presence of multiple divergent gene copies in the same genome (Tagomori et al. 2002) are clear disadvantages for a gene to be used as a sole identification marker. In a previous study to identify COTS isolates, the 16S rRNA gene

was appropriate for allocation of several strains within the family *Vibrionaceae* and discrimination of distant species like *Bacillus* spp. and *Pseudoalteromonas* (Rivera-Posada et al. 2011). However, low genetic distances and BT support values observed in 16S rRNA-based phylogenies (especially for *V. harveyi*-related isolates; Fig. 1) prohibited precise identification at the species level.

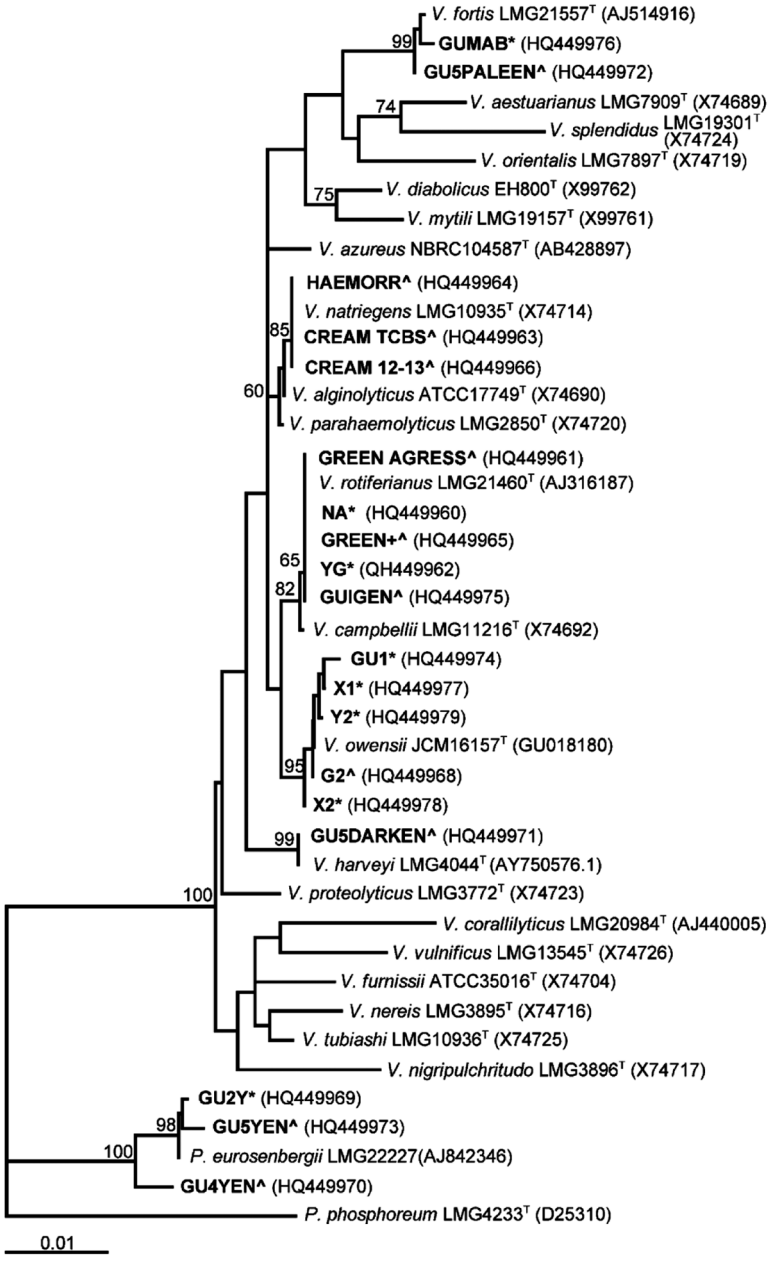

Fig. 2. *Vibrio* spp. Neighbor-joining phylogenetic analysis of isolates within the *V. harveyi* group based on *topA* and *mreB* concatenated gene sequences (1460 nt). *Photobacterium phosphoreum* LMG 4233^T was used as an outgroup. Bootstrap support values after 1000 simulations are shown. Scale bar = 1% (0.01) or 10% (0.1) sequence divergence. X1, X2, Y2, YG, NA, GREEN+, GREEN AGRESS, and G2 were found at Lizard Island (Great Barrier Reef). GUIGEN, GU5DARKEN, and GU1* were found in the Mariana Archipelago (Guam). *: strains isolated from wild *Acanthasther planci*. ^: strains isolated from TCBS-injected moribund *A. planci*

