Colonial choanoflagellate isolated from Mono Lake harbors a microbiome

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ABSTRACT

Choanoflagellates offer key insights into bacterial influences on the origin and early evolution of animals. Here we report the isolation and characterization of a new colonial choanoflagellate species, *Barroeca monosierra,* that, unlike previously characterized species, harbors a stable microbiome. *B. monosierra* was isolated from Mono Lake, California and forms large spherical colonies that are more than an order of magnitude larger than those formed by the closely related *Salpingoeca rosetta*. By designing fluorescence *in situ* hybridization probes from metagenomic sequences, we found that *B. monosierra* colonies are colonized by members of the halotolerant and closely related *Saccharospirillaceae* and *Oceanospirillaceae,* as well as purple sulfur bacteria (*Ectothiorhodospiraceae*) and non-sulfur *Rhodobacteraceae.* This relatively simple microbiome in a close relative of animals presents a new experimental model for investigating the evolution of stable interactions among eukaryotes and bacteria.

IMPORTANCE

The animals and bacteria of Mono Lake (California) have evolved diverse strategies for surviving the hypersaline, alkaline, arsenic-rich environment. We sought to investigate whether the closest living relatives of animals, the choanoflagellates, exist among the relatively limited diversity of organisms in Mono Lake. We repeatedly isolated members of a single species of choanoflagellate, which we have named *Barroeca monosierra,* suggesting that it is a stable and abundant part of the ecosystem. Characterization of *B. monosierra* revealed that it forms large spherical colonies that each contain a microbiome, providing an opportunity to investigate the evolution of stable physical associations between eukaryotes and bacteria.

DISCOVERY REPORT

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A newly identified choanoflagellate species forms large colonies that contain a microbiome

Choanoflagellates are the closest living relatives of animals and, as such, provide
60 insights into the origin of key features of animals, including animal multicellularity and cell biology [1,2]. Over a series of four sampling trips to Mono Lake, California (Fig. 1A; Table S1) we collected single-celled choanoflagellates and large spherical choanoflagellate colonies, many of which were hollow (Fig. 1B) and resembled the blastula stage of animal development. In colonies and single cells, each cell had the
65 typical collar complex observed in other choanoflagellates: an apical flagellum surrounded by a collar of microvilli [1,2]. In these "rosette" colonies, the cells were oriented with the basal pole of each cell pointing inwards and the apical flagellum facing out (Fig. 1). To study the Mono Lake choanoflagellates in greater detail, we established clonal strains from ten independent isolates, two of which were each started from a
70 single-celled choanoflagellate and the remaining eight of which were each started from a single colony (Table S1). The two strains started from single-celled choanoflagellates, isolates ML1.1 and ML1.2, took on the colonial morphology observed in the other isolates after culturing in the laboratory, suggesting that the colonies and single cells isolated from Mono Lake could belong to the same species. We are aware of no prior
75 reports of choanoflagellates having been cultured from any alkaline soda lake, including Mono Lake.

The 18S rDNA genes for six of the Mono Lake isolates were sequenced and found to be >99% identical (Table S1). Phylogenetic analysis confirmed that all of the
80 isolates are members of a single choanoflagellate species (Fig. S1). In further phylogenetic analyses based on 18S rDNA and two protein-coding genes from isolate ML2.1 (Fig. 1C) [3], we found that its closest relatives are the emerging model choanoflagellate *S. rosetta* [4–8], additional *Salpingoeca* spp. [9] and *Microstomoeca roanoka* [3,10]. The phylogenetic distance separating the Mono Lake species from its
85 closest relatives is similar to the distance separating other choanoflagellate genera. Therefore, we propose the name *Barroeca monosierra*, with the genus name inspired by esteemed choanoflagellate researcher Prof. Barry Leadbeater and the species name inspired by the location of Mono Lake in the Sierra Nevada mountain range. (See Supplemental Methods for further details and a formal species description.)
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Although *B. monosierra* and *S. rosetta* form rosette-shaped spherical colonies, they differ greatly in size. *S. rosetta* colonies range from 10-30 μm in diameter while *B. monosierra* forms among the largest choanoflagellate colonies observed [1,11], with a single culture exhibiting colony sizes spanning from 10-120 μm in diameter (Fig. 1D-F).
95 Unlike the rosettes of *S. rosetta*, in which the basal poles of cells are closely apposed in the rosette center [5,7,11,12], cells in large *B. monosierra* rosettes form a shell on the surface of a seemingly hollow sphere. Inside the ostensibly hollow sphere, a branched network of extracellular matrix connects the basal poles of all cells (Fig. S2.)

