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Association of rhizobia with a marine polychaete

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Summary

We report the presence of *Mesorhizobium*, a genus best known for its nitrogen-fixing symbiosis with terrestrial legumes, associated with the marine polychaete *Meganerilla bactericola* (Annelida: Nerillidae). Abundant epibionts were previously described as coating the exterior of *M. bactericola*, which is found within the anoxic sulfide-oxidizing microbial mats of the Santa Barbara Basin, California, USA. 16S rRNA investigation of the bacterial community associated with this polychaete discovered the presence of bacteria belonging to *Mesorhizobium*. We identified these bacteria using phylogenetic analyses of 16S rRNA and three additional functional genes, *nifH, atpD* and *recA*, and group-specific fluorescence *in situ* hybridization (FISH).

Introduction

Nitrogen fixation, the metabolic conversion of atmospheric nitrogen (N_2) into ammonia (NH_3), is restricted to a limited number of *Bacteria* and *Archaea*, collectively referred to as diazotrophs (Zehr *et al*., 2003). Nitrogen fixation introduces significant amounts of bioavailable nitrogen into terrestrial and aquatic habitats, thus supporting ecosystem productivity. All non-diazotrophic organisms rely on alternative ways of acquiring nitrogen: most animals and fungi consume nitrogen in biomolecules through heterotrophy; the majority of plants and bacteria assimilate inorganic nitrogen compounds from soil or water; and finally a variety of eukaryotes including protists, plants, fungi, and invertebrates obtain bioavailable nitrogen from symbioses with diazotrophs. These symbioses range from temporary, non-specific external associations to permanent intracellular interactions with symbionts passed between generations (Fiore *et al*., 2010). Among the best characterized diazotrophs are the rhizobia bacteria living with leguminous plants. This interaction requires coordinated gene expression by the host plant and its symbiotic bacteria to establish a specialized nodule where the latter are protected from oxygen and provided with carbon compounds in exchange for fixed nitrogen compounds (see recent reviews: Kereszt *et al*., 2011; Mortier *et al*., 2012; Wang *et al*., 2012).

In contrast to terrestrial systems, most known examples of nitrogen fixation in the ocean involve free-living, non-symbiotic photoautotrophic diazotrophs such as the marine cyanobacterium *Trichodesmium* (Riemann *et al*., 2010; Kranz *et al*., 2011; Sohm *et al*., 2011; Zehr, 2011; Zehr and Kudela, 2011). In addition, several examples of marine symbioses based on N_2-fixation have been described. These involve such hosts as protists, corals, sponges, molluscs, sea urchins, and tunicates (reviewed in Kneip *et al*., 2007; Foster and O'Mullan, 2008; Fiore *et al*., 2010). The prokaryotes involved in these marine symbioses are commonly cyanobacteria, but may not infrequently belong to other bacterial groups including *Vibrio*, gamma-proteobacteria, *Desulfovibrio*, and alpha-proteobacteria such as *Rhizobiales* (Lesser *et al*., 2004; Sfanos *et al*., 2005; Olson *et al*., 2009; Lema *et al*., 2012).

The current study demonstrates the first definitive association between bacteria with the genetic potential for nitrogen fixation (*nifH*) and a marine polychaete. The annelid, *Meganerilla bactericola*, lives within the deep-water sulfide-oxidizing microbial mats of the Santa Barbara Basin, California, USA. Abundant, unidentified epibiotic bacteria were previously described as covering, but not penetrating, the epidermis of this polychaete (Bernhard *et al*., 2000; Müller *et al*., 2001). Rhizobial epibionts of this annelid were identified using 16S rRNA and three additional functional genes, *nifH*, *atpD* and *recA*, phylogenetic analysis, and group-specific fluorescence *in situ* hybridization (FISH).