Compared to 16S rRNA gene analysis and expensive DNA–DNA hybridization, the MLSA is advantageous in terms of resolution power and reproducibility, respectively. Some loci have recently been described as highly discriminative among species of the Harveyi clade. Pascual et al. (2010) developed an MLSA based on 7 housekeeping genes and found *rpoD*, *rctB*, and *toxR* protein-coding loci as the most discriminative and informative for differentiation of 6 species of the Harveyi clade. More specifically, in an MLSA with 7 different housekeeping genes, Thompson et al. (2007) found *topA*, *mreB*, *ftsZ*, and *pyrH* to be suitable protein-coding genes for differentiation of the sister species *Vibrio harveyi* and *V. campbellii*. Following these studies, a similar MLSA (*rpoA*, *pyrH*, *topA*, *ftsZ*, and *mreB*) aimed at the description of the new species *V. owensii* within the *V. harveyi* species group (now consisting of *V. harveyi*, *V. campbellii*, *V. rotiferianus*, and *V. owensii*). In addition, these authors reduced the number of genes in the analysis for more reliable and yet accurate identification of the 4 species. Results showed that concatenated sequence analysis of only *topA* and *mreB* genes produced consistent clustering of strains compared to full MLSA and 16S rRNA-based analysis. This combination of genes showed high phylogenetic signal, low interspecies sequence similarities, and thus high resolution for precise identification of the cryptic *V. harveyi* group.

In our study, the results showed lower interspecies *topA–mreB* sequence similarities between strains identified as *Vibrio harveyi*, *V. rotiferianus* and *V. owensii* (90.7 to 92.3%) compared to those observed in previous 16S rRNA gene analysis (99 to 99.4%; Table 3). These values were higher when *V. harveyi*-related spp. were compared with the more distant *Vibrio* spp. identified as *V. natriegens* and *V. fortis* (81.2 to 86.6% *topA–mreB* gene sequence similarity).

Table 4. *Vibrio* spp. Biochemical profiles of *Vibrio* isolates from *Acanthasther planci* using API 20NE strips (bioMérieux®). Pos: positive; Neg: negative; ±: variable between strains; w: weak reaction. *P.*: *Photobacterium*

Test	Reaction	*V. owensii* (X1,X2,Y2, G2,GU1)	*V. harveyi* (GU5DARKEN)	*V. rotiferianus* (GREEN AGRESS, NA,YG, GREEN+,GUIGEN)	*V. natriegens* (CREAM TCBS, CREAM 12-13, HAEMORR)	*V. fortis* (GUMAB GU5PAL EEN)	*P. eurosenbergii* (GU2Y, GU5YEN, GU4YEN)
NO_3	Potassium nitrate	Pos	Pos	Pos	Pos	Pos	Pos
TRP	L-tryptophane	Pos	Pos	Pos	Pos	Pos	Pos
GLU	D-glucose	Pos	Pos	Pos	Pos	Pos	Pos
ADH	L-arginine	Neg	Neg	Neg	Neg	Neg	Neg
URE	Urea	Neg	Neg	Neg	Neg	Neg	Neg
ESC	Esculin	Pos	Pos	Pos	Pos	Pos	Pos
GEL	Gelatin	±	Pos	Pos	Pos	Pos	Pos
PNPG	β-galactosidase	Pos	Pos	Pos	±	Pos	Pos
GLU	Glucose	Pos	Pos	Pos	Pos	Pos	Pos
ARA	Arabinose	Neg	Neg	Neg	Neg	Neg	Neg
MNE	Mannose	Pos	Pos	Pos	Neg	w	±
MAN	Mannitol	w	Pos	±	Pos	w	Neg
NAG	N-acetyl-glucosamine	w	Pos	Pos	Pos	w	w
MAL	Maltose	w	±	Pos	Pos	w	Neg
GNT	Potassium gluconate	Pos	Pos	Pos	Pos	Pos	Neg
CAP	Capric acid	Neg	Neg	Neg	±	Neg	Neg
ADI	Adipic acid	Neg	Neg	Neg	Neg	Neg	Neg
MLT	Malate	Pos	Pos	Pos	Pos	Pos	±
CIT	Citrate	Neg	Pos	±	Pos	Neg	Neg
PAC	Phenylacetic acid	Neg	±	±	Neg	Neg	w
OX	Oxidase	Pos	Pos	Pos	Pos	Pos	Pos

These results and the congruence in phylogenies based on 16S rRNA and protein-coding genes support the usefulness of *topA* and *mreB* genes for *V. harveyi*-related species identification (Cano-Gomez et al. 2011). Our study contributes to the databases with additional sequences from strains of different sources and geographic locations, and has revealed a wider distribution range of the potentially pathogenic species *V. owensii*, recently described in diseased larvae of the Australian ornate spiny lobster *Panulirus ornatus*.

