α -Proteobacterial Symbionts of Marine Bryozoans in the Genus *Watersipora*[▽]

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Bacterial symbionts that resembled mollicutes were discovered in the marine bryozoan *Watersipora arcuata* in the 1980s. In this study, we used PCR and sequencing of 16S rRNA genes, specific fluorescence in situ hybridization, and phylogenetic analysis to determine that the bacterial symbionts of “*W. subtorquata*” and “*W. arcuata*” from several locations along the California coast are actually closely related α -Proteobacteria, not mollicutes. We propose the names “*Candidatus Endowatersipora palomitas*” and “*Candidatus Endowatersipora rubus*” for the symbionts of “*W. subtorquata*” and “*W. arcuata*,” respectively.

Bacterial symbionts of bryozoans in the genus *Watersipora* were originally reported in 1983 (38). The bacteria were consistently observed in a surface groove with an overhanging flange unique to *Watersipora* larvae (38). Inside adult colonies of *Watersipora*, the bacterial symbionts were found in clusters surrounded by host mesodermal cells (38). The bacterial symbionts were present in *Watersipora* larvae immediately upon their release into the water column, indicating vertical transmission of the bacteria, although transmission has not been observed directly. With their irregular shape and apparent lack of a cell wall in transmission electron micrographs, the bacterial symbionts resembled mollicutes and were called mycoplasma-like organisms (38). In 1989, an *Acholeplasma* sp., a type of mollicute thought to be the symbiont, was isolated from *Watersipora* larvae on mycoplasma agar amended with filter-sterilized adult *Watersipora* extract (5). However, the molecular techniques necessary to confirm that the isolated *Acholeplasma* sp. was actually the symbiont were unavailable at the time.

Calcareous marine bryozoans in the genus *Watersipora* are found worldwide in the tropics and subtropics (31). Along the coast of California, they are common on floating docks, boat bottoms, and other sturdy substrates in bays and harbors and are considered successful invaders that probably arrived in the 1960s (3). The systematics of the genus *Watersipora* are unresolved (15, 29, 31), making classification of host samples to the species level problematic. This is illustrated by the first report of symbionts in *Watersipora*, which identified the bryozoan host as *Watersipora cucullata* (38). Later, the identification was revised to *Watersipora arcuata* (37). Because of the confusion in systematics, it is critical to keep thorough records of the *Watersipora* samples, including host gene DNA sequences, light micrographs, and in some cases, scanning electron micrographs (SEMs), in order to study this symbiosis.

Examples of associations between bryozoans and bacteria are abundant (2, 24, 36, 38). In most cases, the roles of the bacteria in the lives of their host bryozoans are unknown. The symbiosis between the γ -proteobacterium “*Candidatus Endobugula sertula*” and the bryozoan *Bugula neritina* is an exception for which there is extensive evidence indicating that “*Candidatus Endobugula sertula*” is the source of the bryostatins, a family of polyketides which provide chemical defense for *B. neritina* larvae (9, 23). The closely related bacterial symbiont of the bryozoan *Bugula simplex*, “*Candidatus Endobugula glebosa*,” appears to have a similar role (22).

The objective of this study was to identify the bacterial symbionts of *Watersipora* species from samples collected at several locations along the California coast by amplifying their 16S rRNA genes by PCR from *Watersipora* larvae, directly sequencing the PCR products, confirming that the sequenced genes belonged to the symbiont by fluorescence in situ hybridization (FISH), and conducting a 16S rRNA gene-based phylogenetic analysis of the bacterial symbionts. The molecular work in this study focused on the nonfeeding larvae of *Watersipora*, taking advantage of the comparative simplicity of the associated microbial community relative to that in filter-feeding adult *Watersipora* colonies.

MATERIALS AND METHODS

Sample collection. “*W. subtorquata*” colonies were collected from floating docks at Shelter Island, San Diego, CA (September 2003, March 2004, February 2005, and June 2006), Morro Bay, CA (June 2005), San Francisco, CA (July 2005), and Bodega Bay, CA (September 2004), and from a boat bottom in Ventura, CA (July 2005). “*W. arcuata*” colonies were collected from floating docks in Oceanside Harbor, Oceanside, CA (August 2005, November 2005, and June 2006). Adult colonies were maintained in aerated aquaria with running seawater on a 12-h light–12-h dark cycle. *Watersipora* larvae were released in the morning when the lights turned on. Upon release, larvae were transferred from the aquaria to clean seawater and then to sterile artificial seawater on ice with a Pasteur pipette. In ice-cold seawater, the larvae sank to the bottom of the tube, and the seawater was removed. Larvae to be used for FISH experiments were fixed by incubation in 4% paraformaldehyde-morpholinepropanesulfonic acid (MOPS) buffer (4% paraformaldehyde, 0.1 M MOPS, 0.5 M sodium chloride, pH 7.5) for 2 h at room temperature. The paraformaldehyde buffer was removed, and the larvae were rinsed with 70% ethanol and then stored in 70% ethanol at -20°C .