Results and discussion

Specimens of *M. bactericola* and bacterial mat samples were collected with a Soutar box corer onboard the R/V *Melville* from ~ 585 m depth in the Santa Barbara Basin (34°10-17′N, 120°1′W). Animals were sorted under a stereomicroscope and fixed in 95% ethanol, 4% paraformaldehyde, or 2% glutaraldehyde in sodium

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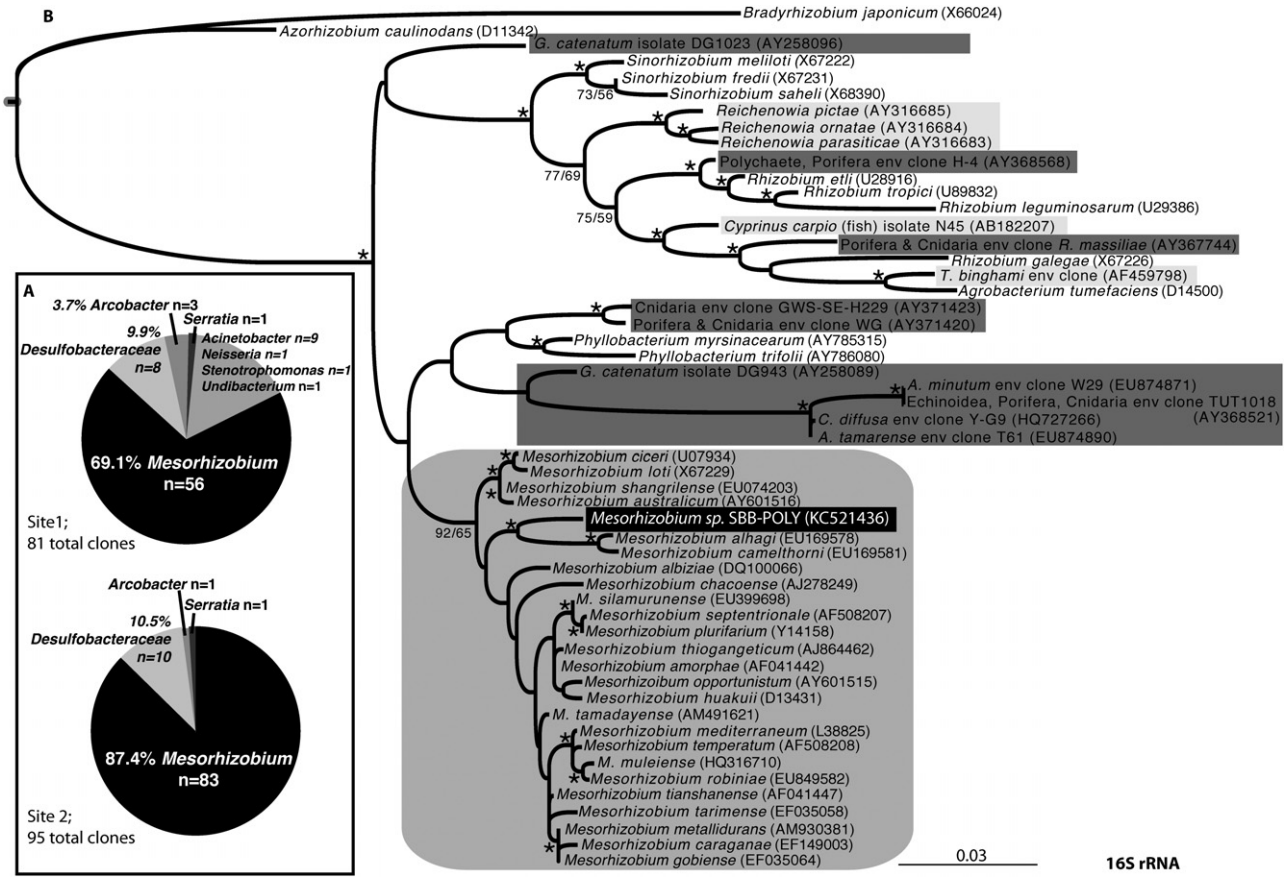

Fig. 1. 16S rRNA gene taxonomic identities of bacteria found associated with *Meganerilla bactericola* at two sites in the Santa Barbara Basin (34°10′N, 120°1′W; 34°17′N, 120°1′W) (A) and 16S rRNA maximum likelihood tree of *Rhizobiales*, with emphasis on *Mesorhizobium*, highlighted in medium grey (B). Jackknife (parsimony) and bootstrap (likelihood) support of greater than 70 are indicated with an asterisk (*). Support for other clades is shown as jackknife/bootstrap. A representative 16S rRNA gene sequence of the most abundant clone found associated with *M. bactericola* is referred to as *Mesorhizobium* sp. SBB-POLY (highlighted in black). Environmental clones and isolates from eukaryotes are highlighted in dark grey (marine) and light grey (terrestrial).