100   Upon staining *B. monosierra* with the DNA dye Hoechst 33342, we observed the expected toroidal nuclei in each choanoflagellate cell [12,13], but were surprised to detect Hoechst-positive material in the interior of *B. monosierra* colonies (Fig. 2A, A'). Transmission electron microscopy revealed the presence of 1 µm and smaller cells with diverse morphologies bounded by cell walls in the centers of rosettes (Fig. 2B, B'; Fig.

105   S3). Together, these observations led us to hypothesize that the centers of *B. monosierra* colonies contain bacteria.

        By performing hybridization chain reaction fluorescence *in situ* hybridization (HCR-FISH [14–16]) with a broad-spectrum probe of bacterial 16S rRNA, EUB338 [17],

110   we confirmed that the cells in the center of colonies are bacteria (Fig. 2C). A second probe that specifically targeted 16S rRNA sequences from Gammaproteobacteria, GAM42a, revealed that the majority of the bacteria inside the colonies are Gammaproteobacteria (Fig. 2C')[18]. Finally, by incubating *B. monosierra* cultures with fluorescently labeled D-amino acids, which are specifically incorporated into the cell

115   walls of growing bacteria, we found that the bacteria in *B. monosierra* colonies are alive and growing (Fig. S4)[19]. Therefore, *B. monosierra* contains a microbiome as defined in [20].

        To visualize the spatial distribution of choanoflagellate and bacterial cells in a

120   representative colony, we generated a 3D reconstruction from serial sections imaged by TEM. The colony contained 70 choanoflagellate cells that were tightly packed, forming a largely continuous monolayer of cells (Fig. 2D). As observed by immunofluorescence microscopy (Figs. 2A and 2A'), all cells were highly polarized and oriented with their apical flagella and collars extending away from the centroid of the rosette. Many cells

125   were connected by fine intercellular bridges (Fig. S5) that have been previously observed in other colonial choanoflagellates, including *S. rosetta* [5,12].

        The 3D reconstruction also revealed at least 200 bacterial cells in the center of the rosette (Fig. 2D', 2D''), some of which were physically associated with and wrapped

130   around the choanoflagellate ECM (Fig. S6). A small number of bacterial cells were observed between the lateral surfaces of choanoflagellate cells, although it was not possible to determine whether they were entering or exiting the colony (Fig. 2D''; Fig. S7). Colonies failed to incorporate bacteria-sized bovine serum albumin (BSA)-coated latex microspheres (0.2 µm and 1 µm) into their centers, suggesting that environmental

135   bacteria may not be capable of passively accessing the centers of *B. monosierra* colonies (Fig. S8).

Gammaproteobacteria and Alphaproteobacteria in the *B. monosierra* microbiome

140   We next sought to identify which bacteria comprise the microbiomes of *B. monosierra.* To identify candidate bacteria for which to design FISH probes, we first sequenced and assembled metagenomes and 16S rDNA sequences from choanoflagellate-enriched samples and from environmental bacteria-enriched samples. These samples were derived from two co-cultures of *B. monosierra* with Mono Lake

145   bacteria, ML2.1E and ML2.1G (Fig. S9), with the enrichment for choanoflagellates or

bacteria performed by centrifugation. A total of 24 different bacterial species were identified via two complementary bioinformatic approaches (EukRep Metagenomic Analysis and EMIRGE 16S rRNA Analysis; Table S2), of which 22 species were present in fractions enriched with *B. monosierra* colonies (Table S3). The phylogenetic
150 relationships among these and other bacterial species were determined based on analysis of highly conserved ribosomal proteins and 16S rDNA sequences (Fig. 2E and S10).