DNA isolation. Genomic DNAs were isolated from fresh *Watersipora* larvae (all collections) and from embryo-free regions of *Watersipora* adult colonies (San

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Diego, June 2006; Oceanside, June 2006) using a DNeasy tissue kit (QIAGEN Inc.) following the manufacturer's protocol for animal tissue, including the optional RNase step, except that DNA was eluted twice with 40 μ l of elution buffer.

16S rRNA and mitochondrial COI PCR and sequencing. *Watersipora* larval DNA, which contains bryozoan DNA and bacterial symbiont DNA, was amplified from collections obtained from March 2004 to June 2006 with the following primers (all synthesized by Integrated DNA Technologies): universal bacterial 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (21) and 1492R (5'-TACGGYTACCTTGTACGACTT-3') (21) and mitochondrial cytochrome *c* oxidase subunit I (COI) primers LCO1490 (5'-GGTCAACAAA TCATAAAGATATTGG-3') (12) and HCO2198 (5'-TAAACTTCAGGGTGA CCAAAAAATCA-3') (12). "*W. subtorquata*" adult DNA was amplified with bacterial symbiont 16S rRNA gene primers Ws100F (5'-CGGTAGGGAATAA CGCATAG-3') and Wpa1365R (5'-CACAACGCCTTCAAGTAG-3'). "*W. arcuata*" adult DNA was amplified with Ws100F and 1492R. Each 50- μ l PCR mixture included 125 to 400 ng *Watersipora* DNA, 1 μ M of each primer, 1.25 units *Taq* DNA polymerase (Roche), 1 $\times$ PCR buffer (Roche), a 200 μ M concentration of each deoxynucleoside triphosphate (Invitrogen), and 0.2 mg/ml bovine serum albumin. PCR consisted of 1 cycle of 94°C for 90 s; 30 cycles of 94°C for 60 s, the annealing temperature for 60 s, and 72°C for 60 s; and 1 cycle of 72°C for 7 min. The annealing temperature was 55°C for the 27F/1492R primers, 50°C for the LCO1490/HCO2198 primers, and 60°C for the adult *Watersipora* samples.

PCR products were cleaned up with a Rapid PCR purification system kit (Marpigen Bioscience Inc.) according to the manufacturer's protocol, except that the elution volume was 30 μ l, or with a QIAquick PCR purification kit (QIAGEN Inc.) according to the manufacturer's protocol. The purified PCR products were then directly sequenced using 2 μ l BigDye Terminator v3.1 (Applied Biosystems), 5 picomoles oligonucleotide primer, and 3.5 to 5 μ l purified PCR product in a total volume of 10 μ l. The same primers used for amplification were used for sequencing as well as internal primers for the 16S rRNA gene. The sequencing reaction consisted of 1 cycle of 96°C for 60 s and 28 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. After the sequencing reaction was complete, 40 μ l of 75% isopropanol was added to each reaction, mixed by pipetting, and incubated for 15 min at room temperature. The samples were then centrifuged for 30 min at 2,000 $\times$ g, the supernatant was removed by inverting the tube, and the sample was dried by inverted centrifugation for 1 min at 700 $\times$ g. Sequencing reactions were then run on a capillary electrophoresis DNA sequencer (ABI PRISM 3100 genetic analyzer) by SeqXcel, Inc. (San Diego, CA). Sequences were evaluated and assembled with Sequencher 4.1.4 (Gene Codes Corporation).

Mollusc-specific 16S rRNA PCR. PCR with primers MGSO (5'-TGCACCA TCTGTCACTCTGTTAACTC-3') (35) and GPO-3 (5'-GGGAGCAAACAG GATTGATACCT-3') (34), which were designed to specifically amplify part of the 16S rRNA genes of molluscs, was performed on "*W. subtorquata*" larval DNA (San Diego, September 2003 and March 2004). PCR reagents and concentrations were the same as those described above. The PCR conditions described by van Kuppeveld et al. (35), i.e., 1 cycle of 94°C for 60 s and 40 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 2 min, were used. Products were electrophoresed in 1.2% agarose gels, cut out, purified with a QIAquick gel extraction kit (QIAGEN Inc.), cloned with a TOPO TA cloning kit (Invitrogen), transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen), and sequenced.