A. Genomic DNA was extracted from six ethanol-fixed animals per site by grinding of tissue together followed by phenol-chloroform extraction (Sambrook and Russell, 2001). 16S rRNA genes were amplified using bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′) (Lane, 1991), cloned, and sequenced to produce two independent libraries of 81 and 95 sequences. We identified closest bacterial ribotypes using BLAST (http://blast.ncbi.nlm.nih.gov/) and Greengenes (http://greengenes.lbl.gov/) and assigned taxonomic identities using the Ribosomal Database Project (http://rdp.cme.msu.edu/). Direct distance calculations among sequences assigned to *Mesorhizobium* were calculated using PAUP*4.0b10 (Swofford, 2002) and found to be 99% identical.

B. 16S molecular data for a total of 53 taxa were analysed using phylogenetics. This sampling covers all species within *Mesorhizobium*, representative strains of *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Phyllobacterium*, and environmental clones and isolates associated with eukaryotes. *Azorhizobium caulinodans* and *Bradyrhizobium japonicum* were chosen as out-groups based on previous work (Peter *et al.*, 1996; Vidal *et al.*, 2009). 16S sequences were aligned using MUSCLE (Edgar, 2004), resulting in 1459 terminals, 228 parsimony informative and 100 parsimony uninformative. A maximum parsimony analysis was carried out using PAUP*4.0b10 (Swofford and Sullivan, 2003) with the heuristic search option with 100 random additions. Strict consensus trees were generated and clade supports determined using 100 jackknife replicates (Farris *et al.*, 1996). This analysis produced 32 most parsimonious trees (length = 906). Maximum likelihood analysis was completed using RAxML GUI v. 0.93 (Stamatakis, 2006; Silvestro and Michalak, 2010) with the GTRGAMMA model based on author recommendations (Stamatakis, 2008). Thorough bootstrap analysis was carried out with 1000 pseudoreplicates using the same model. The alignment and phylogenetic analyses are available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S13642).

cacodylate buffer. Bacterial mat samples were fixed in 95% ethanol and frozen at –80°C.

Identification and phylogenetic analysis of nerillid-associated bacteria

To identify the composition of epibionts associated with *M. bactericola*, we used bacterial primers 27F and 1492R (Lane, 1991) to construct two 16S rRNA gene clone libraries from animals at two independent sites approximately 13 km apart within the Santa Barbara basin. Ribotypes were assigned to three main bacterial groups: *Mesorhizobium* (69.1 and 87.4% of recovered ribotypes), *Desulfobacteracea* (9.9 and 10.5%), and *Arcobacter* (3.7 and 1.1%) (Fig. 1A). Based on the clustering of sequences at 99% identity, all 139

Mesorhizobium sequences represented a single clone, referred to here as *Mesorhizobium* sp. SBB-POLY. *Mesorhizobium* sp. SBB-POLY has a sequence identity (uncorrected distance) of 98.2% to the type strain of *Mesorhizobium shangrilense* and 99.9% to environmental sequences from arsenic-enriched mine tailings (Macur *et al*., 2001) and a microbial electrolysis cell inoculated with anaerobic paper mill sludge (Croese *et al*., 2011) (GenBank Accessions AF331662 and FR669221). To further assess the evolutionary relationship of *Mesorhizobium* sp. SBB-POLY to previously known taxa, a comprehensive phylogenetic analysis was performed that incorporated all species of *Mesorhizobium* as well as rhizobia-like environmental clones and isolates from eukaryotes available on GenBank (Fig. 1B). We found moderate support for the clade comprising *Mesorhizobium*, with *Mesorhizobium.* sp. SBB-POLY strongly supported as sister to *Mesorhizobium camelthorni* and *Mesorhizobium alhagi.* Previous isolates and environmental clones were not within *Mesorhizobium*. A 16S rRNA or metagenomic survey of bacteria within the microbial mat or adjacent waters of the Santa Barbara Basin has not been completed, however rhizobia were sequenced from sediment in methane-producing clam fields in the basin (Orphan *et al*., 2001).