The species *Vibrio harveyi* and *V. campbelli* are almost indistinguishable phenotypically, while *V. owensii* has been reported to differ from other species of the Harveyi clade in its ability to use citrate and to produce acid from amygdalin, arabinose, and sucrose (API 20E; Cano-Gomez et al. 2010). Of these tests, the API 20NE used in this study only included citrate utilization and, being negative for all *V. owensii* strains, resulted as the only discriminatory character found between *V. owensii* and *V. harveyi* isolates (Table 4). The species *V. harveyi* and *V. rotiferianus* were characterized as positive for citrate use, although 2 of our 5 *V. rotiferianus* isolates had negative results. Aside from their high interspecies similarities, intraspecies variability of biochemical profiles has previously been considered as a disadvantage of phenotypic methods for precise identification of *V. harveyi*-related strains (Gomez-Gil et al. 2004).

Vibrios constitute a natural and important part of the microflora in a wide array of echinoderms and numerous marine animals (Bensoussan et al. 1984, Harris 1993, Oxley et al. 2002). Not surprisingly, species belonging to the phylum Echinodermata also show susceptibility to this bacterium (Table 5).

The Harveyi clade can be considered as the most pathogenic vibrio group for echinoderms, followed by the Splendidus clade. Nearly all members belonging to the Harveyi clade have been previously reported as etiological agents of echinoderm diseases, except *Vibrio mytili* (Table 5). Additionally, in this study, *V. owensii, V. rotiferianus, V. harveyi,* and *V. natriegens* were also isolated from infected tissues of COTS, confirming the pathogenicity of the Harveyi clade. On the other hand, the Splendidus clade showed that 4 of its 8 members (*V. splendidus, V. lentus, V. pomeroyi,* and *V. tasmaniensis*) were also identified as causal agents of echinoderm diseases (Table 5). Furthermore, our study includes a first-time report of *V. fortis* in COTS tissues.

COTS bacterial flora and physicochemical conditions like temperature and pH of the areas studied

were comparable during the months of February at Lizard Island and November at Guam. In addition, Guam lies relatively close to the Indo-Pacific center of coral reef biodiversity (Veron 2000). Over 150 species of scleractinian coral were documented at Haputo Environmental Reserve Area (Guam collection area), where coral cover was dominated by *Montipora* and *Porites* (Amesbury et al. 2001). However,

Table 5. Vibrios isolated from diseased echinoderms

Echinoderm	Area	Bacteria identified	Disease induced and isolation site	Source
Acanthasther planci	Australia, Guam (USA), Okinawa (Japan)	*Vibrio owensii, V. rotiferianus, V. harveyi, V. natriegens V. fortis, Photobacterium eurosenbergii, V. tubiashi, V. campbellii, V vulnificus, V. cholerae*	Sick and healthy *A. planci*	Rivera-Posada et al. (2011), Sutton et al. (1988), Reed et al. (1999)
Amphipholis gracillima	Georgetown, Texas, USA	*V. parahaemolyticus*	Dermal tissue	Strahl et al. (2002)
Amphipholis squamata		*Vibrio* sp.	Dermal tissue	Walker & Lesser (1989)
Apostichopus japonicus	China	*V. harveyi, V. cyclitrophicus, V. splendidus, V. tasmaniensis,V. tapetis, V. lentus, V. pomeroyi, V. gigantis* and other *Vibrio* sp.	Skin ulceration disease, peristome edema disease, and infected dermal tissues	Y. Ma et al. (2006a,b), H. Ma et al. (2009), Deng et al. (2009)
Archaeopneustes hystrix and *Paleopneustes cristatus*	Bahamas	*V. alginolyticus*	Epidermal vibriosis, isolated from gut gonads and infected lesions	Bauer & Young (2000)
Astropecten jonstoni	Sardinia, Italy	*V. vulnificus, V. lentus, Vibrio* sp.	Swabs of live and dead animals	Staheli et al. (2009)
Echinometra mathaei	Okinawa	*V. vulnificus, V. cholerae*	Spines	Reed et al. (1999)
Holothuria scabra	Bribie Island, Australia, Toliara, Madagascar	*V. harveyi, V. alginolyticus, V. natriegens*, other *Vibrio* sp.	Skin ulceration disease, infected tissues	Morgan (2000), Becker et al. (2004)
Ophiactis balli	Unspecified	*V. harveyi*	Subcuticular bacteria of arms	Burnett & McKenzie (1997)
Paracentrotus lividus	France, Mediterranean and Atlantic Ocean, Brittany	*Vibrio* sp.	Bald sea urchin disease, isolated from infected dermal tissues	Becker et al. (2008), Maes & Jangoux (1985)
Strongylocentrotus intermedius	Japan	*Vibrio* sp.	Spotting disease	Tajima et al. (1997a,b), Takeuchi et al. (1999)
Strongylocentrotus purpuratus	California (USA)	*V. anguillarum*	Infected epithelium	Gilles & Pearse (1986)
Strongylocentrotus nudus and *S. intermedius*	Japan	*Vibrio* sp.	Guts	Sawabe et al. (1995)
Tripneustes gratilla	Toliara, Madagascar	*V. harveyi, V. parahaemolyticus, V. nigripulchritudo*	Body wall lesions	Becker et al. (2007)