The 22 bacterial species detected in cultures with *B. monosierra* may have co-
155 sedimented with the *B. monosierra* colonies due their community-structure densities (e.g. biofilms), a transient association with the choanoflagellate colonies (e.g. as prey), or through a stable association with the choanoflagellate colonies. Upon investigation by FISH microscopy, we detected ten or eleven of these species in the centers of *B. monosierra* colonies (Table S4, Fig. S11). (The uncertainty regarding the precise
160 number of choanoflagellate-associated bacterial species stems from the inability to disambiguate 16S rDNA sequences corresponding to one or two of the species.) Of these microbiome bacteria, nine were Gammaproteobacteria from the families *Oceanospirillaceae* (Fig. S11A; OceaML1, OceaML2, OceaML3, OceaML4, OceaML4)*, Ectothiorhodospiraceae* (Fig. S11B; EctoML1, EctoML2, EctoML3, EctoML4), and
165 *Saccharospirillaceae* (Fig. S11C; SaccML), matching our original observation that the majority of the bacteria were Gammaproteobacteria (Fig. 2C, C'). The remaining species was a *Roseinatronobacter* sp*. (RoseML; Alphaproteobacteria) (Fig. S11D). The microbiome bacteria exhibited an array of morphologies, from long and filamentous to rod shaped (Fig. S3 and Fig. S12). Intriguingly, with the exception of OceaML3, which
170 was exclusively detected inside *B. monosierra* colonies of ML2.1E (Fig. S13), all other microbiome species identified in this study were detected both inside and outside the colonies.

In animals, the microbiome often contains a core set of host-adapted bacteria
175 that are present in many or all individuals in a host species, as well as a more flexible set of bacteria that may be found in only a subset of individuals [21–23]. To identify core members of the *B. monosierra* microbiome, we measured the frequency with which colonies of ML2.1EC contained or lacked a number of representative microbiome bacteria (Fig. S14A) and estimated the abundance of each species relative to the total
180 microbiome (Fig. S14B). Only one bacterium tested, OceaML1, was found in the microbiome of all *B. monosierra* colonies (Fig. S14A). The other most frequently observed members of the microbiome were SaccML (93.3% of colonies), EctoML3 (91.8% of colonies) and EctoML1 (82.4% of colonies; Fig. S14A). Two other Gammaproteobacterial species, OceaML2 and EctoML2, were found in ~50 - 60% of
185 colonies, while the Alphaproteobacterium RoseML was found in only 13.9% of rosettes.

The most common resident of the *B. monosierra* microbiome, OceaML1, was also the most abundant, representing on average 66.4% of the total bacterial load per colony (Fig. S14B). Other abundant bacteria, some found in >80% of colonies, represented
190 approximately smaller percentages of the average bacterial biomass in the microbiomes in which they were found. For example, SaccML was found in 93.3% of

microbiomes but represented only 30.3% of total bacteria in the *B. monosierra* rosettes in which it was found. EctoML1, which was found in 82.4% of *B. monosierra* rosettes represented less than 10% of the bacteria in the bacteria in which was detected. Thus, only OceaML1 appears to be a core member of the *B. monosierra* microbiome. Other symbionts detected in *B. monosierra* (e.g. SaccML and OceaML2-4) are close relatives of OceaML1 and may engage in similar metabolic or developmental functions in their interactions with *B. monosierra* [24].

Discussion

Interactions with bacteria are essential to choanoflagellate nutrition and life history. Bacteria are the primary food source for choanoflagellates, and the choanoflagellate *S. rosetta* responds to different secreted bacterial cues to undergo either multicellular developmental or mating [25–28]. We report the isolation and characterization of a new choanoflagellate species, *B. monosierra,* that forms large colonies and contains a microbiome consisting of at least ten different bacterial symbionts. To our knowledge, this is the first example of a stable interaction between choanoflagellates and ectosymbiotic bacteria. Future studies will be important to determine how the *B. monosierra* colonies and their bacterial symbionts interact.