To bolster the phylogenetic placement of *Mesorhizobia* sp. SBB-POLY, we targeted three additional phylogenetically informative genes for analysis: *nifH*, *atpD* and *recA*. Each gene was PCR amplified from *M. bactericola* DNA extracts and sequenced directly, providing further evidence in support of *Mesorhizobium* sp. SBB-POLY being a single clonal population. Phylogenetic analyses of each of these genes are congruent with the 16S rRNA gene-based placement of SBB-POLY within the *Mesorhizobium* (Fig. 2). These trees show strong support for the placement of *Mesorhizobium* sp. SBB-POLY within the genus *Mesorhizobium* and distinctiveness from previous eukaryote-associated *nifH* clones. The successful amplification of the nitrogenase reductase gene *nifH*, which encodes a key component of the nitrogenase enzyme complex (Mortenson and Thorneley, 1979), provides evidence supporting the genetic potential for nitrogen fixation in *Mesorhizobium* sp. SBB-POLY.

To investigate the possibility of free-living *Mesorhizobium* sp. SBB-POLY in the microbial mat, we attempted, without success, to directly amplify rhizobial *nif-H*, *atpD*, *recA* (primers described in Fig. 1), and 16S rRNA gene sequences (Mes16S-F 5′-TGGGCGCAAGCCTGATCCAG-3′ and Mes16S-R 5′-CGTTAGCTGCGCCACCGACA-3′) from genomic DNA extracted from the mat. While not conclusive, these results suggest that *Mesorhizobium* sp. SBB-POLY is more abundant in association with the polychaete than free-living in the microbial mat.

Association of Mesorhizobium *sp. SBB-POLY with* Meganerilla bactericola

To confirm the association of *Mesorhizobium* sp. SBB-POLY with *M. bactericola*, a 16S rRNA gene probe specific to the genus *Mesorhizobium* was designed for FISH. This probe, Meso163 (5′-CCCGAAGGACGTATACGGTATTAGCTCCAG-3′) was hybridized to 15 whole-mounts of ethanol and paraformaldehyde-fixed specimens of *M. bactericola* (Fig. 3B–E). Two additional FISH probes, the universal *Bacteria* probe set EUB338 (EUB338, EUB338II, and EUB338III) (Amann *et al*., 1990; Daims *et al*., 1999) and the nonsense probe NONEUB (Wallner *et al*., 2005) were used as controls. Based on the FISH results, we estimate that *Mesorhizobium* sp. SBB-POLY accounts for approximately 80% of the universal bacterial signal (Fig. 3C–E), a result consistent with the 16S rRNA gene library analyses. Both the group-specific Meso163 and universal bacteria probes showed discrete patches of hybridization on the exterior of the animal (Fig. 3B). This result was unexpected since the epibionts were previously shown to coat the entirety of the animal (Bernhard *et al*., 2000; Müller *et al*., 2001). The patchiness of both universal EUB338 and Meso163 FISH hybridizations suggests that some bacteria were disassociated during fixation in ethanol and paraformaldehyde. Examination of bacterial coverage on specimens fixed in glutaraldehyde via transmission electron microscopy (TEM), revealed bacteria of the same size and shape covering the outside of the animal and lining the pharynx (Fig. 3F–G). These observations match those previously reported (Bernhard *et al*., 2000; Müller *et al*., 2001).

Bacterial signals detected on the exterior of the worm demonstrate that *Mesorhizobium* sp. SBB-POLY, unlike other symbiotic rhizobia, is an ectobiont on the *M. bactericola* host. The bacteria lining the pharynx remain unidentified, presenting the possibility that these may or may not be different bacteria. Symbiotic associations involving freshwater and terrestrial rhizobia (e.g. leeches, termites, plants) are endosymbiotic (Siddall *et al*., 2004; Fröhlich *et al*., 2007; Kvist *et al*., 2011). Rhizobia found with plants are localized in root nodules to allow the physical separation of oxygen from the nitrogenase enzyme (Soupène *et al*., 1995). Considering the marked anoxic conditions of the Santa Barbara Basin habitat, an ectobiotic lifestyle may not inhibit nitrogenase activity in this environment.