***pufLM* PCR.** PCR with primers *pufL* (5'-CTKTTCTGACTTCTGGGTSGG-3') (27) and *pufM* (5'-CCATSGTCCAGCGCCAGAA-3') (4), which were designed to amplify ~1,500 bp of the *pufL* and *pufM* reaction center complex genes from aerobic, anoxygenic, phototrophic bacteria, was performed on "*W. subtorquata*" (Ventura, August 2005) and "*W. arcuata*" (Oceanside, June 2006) larval DNAs. Strain DG1128 (16) was used as a positive control. The PCR reagents and concentrations were the same as those used for 16S rRNA PCR. The PCR conditions described by Green et al. (16), i.e., 1 cycle of 94°C for 2 min, 30 cycles of the annealing temperature for 30 s, 72°C for 2.5 min, and 94°C for 10 s, and 1 cycle of 72°C for 10 min, were used. Annealing temperatures ranged from 48°C to 58°C.

Symbiont-specific probe design and FISH. Cy3-labeled oligonucleotide probes specific for the "*W. subtorquata*" bacterial symbiont and the "*W. arcuata*" bacterial symbiont were designed based on the 16S rRNA gene sequences of the 27F/1492R PCR products from "*W. subtorquata*" and "*W. arcuata*" larvae, respectively. Potential probes complementary to the obtained 16S rRNA gene sequences were tested against The Ribosomal Database Project II (RDP-II) database using Probe Match (8). Epa1365R-Cy3 (5'-Cy3-CACAACGCCTTCA AGTAG-3'), the "*W. subtorquata*" symbiont-specific probe, had 0 matches with zero or one mismatches allowed and 180 matches with two mismatches allowed in the RDP-II database as of 29 July 2006. A one-base mismatch probe, Mis-

match Epa1365R-Cy3 (5'-Cy3-CACAACGCCTTGAAGTAG-3'), was also designed to ensure that it was possible to resolve a one-base mismatch with the hybridization conditions used. Successful hybridization of "*W. subtorquata*" bacterial symbionts with Epa1365R-Cy3 required the addition of unlabeled helper oligonucleotides designed by the method of Fuchs et al. (13) to bind to the 16S rRNA molecule adjacent to the Epa1365R-Cy3 target (H1347 [5'-AACCAAC TCCCATGGTGT-3'] and H1383 [5'-TTGCCTCTTACGGTTAG-3']) and to be identical to the Epa1365R-Cy3 target (H1365 [CTACTTGAAGCGTT GTG-3']). Eru1365R-Cy3 (5'-Cy3-CTCAACGCCTTCAAGTAG-3'), the "*W. arcuata*" symbiont-specific probe, had 0 matches with zero or one mismatches allowed and 31 matches with two mismatches allowed in the RDP-II database as of 29 July 2006. The unlabeled helper oligonucleotides H1365ru (5'-CTACCT GAAGGCGTTGAG-3') and H1383ru (5'-TGCCTCTCAACGGTTAG-3') were designed to bind the 16S rRNA molecule of the "*W. arcuata*" symbiont.

The FISH protocol was based on the work of Glockner et al. (14). Fixed "*W. subtorquata*" (San Diego, June 2006) and "*W. arcuata*" (Oceanside, June 2006) larvae were incubated with 5 ng/ μ l Eub338-Cy5 (1) and one of the following Cy3-labeled probes in 35% formamide hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 35% formamide, 0.01% sodium dodecyl sulfate) for 4 h at 46°C: 0.5 ng/ μ l Epa1365R-Cy3, 0.5 ng/ μ l Mismatch Epa1365R-Cy3, or 0.5 ng/ μ l or 5 ng/ μ l Eru1365R-Cy3. H1347 (5 ng/ μ l), H1365 (5 ng/ μ l), and H1383 (5 ng/ μ l) were also used in the Epa1365R-Cy3 and Mismatch Epa1365R-Cy3 hybridizations. H1347 (5 ng/ μ l), H1365ru (5 ng/ μ l), and H1383ru (5 ng/ μ l) were used with 0.5 ng/ μ l Eru1365R-Cy3. The larvae were washed for 40 min at 48°C in wash buffer (70 mM NaCl, 20 mM Tris-HCl, pH 7.2, 5 mM EDTA, pH 8.0, 0.01% sodium dodecyl sulfate). Larvae were then washed with phosphate-buffered saline, placed on a slide, covered with VectaShield (Vector Laboratories, Burlingame, CA) and a coverslip, viewed by confocal microscopy (Zeiss LSM 5 Pascal), and photographed.