Table 6. Similarities/differences in the microflora of the different crown-of-thorns starfish (COTS)

Isolate	*Vibrio* identified	Wild COTS	Sick COTS	Swab site	Location
X1, X2,Y2	*V. owensii*	X		Digestive tract	Lizard Island
G2	*V. owensii*		X	Open sores	Lizard Island
GU1*	*V. owensii*	X		Dermal tissue	Guam
GU5DARKEN	*V. harveyi*		X	Open sores	Guam
GREEN+, GREEN AGRESS	*V. rotiferianus*		X	Digestive tract, mucus	Lizard Island
NA,YG	*V. rotiferianus*	X		Digestive tract	Lizard Island
GUIGEN	*V. rotiferianus*		X	Open sores	Guam
GUMAB	*V. fortis*	X		Digestive tract	Guam
GU5PALEEN	*V. fortis*		X	Open sores	Guam
CREAM TCBS, CREAM 12-13	*V. natriegens*		X	Digestive tract, mucus	Lizard Island
HAEMORR	*V. natriegens*		X	Open sores	Lizard Island
GU2Y	*Photobacterium eurosenbergii*	X		Digestive tract	Guam
GU5YEN, GU4YEN	*P. eurosenbergii*		X	Open sores	Guam

the Guam COTS were collected during the late stages of a devastating outbreak after COTS shifted their diet towards non-preferred species like *Porites* and faviids in the absence of *Acropora* and *Montipora*. These changes in food do not seem to have a major influence on bacterial microflora of COTS when compared with those from Lizard Island found feeding on their preferred corals, *Acropora* (Pratchett 2001, 2010). *Vibrio owensii* and *V. rotiferianus* were found at both locations. *V. fortis*, *Photobacterium eurosenbergii*, and *V. harveyi* were found at Guam only (Table 6). However, Sutton et al. (1988) previously isolated *V. harveyi, V. campbellii*, and *V. tubiashi* from *Acanthaster planci* and acknowledged these organisms as potential pathogens. The results of our study support those reported by Sutton et al. (1988), confirming that members of the Harveyi group are normal COTS inhabitants that play important roles in the induction of diseases in COTS and many other echinoderms (Table 5). Furthermore, our study suggests that host and digestive morphology may exert an influence over which bacterial species colonize the gut. These characteristics have been reported in other marine invertebrates and omnivorous animals (Harris 1993, Oxley et al. 2002, Egert et al. 2005).

All COTS, irrespective of their site of collection (Lizard Island or Guam), had similar bacterial microflora and responded in the same way to the TCBS agar (Rivera-Posada et al. 2011), indicating that a common mechanism of disease induction affects their bacterial communities and immune responses. Pathogenic and non-pathogenic vibrio isolates identified as *V. owensii*, *V. rotiferianus*, *V. fortis*, and *Photobacterium eurosenbergii* were recovered from sick and wild COTS at 2 different locations (Guam and Lizard Island), supporting the hypothesis that TCBS coupled with adequate environmental conditions could trigger vibrio virulence factors turning normal vibrios pathogenic (Rivera-Posada et al. 2011).

The species *Photobacterium eurosenbergii* is of particular significance because it can infect both COTS and coral, although it was found only in COTS from Guam. This vibrio is also recognized as a coral pathogen isolated from the mucus of the Caribbean elkhorn coral *Acropora palmata* (Ritchie 2006). *P. eurosenbergii* was also recovered from mucus and water surrounding bleached *Barabattoia amicorum* corals at Magnetic Island, GBR (Munn et al. 2008). Due to the feeding preferences of COTS, studying the transmission of bacteria between corals and COTS is necessary.

Studies of colonization sites within gut regions and growth patterns of different bacterial populations will allow a more focused approach to the types of associations within COTS. Density, production and turnover estimation of gut microbes are also required to establish bacterial associations and to make comparisons of microbial processes between sick and wild COTS.

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