Nature of the symbiosis

The external association of the rhizobia *Mesorhizobium* sp. SBB-POLY with the marine polychaete *M. bactericola* is supported by multiple lines of evidence, including reproducible genetic surveys, phylogenetic analyses, FISH, and TEM. The possibility of nitrogen-fixation by

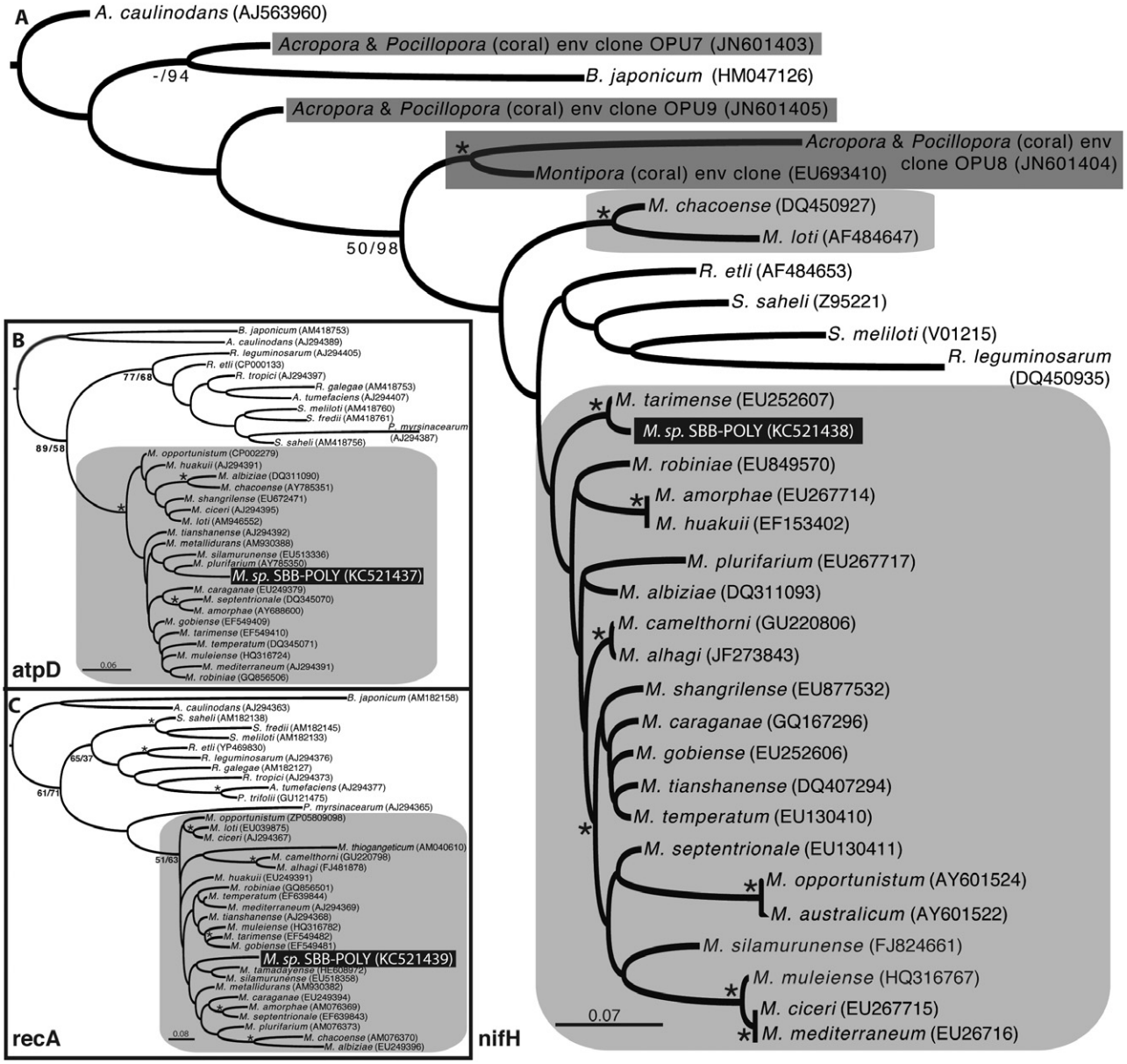

Fig. 2. Maximum likelihood trees of nerillid-associated *nifH* (A), *atpD* (B), and *recA* (C) of *Rhizobiales*, with emphasis on the genus *Mesorhizobium*, highlighted in medium grey. *Mesorhizobium* sp. SBB-POLY is highlighted in black. Jackknife (parsimony) and bootstrap (likelihood) support of greater than 70 are indicated with an asterisk (*). Support for other clades is shown as jackknife/bootstrap, with minus symbol (–) indicating a node not present in parsimony analysis. Sequences for each gene were aligned using MUSCLE (Edgar, 2004). Maximum parsimony and maximum likelihood analyses were carried out for each gene as described in Fig. 1. The alignments and phylogenetic analyses are available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S13642).
A. *nifH* was amplified using the primers nifHF (5′-TACGGNAARGGSGGNATCGGCAA-3′) and nifHI (5′-AGCATGTCYTCSAGYTCNTCCA-3′) (Laguerre *et al.*, 2001). Phylogenetic analyses included 33 taxa and 704 characters (235 parsimony informative/94 parsimony uninformative). Maximum parsimony analysis produced 46 most parsimonious trees (length = 974).
B. *atpD* was amplified using the primers atpD-F (5′-ATCGGCGAGCCGGTCGACGA-3′) and atpD-R (5′-GCCGACACTTCCGAACCNGCCTG-3′) (Gaunt *et al.*, 2001). Phylogenetic analyses included 32 taxa and 512 characters (156 parsimony informative/46 parsimony uninformative). Maximum parsimony analysis produced 12 most parsimonious trees (length = 704).
C. *recA* was amplified using the primers recA-F (5′-ATCGAGCGGTCGTTCGGCAAGGG-3′) and recA-R (5′-TTGCGCAGCGCCTGGCTCAT-3′) (Gaunt *et al.*, 2001). Phylogenetic analyses included 36 taxa and 536 characters (190 parsimony informative/48 parsimony uninformative). Maximum parsimony analysis produced 25 most parsimonious trees (length = 941).