Phylogenetic analysis. The 16S rRNA gene sequences of the bacterial symbionts were aligned by eye with the *Mesorhizobium loti* 16S rRNA (GenBank accession no. AP003001) sequence and secondary structure (<http://www.rna.icmb.utexas.edu/>). Top matches from the NCBI BLASTN server and from Sequence Match by RDP-II, other α -Proteobacteria, and *Marinobacter bryozoorum*, a γ -proteobacterium used as an outgroup, all of which were at least 1,212 nucleotides long, were downloaded in alignment from RDP-II (8). The *Watersipora* symbiont 16S rRNA genes were added to the alignment, using *M. loti* for reference. The alignment was exported in Nexus format, and phylogenetic trees were made using MrBayes 3.1 (18, 28) and PAUP* 4.0b10 (32). For MrBayes, the evolutionary model was set to the general time reversible model, with rates equal to the inverse of gamma. Three million generations were run, with sampling every 100 generations, and burn-in was set to 20,000. For PAUP*, the criterion was set to parsimony, transversions were weighted 1.5 times more than transitions, and 2,000 bootstrap replicates were performed.

Watersipora COI gene sequences were aligned with Sequencher 4.1.4 (Gene Codes Corporation), using *B. neritina* as the outgroup. Host phylogenetic trees were constructed with the 705-bp (including gaps) COI gene sequence alignment, using MrBayes 3.1 as described above. PAUP* 4.0b10 was used as described above, except that transversions were weighted 2.2 times more than transitions.

SEM. "*W. subtorquata*" (San Diego, February 2005) and "*W. arcuata*" (Oceanside, November 2005) adults from the same samples from which larvae were obtained for DNA sequencing were placed in 10% bleach for 4 h at room temperature and rinsed with distilled water. The samples were then critical point dried and sputter coated. SEM images were obtained with a Quanta 600 scanning environmental microscope (FEI Company).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of "*Candidatus Endowatersipora palomitas*" and "*Candidatus Endowatersipora rubus*" were deposited in GenBank under accession numbers DQ417460 and DQ417461, respectively. The *Watersipora* COI gene sequences from this study were deposited in GenBank under accession numbers DQ417453 to DQ417459.

RESULTS

16S rRNA gene sequences. Directly sequencing the 27F/1492R 16S rRNA PCR product from each "*W. subtorquata*" larval sample yielded a single α -proteobacterial 16S rRNA gene sequence. Sequences from three different 16S rRNA PCR products from "*W. subtorquata*" (San Diego, March 2004) larval DNAs were assembled to yield 1,422 bp of good sequence with 1 $\times$ to 11 $\times$ coverage (GenBank accession number

FIG. 1. “*W. subtorquata*” and “*W. arcuata*” larvae dually labeled with Eub338-Cy5 and a Cy3-labeled symbiont-specific probe have strong signals in the supracoronal groove. Bars, 100 μ m. Posterior (A) and anterior (B) views of “*W. subtorquata*” larvae hybridized with Eub338-Cy5 and Epa1365R-Cy3 are shown. (C) Posterior view of “*W. arcuata*” larva hybridized with Eub338-Cy5 and 0.5 ng/ μ l Eru1365R-Cy3 with helper oligonucleotides. (D) Anterior view of “*W. arcuata*” larva hybridized with Eub338-Cy5 and 5 ng/ μ l Eru1365R-Cy3 without helper oligonucleotides.

DQ417460). Coverage of 16S rRNA genes from all other “*W. subtorquata*” larval samples was lower (1 $\times$ to 2 $\times$ from one PCR product, unless otherwise stated), but all sequences matched sequence DQ417460 over the range that good sequence was obtained, as follows: Bodega Bay, September 2004, 1,234 bp, 1 $\times$ to 3 $\times$ coverage from two PCR products; San Diego, February 2005, 1,323 bp, 1 $\times$ to 3 $\times$ coverage from one PCR product; Morro Bay, June 2005, 1,156 bp; Ventura, July 2005, 1,234 bp; San Francisco, July 2005, 1,238 bp; and San Diego, June 2006, 1,233 bp. The 16S rRNA PCR product obtained from an embryo-free region of adult “*W. subtorquata*” (San Diego, June 2006) matched DQ417460 over 1,145 bp with 1 $\times$ to 2 $\times$ coverage.

A single α -proteobacterial 16S rRNA gene sequence which was 96.9% identical over 1,377 bp to that from “*W. subtorquata*” was obtained by sequencing the 27F/1492R 16S rRNA PCR product from each “*W. arcuata*” larval sample. Sequences from two different 16S rRNA PCR products from “*W. arcuata*” (Oceanside, August 2005) larval DNAs were assembled to yield 1,377 bp of good sequence with 1 $\times$ to 10 $\times$ coverage (GenBank accession number DQ417461). Coverage of 16S rRNA genes from all other “*W. arcuata*” larval samples was lower (1 $\times$ to 2 $\times$ from one PCR product), but all sequences matched sequence DQ417461 over the range that good sequence was obtained, as follows: Oceanside, November 2005,

1,282 bp, with one ambiguous base pair; and Oceanside, June 2006, 1,287 bp, with one ambiguous base pair. The sequence of the 16S rRNA gene PCR product from an embryo-free region of adult “*W. arcuata*” (Oceanside, June 2006) matched sequence DQ417461 over 1,067 bp with 1 $\times$ to 2 $\times$ coverage.