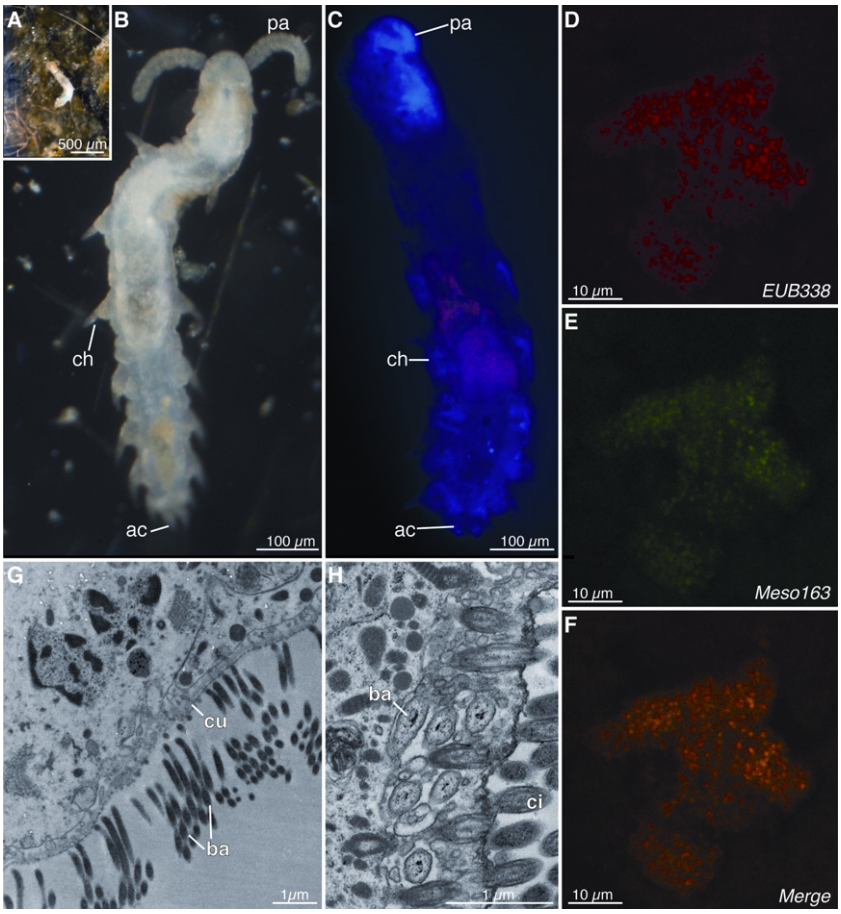

Fig. 3. Live photography (A–B), FISH (C–F), and TEM (G–H) of the marine annelid *Meganerilla bactericola* and its associated bacteria.
A and B. Live *M. bactericola*, *in situ* within the microbial mat of the Santa Barbara Basin (A). Animals were examined using a Leica S8 Apo dissecting microscope and photographs acquired with a Canon Rebel 2Ti camera.
C–F. Whole-mounts of *M. bactericola* preserved in ethanol and paraformaldehyde in 1% low-melt agarose, followed by FISH treatment (Fuchs *et al.*, 2007). Hybridizations were performed at 48°C with EUB388 (5′-GCTGCC TCCCGTAGGAGT-3′) (Daims *et al.*, 1999), a designed probe (Meso163 5′-CCCGAAGGACGTATACGGTATTAGCTCCAG-3′) with two helper probes (H145 5′-TTTCCCGGAGTTGTTCCG-3′ & H193 5′-CCGATAAATCTTTCT-3′), and NONEUB (5′-ACTCCTACGGGAGGCAGC-3′) (Wallner *et al.*, 2005) as a negative control. The specificity of the Meso163 probe, alone and in combination with the two helper probes, was tested *in silico* using ProbeCheck (http://www.microbial-ecology.net/probecheck). The probe set of Meso163, H145, and H193 matched only the target clone. Meso163 alone was specific to members of *Phyllobacteracae* until 3 mismatches. Hybridization stringencies were tested *in situ* by applying formamide concentrations from 0 to 45% in 5% increments on both *Mesorhizobium loti* (DSMZ 2626) (0 bp mismatch to Meso163) and *Phyllobacterium myrsinacearum* (DSMZ 5892) (3 bp mismatch to Meso163). At 35% formamide concentration, less than 1% of *P. myrsinacearum* was hybridized. NONEUB treatments conducted simultaneously and under the same conditions as EUB388/Meso163 treatments showed no hybridization (data not shown).
C. Ethanol-fixed whole-mount; red fluorescence of bacteria hybridized with genus-specific Alexa 555 probe Meso163. Blue fluorescence of animal nuclei stained with Hoescht. Colocalization with universal probe (EUB 388) not shown. Specimen examined using Zeiss ApoTome.2 and photograph acquired with Zeiss AxioCamHRm camera.
D–F. Patch of bacteria on the outside of PFA-fixed specimen.
D. Red fluorescence of bacteria hybridized with Cy3 universal EUB338 probe set.
E. Green fluorescence of bacteria hybridized with 5-Fam Meso163 probe.
F. Merge colocalization of EUB388 and Meso163. Specimen examined using Leica DM6000CS confocal laser microscope.
G–H. TEM images of *M. bactericola*. Samples for TEM were post-fixed in 1% osmium tetroxide for 2 h, dehydrated, and embedded in Spurr's epoxy resin. 70 nm sections were examined using a JEOL 120 EXII TEM.
G. Apices neighbouring epidermal cells with numerous bacteria associated with the surface facing the environment (lower right).
H. Small clusters of bacteria in phagocytic vesicles and/or internalized vacuoles in the apical cytoplasm of the multiciliated pharyngeal endoderm cells; lumen toward right.
ac, anal cirri; ba, bacteria; ch, chaetae; ci, cilia; cu, cuticle; pa, palps.

Mesorhizobium sp. SBB-POLY warrants further investigation, especially in regard to traditional hypotheses concerning ectosymbiotic relationships (e.g. detoxification, protection, or nutrition) (see reviews: Cavanaugh, 1994; Lee and Childress, 1994; Dubilier *et al*., 2008; Goffredi, 2010). Nitrogen fixation and possible trophic transfers to the host should be investigated through a combination of genetic (e.g. *nif* gene expression based on mRNA detection), nitrogenase enzyme activity (e.g. acetylene reduction assays with live animals), and nitrogen isotopic studies (e.g. nano-SIMS). Additional metabolic features of *Mesorhizobium* sp. SBB-POLY may also explain its association with a marine polychaete. Cultivation of the bacteria, and if that is not possible, single-cell genomic investigation could answer many of these questions. Evidence for rhizobia-related bacteria associated with an animal in an anoxic microbial mat community also raises questions about the dynamics of nitrogen cycling phenomena in this habitat. Further biogeochemical, microbiological, and zoological analysis of these dynamic microbial mat ecosystems will undoubtedly provide important contributions to the understanding of novel host–microbe associations in marine anoxic environments.

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