Mollicute 16S rRNA PCR. A mollicute 16S rRNA gene fragment of the expected size ($\sim$ 270 bp) was obtained from “*W. subtorquata*” larval DNA (San Diego, September 2003) (data not shown). The mollicute 16S rRNA product was verified by DNA sequencing. No mollicute 16S rRNA gene product was obtained from “*W. subtorquata*” larval DNA (San Diego, March 2004), indicating the lack of a consistent association between “*W. subtorquata*” larvae and mollicutes.

pufLM PCR. The anoxygenic photosynthetic reaction center genes *pufLM* were not amplified from “*W. subtorquata*” (Ventura, July 2005) or “*W. arcuata*” (Oceanside, June 2006) larval DNA. At an annealing temperature of 48°C, differently sized products under 500 bp, which is much smaller than the expected size of an actual *pufLM* product ($\sim$ 1,500 bp), were observed from each type of *Watersipora*. At higher annealing temperatures of up to 58°C, no products were observed from either *Watersipora* sample. The positive control, strain DG1128, yielded products of the expected size ($\sim$ 1,500 bp) at all annealing temperatures tested from 48°C to 58°C.

FISH. The signals from Epa1365R-Cy3, a “*W. subtorquata*” symbiont-specific probe (see Fig. 2C), and Eub338-Cy5, a general eubacterial probe (see Fig. 2B) colocalized (Fig. 1A and B and 2A) to cells in the supracoronal groove of “*W. subtorquata*” larvae. Bacterial symbionts in the supracoronal groove of “*W. subtorquata*” larvae which were hybridized with Eub338-Cy5 (Fig. 2E and H) and Mismatch Epa1365R-Cy3, a single-base-mismatch probe (Fig. 2F), or Eru1365R-Cy3, a “*W. arcuata*” symbiont-specific probe (Fig. 2I), showed a strong Cy5 signal but lacked a Cy3 signal. The signals from Eru1365R-Cy3, a “*W. arcuata*” symbiont-specific probe (Fig. 2L), and Eub338-Cy5 (Fig. 2K) colocalized to cells in the supracoronal groove of “*W. arcuata*” larvae (Fig. 1C and D and 2J). Bacterial symbionts in the supracoronal groove of “*W. arcuata*” larvae which were hybridized with Eub338-Cy5 (Fig. 2N) and Epa1365R-Cy3 (Fig. 2O) showed a strong Cy5 signal but lacked a Cy3 signal.

The presence of a Cy3 signal in “*W. subtorquata*” larvae hybridized with Epa1365R-Cy3 (Fig. 2C) compared with the lack of a Cy3 signal in “*W. arcuata*” larvae hybridized with Epa1365R-Cy3 (Fig. 2O) and the presence of a Cy3 signal in “*W. arcuata*” larvae hybridized with Eru1365R-Cy3 (Fig. 2L) compared with the lack of a Cy3 signal in “*W. subtorquata*” larvae hybridized with Eru1365R-Cy3 (Fig. 2I) demonstrate the specificity of the symbiont-targeting probes. The presence of a Cy3 signal in “*W. subtorquata*” larvae hybridized with Epa1365R-Cy3 (Fig. 2C) compared with the lack of a Cy3 signal in “*W. subtorquata*” larvae hybridized with Mismatch Epa1365R-Cy3 (Fig. 2F) shows that the hybridization conditions were stringent enough to distinguish a one-nucleotide difference. The specific binding of Epa1365R-Cy3 and Eru1365R-Cy3 to their targets and their colocalization with Eub338-Cy5 confirm that the α -proteobacterial 16S rRNA gene sequences obtained from “*W. subtorquata*” and “*W. arcuata*” larvae are from the most abundant, and perhaps only, bacterial symbionts present in “*W. subtorquata*” and “*W. arcuata*” larvae, respectively.

Although Epa1365R-Cy3 and Eru1365R-Cy3 target the same region of the 16S rRNA molecule, Epa1365R-Cy3 requires helper oligonucleotides to successfully hybridize with “*W. subtorquata*” bacterial symbiont 16S rRNA (data not shown), while Eru1365R-Cy3 does not require helper oligonucleotides to hybridize with “*W. arcuata*” bacterial symbiont 16S rRNA (Fig. 1D).

The bacterial symbionts of “*W. subtorquata*” have an unusual, irregular cell shape (Fig. 2P). The bacterial symbionts of “*W. arcuata*” are rounder, with smoother edges (Fig. 2Q). Both bacterial symbiont types have dark areas near their centers in FISH images (Fig. 2P and Q), indicating a compartment or

region where rRNA is excluded. This dark region seems to correspond with the “central, spherical, electron-opaque” region in transmission electron micrographs of “*W. arcuata*” bacterial symbionts in the original report (38).

Bacterial symbiont phylogeny. The symbionts of “*W. subtorquata*” and “*W. arcuata*” cluster together within the *Phyllobacteriaceae* family of the α -*Proteobacteria* subdivision in the 16S rRNA gene sequence-based phylogenetic tree (Fig. 3). The bacterial symbiont 16S rRNA gene sequences from all “*W. subtorquata*” samples (March 2004 to June 2006) were identical, and the bacterial symbiont 16S rRNA gene sequences from all “*W. arcuata*” samples (August 2005 to June 2006) were identical. The bacterial symbiont 16S rRNA gene sequence from “*W. arcuata*” was about 3.1% different from that of the “*W. subtorquata*” bacterial symbiont over 1,377 bp.

We propose the name “*Candidatus Endowatersipora palomitas*” (“palomitas,” which means popcorn in Spanish, for the shape of the cells) for the bacterial symbiont of “*W. subtorquata*,” with the following description (26): “*Candidatus Endowatersipora palomitas*” (α -*Proteobacteria* NC; gram negative; O; GenBank accession number DQ417460 for oligonucleotide sequence complementary to diagnostic region of 16S rRNA [5'-CACAACGCCTTCAAGTAG-3']; S [*Watersipora subtorquata*,” supracoronal groove of larvae]; M). We propose the name “*Candidatus Endowatersipora rubus*” (“rubus,” the genus name of raspberries and their allies, for the shape of the cells) for the bacterial symbiont of “*W. arcuata*,” with the following description (26): “*Candidatus Endowatersipora rubus*” (α -*Proteobacteria* NC; gram negative; O; GenBank accession number DQ417461 for oligonucleotide sequence complementary to diagnostic region of 16S rRNA [5'-CTCAACGCCTTCAGGTAG-3']; S [*Watersipora arcuata*,” supracoronal groove of larvae]; M).

Based on the 16S rRNA gene phylogeny, the closest relatives of the *Watersipora* symbionts are the uncultured bacterium IndB3-43 (92.8% identical to “*Candidatus Endowatersipora palomitas*” over 1,196 nucleotides; 92.8% identical to “*Candidatus Endowatersipora rubus*” over 1,169 nucleotides), the *Ophiactis balli* (brittle star) symbiont (92.7% identical to “*Candidatus Endowatersipora palomitas*” over 1,351 nucleotides; 92.0% identical to “*Candidatus Endowatersipora rubus*” over 1,333 nucleotides) and *Mesorhizobium* sp. strain DG943 (92.2% identical to “*Candidatus Endowatersipora palomitas*” over 1,380 nucleotides; 92.1% identical to “*Candidatus Endowatersipora rubus*” over 1,369 nucleotides).

***Watersipora* mitochondrial COI gene sequences and phylogeny.** All “*W. subtorquata*” samples cluster together, excluding the cluster of “*W. arcuata*” samples, in the phylogenetic tree of

FIG. 2. FISH experiments on *Watersipora* larvae. The 633-nm λ laser used to visualize Cy5 signals (red) was set to 1.1% transmission, and the 543-nm λ laser used to visualize Cy3 signals (yellow) was set to 3.9% transmission for all samples. All Cy-3-labeled probes were used at a concentration of 0.5 ng/ μ l with the corresponding helper oligonucleotides. Bars, 10 μ m (A to O) and 5 μ m (P and Q). (A to C) “*W. subtorquata*” larva hybridized with Eub338-Cy5 (B) and Epa1365R-Cy3 (C), with merged signals shown in panel A. (D to F) “*W. subtorquata*” larva hybridized with Eub338-Cy5 (E) and Mismatch Epa1365R-Cy3 (F), with merged signals shown in panel D. (G to I) “*W. subtorquata*” larva hybridized with Eub338-Cy5 (H) and 0.5 ng/ μ l Eru1365R-Cy3 (I), with merged signals shown in panel G. (J to L) “*W. arcuata*” larva hybridized with Eub338-Cy5 (K) and 0.5 ng/ μ l Eru1365R-Cy3 (L), with merged signals shown in panel J. (M to O) “*W. arcuata*” larva hybridized with Eub338-Cy5 (N) and Epa1365R-Cy3 (O), with merged signals shown in panel M. (P) Close-up of “*Candidatus Endowatersipora palomitas*” labeled with Eub338-Cy5 in “*W. subtorquata*” larvae. (Q) Close-up of “*Candidatus Endowatersipora rubus*” labeled with Eub338-Cy5 in “*W. arcuata*” larvae.

FIG. 3. Phylogenetic tree of *Watersipora* bacterial symbionts “*Candidatus Endowatersipora palomitas*” and “*Candidatus Endowatersipora rubus*” and other α -*Proteobacteria* constructed with MrBayes 3.1, using 16S rRNA genes. *Marinobacter bryozoorum*, a γ -*proteobacterium*, was used as the outgroup. Posterior probabilities (top numbers) are given with bootstrap values from maximum parsimony analysis using PAUP* (bottom numbers) when values are >60. Bar, 0.1 expected change per site.

host COI genes (Fig. 4A). Within the “*W. subtorquata*” cluster, there are two distinct clades of “*W. subtorquata*” (Fig. 4A).

The clade of Morro Bay, San Francisco, and Bodega Bay “*W. subtorquata*” samples probably represents a single *Watersipora* COI sequence, since samples from San Francisco and Bodega Bay are identical over 621 nucleotides and the sample from Morro Bay is 99.8% identical to the San Francisco and Bodega Bay samples over 619 nucleotides. Within the San Diego and Ventura “*W. subtorquata*” clade, the March 2004 San Diego sample and the Ventura sample are identical over 625 nucleotides. The February 2005 San Diego sample is 95.4% identical to the March 2004 San Diego and Ventura samples over 655 and 625 nucleotides, respectively, without counting the single ambiguity in the February 2005 San Diego sequence as a difference. Within the entire “*W. subtorquata*” COI cluster, there is as much as 14.8% difference over 622 nucleotides between samples.

The “*W. arcuata*” clade may represent a single COI sequence, since the August 2005 and November 2005 Oceanside samples are 99.5% identical over 608 nucleotides when the four ambiguities in the August 2005 sequence are not counted as differences.

Between the “*W. subtorquata*” and “*W. arcuata*” COI clades, the differences between samples range from 15.0% over 635 nucleotides to 17.9% over 605 nucleotides when any sequence ambiguities are not counted as differences.

SEMs. The SEMs of “*W. subtorquata*” (San Diego, February 2005) and “*W. arcuata*” (Oceanside, November 2005) adult colonies illustrate characteristic morphological differences be-

tween the two types of *Watersipora* which were used for tentative identification of host samples (Fig. 4B to F). One of the most easily recognized differences between adults of “*W. subtorquata*” and “*W. arcuata*” is the shape of the orifice through which the lophophore, the filter-feeding structure, extends (Fig. 4B and D). Another distinguishing feature is the opening in the basal wall of each adult “*W. arcuata*” individual (Fig. 4F), which is absent in “*W. subtorquata*” zooids.

DISCUSSION

The bacterial symbionts of “*W. arcuata*” and “*W. subtorquata*” are closely related but genetically and morphologically distinct members of the α -*Proteobacteria*, not mollicutes, as their appearance originally suggested. Because less abundant bacterial symbionts can be overlooked by directly sequencing 16S rRNA PCR products rather than cloning the PCR product and sequencing many clones (11), it is possible that there are other bacterial symbionts present in *Watersipora* larvae. However, the fact that all cells observed to be labeled with Eub338-Cy5 were also specifically labeled with Epa1365R-Cy3 in “*W. subtorquata*” larvae or with Eru1365R-Cy3 in “*W. arcuata*” larvae is strong evidence that the most abundant bacterial symbiont in each type of *Watersipora* larva was represented by the 16S rRNA gene sequence obtained by direct sequencing of the PCR products. The presence of the bacterial symbionts in adult *Watersipora* was confirmed by the detection of “*Candidatus Endowatersipora palomitas*” and “*Candidatus Endo-*

FIG. 4. (A) Phylogenetic tree of *Watersipora* samples constructed with MrBayes 3.1 using host COI genes, with *B. neritina* as an outgroup. Posterior probabilities are given, followed by bootstrap values (in parentheses) from maximum parsimony analysis using PAUP*. ♦, samples for which SEMs were obtained. Bar, 0.1 expected change per site. Sample collection locations are indicated by lines to the map. (B and C) SEMs showing frontal views of adult *W. subtorquata* colonies (San Diego, February 2005). (D and E) SEMs showing frontal views of adult *W. arcuata* colonies (Oceanside, November 2005). (F) SEM of basal wall of *W. arcuata* colony (Oceanside, November 2005).

watersipora rubus" 16S rRNA genes in embryo-free regions of adult "*W. subtorquata*" and "*W. arcuata*" colonies, respectively.

If there are other bacterial symbionts consistently associated with *Watersipora* larvae, it is unlikely that they are mollicutes since mollicutes were not consistently detected in "*W. subtorquata*" larvae by a mollicute PCR screen. The isolated *Acholeplasma* sp. thought to be the symbiont of "*W. arcuata*" (5) was probably an incidental environmental bacterium that passed through small-pore-size filters (an ability of many mollicutes) used to prepare adult *Watersipora* extracts, which were required for isolation but not maintenance of the *Acholeplasma* sp. Another possibility is that the actual symbiont of *Watersipora* was isolated but was originally misclassified.

Remarkably, "*W. subtorquata*" samples that vary by as much as 14.8% over 622 nucleotides of COI gene sequence contain bacterial symbionts with identical 16S rRNA gene sequences.

Point Conception, located between Ventura and Morro Bay, CA, is a biogeographical boundary for many marine organisms based on species ranges (6, 10, 33). It is hypothesized that Point Conception is also a phylogeographic boundary, causing a genetic divide in species that span Point Conception, although several studies conflict with this idea (7, 25). Although more sampling would be required to be conclusive, the split in COI gene sequences from our "*W. subtorquata*" samples (Fig. 4A) is consistent with Point Conception being a phylogeographic boundary.

Documenting host *Watersipora* samples is an important part of describing this symbiosis, since the systematics of the genus *Watersipora* are currently incoherent. Our samples consisted of two distinct host morphologies, tentatively identified as "*W. subtorquata*" and "*W. arcuata*," which each contains a consistent and distinct α -proteobacterial symbiont. Although sorting out *Watersipora* systematics is beyond the scope of our work, by recording *Watersipora* COI sequences and SEMs of the two distinct adult *Watersipora* morphologies, we hope to provide the information necessary to fit this research into a future revised version of *Watersipora* systematics.

The consistent association of specific bacterial symbionts with "*W. subtorquata*" and "*W. arcuata*," their apparent vertical transmission from *Watersipora* adults to larvae, and the unique symbiont-containing groove with overhanging flange of *Watersipora* larvae (38), which seems to facilitate transmission, all imply an important function for the bacterial symbionts. However, the phylogeny of the *Watersipora* symbionts fails to deliver obvious clues about the role of the bacteria in the lives of their hosts. The most closely related 16S rRNA gene sequences in the databases, a brittle star symbiont 16S rRNA gene and a 16S rRNA gene found at an inactive deep-sea hydrothermal vent chimney (IndB3-43), represent uncultivated bacteria with unknown metabolic capabilities. The 16S rRNA gene from *Mesorhizobium* sp. strain DG943, the closest gene from a cultivated bacterium to cluster with the *Watersipora* symbionts, is only about 92% identical. *Mesorhizobium* sp. strain DG943 was isolated from a cultured strain of *Gymnodinium catenatum*, a dinoflagellate implicated in paralytic shellfish poisoning, for which the paralytic shellfish toxin content was below the detection limit (16). Pink pigmentation of colonies after aerobic growth in the dark, suggesting the presence of bacteriochlorophyll *a*, and the detection of photosynthetic reaction center genes *pufLM* by PCR indicated that *Mesorhizobium* sp. strain

DG943 may be capable of aerobic, anoxygenic photosynthesis (16). However, *pufLM* genes were not detected in "*W. subtorquata*" or "*W. arcuata*" larval DNA, so there is no evidence that the *Watersipora* bacterial symbionts are capable of aerobic, anoxygenic photosynthesis.

One demonstrated role for γ -proteobacterial bryozoan symbionts from *Bugula neritina* is chemical defense of host larvae. We hypothesize that bacterial symbionts of *Watersipora* are also involved in chemical defense of host larvae. *Watersipora* larvae are free-swimming, soft-bodied, and bright orange-red, making them an appealing target for predators and likely to be chemically defended. Bioactive anthraquinones, including 1,8-dihydroxyanthraquinone, have been isolated from Japanese *Watersipora* species (19, 30) and are one possibility for symbiont-produced chemical defense compounds. A compound similar to those isolated from Japanese *Watersipora* species, 1,8-dihydroxy-3-methylanthraquinone (chrysophanol), protects beetle eggs from ant predators (17). Both 1,8-dihydroxyanthraquinone and chrysophanol have been found in the eggs and larvae of two different leaf beetles, namely, *Trirhabda geminata* and *Galeruca tanacetii* (17, 20). Chrysophanol and 1,8-dihydroxyanthraquinone do not come from the diets of these leaf beetles, and it has been proposed that they could be produced by symbiotic microorganisms as chemical defense for the host offspring (17, 20).

Elucidating the true roles of "*Candidatus Endowatersipora palomitas*" and "*Candidatus Endowatersipora rubus*" will require a combination of chemical analysis of *Watersipora* adults and larvae and an exploration of bacterial symbiont biosynthetic and metabolic genes. The 16S rRNA gene-based identification of the *Watersipora* symbionts "*Candidatus Endowatersipora palomitas*" and "*Candidatus Endowatersipora rubus*" as α -*Proteobacteria* specifically associated with *Watersipora* species from several locations along the California coast lays the groundwork for a better understanding of this symbiosis.

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