We detected 10 – 11 bacterial species from four different families (*Saccharospirillaceae, Oceanospirillaceae, Ectothiorhodospiraceae,* and *Rhodobacteraceae)* in the *B. monosierra* microbiome. Comparisons with other symbioses suggest that their interactions with *B. monosierra* may relate to metabolism and detoxification of environmental sulfur. Such functions have been reported for *Oceanospirillaceae* species that are symbionts of diverse marine animals, from corals to snails [29–36] and for *Ectothiorhodospiraceae* that are symbionts of diverse ciliates and animals [37–40].

Although the composition of the animal gut microbiome often varies between individuals in a species, many host species harbor a core set of microbes, with which they participate in stable metabolic interactions and may coevolve [22,23]. Indeed, choanoflagellates express homologs of Toll-like receptors that, in animals, mediate interactions with gut bacteria to maintain homeostasis [10,41]. In *B. monosierra*, OceaML1 was found in 100% of rosettes assessed and was the most abundant bacterium in the microbiome, suggesting that its interactions with *B. monosierra* may be essential for *B. monosierra* biology. Interestingly, the related bacterium *Endozoicomonas sp.* (Order Oceanospirillales), is a core member of the gut microbiome of the ascidian *Ciona intestinalis*, where it represents up to 54% of the bacterial biomass [32].

B. monosierra and its associated microbiome provide a unique opportunity to characterize a new symbiotic interaction between a single choanoflagellate species and a microbial community. Due to the phylogenetic relevance of choanoflagellates, this symbiotic relationship has the potential to illuminate the ancestry of and mechanisms

underlying stable associations between animals and bacteria, colonization of organisms by diverse microbial communities, and insights into one of the most complex animal-

240   bacterial interactions, the animal gut microbiome.

Acknowledgements

Supplement Contents

280   **Text S1:** Supplemental Materials and Methods

    Figure S1: Ultrastructure and phylogeny of choanoflagellate isolates from Mono Lake
    Figure S2. *B. monosierra* colonies contain an extracellular matrix (ECM).
    Figure S3: Bacterial symbionts in *B. monosierra* colonies exhibit a range of
285   morphologies.
    Figure S4: Bacteria inside *B. monosierra* colonies are alive and growing.
    Figure S5: Intercellular bridges connect cells in *B. monosierra* colonies.
    Figure S6: Bacterial symbionts physically associate with and wrap around the
    choanoflagellate ECM.
290   **Figure S7:** Bacteria are found wedged between the lateral surfaces of *B. monosierra*
    cells.
    Figure S8: *B. monosierra* colonies cannot be passively penetrated by sub-micron
    particles.
    Figure S9: Schematic describing the establishment of *B. monosierra* cultures.
295   **Figure S10:** Phylogenetic analysis of 16S rRNA tree of EMIRGE sequences.
    Figure S11: Diverse bacteria are found in the microbiome of *B. monosierra.*
    Figure S12: *B. monosierra* symbionts exhibit filamentous and rod morphologies.
    Figure S13: The symbiont OceaML3 is present exclusively inside rosettes.
    Figure S14: OceaML1 is a core member of the *B. monosierra* microbiome.

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    Table S1: Date, location, phenotype, and isolate designations for *Barroeca monosierra*
    isolates.
    Table S2: Nine genera of bacteria identified in *B. monosierra* cultures through two
    independent analyses: comparison of ribosomal proteins detected through
305   metagenomic assembly and by 16s rRNA assembly and analysis.
    Table S3: Predicted targets of HCR-FISH probes based on 16S rRNA sequences.
    Bacteria in bold were identified inside *B. monosierra* colonies.
    Table S4: Full length probes, with spacer and initiator sequences, used for HCR-FISH.
    Table S5: Growth Media Recipes.
310   **Table S6:** Bacterial 16S rDNA – Genbank accession numbers.
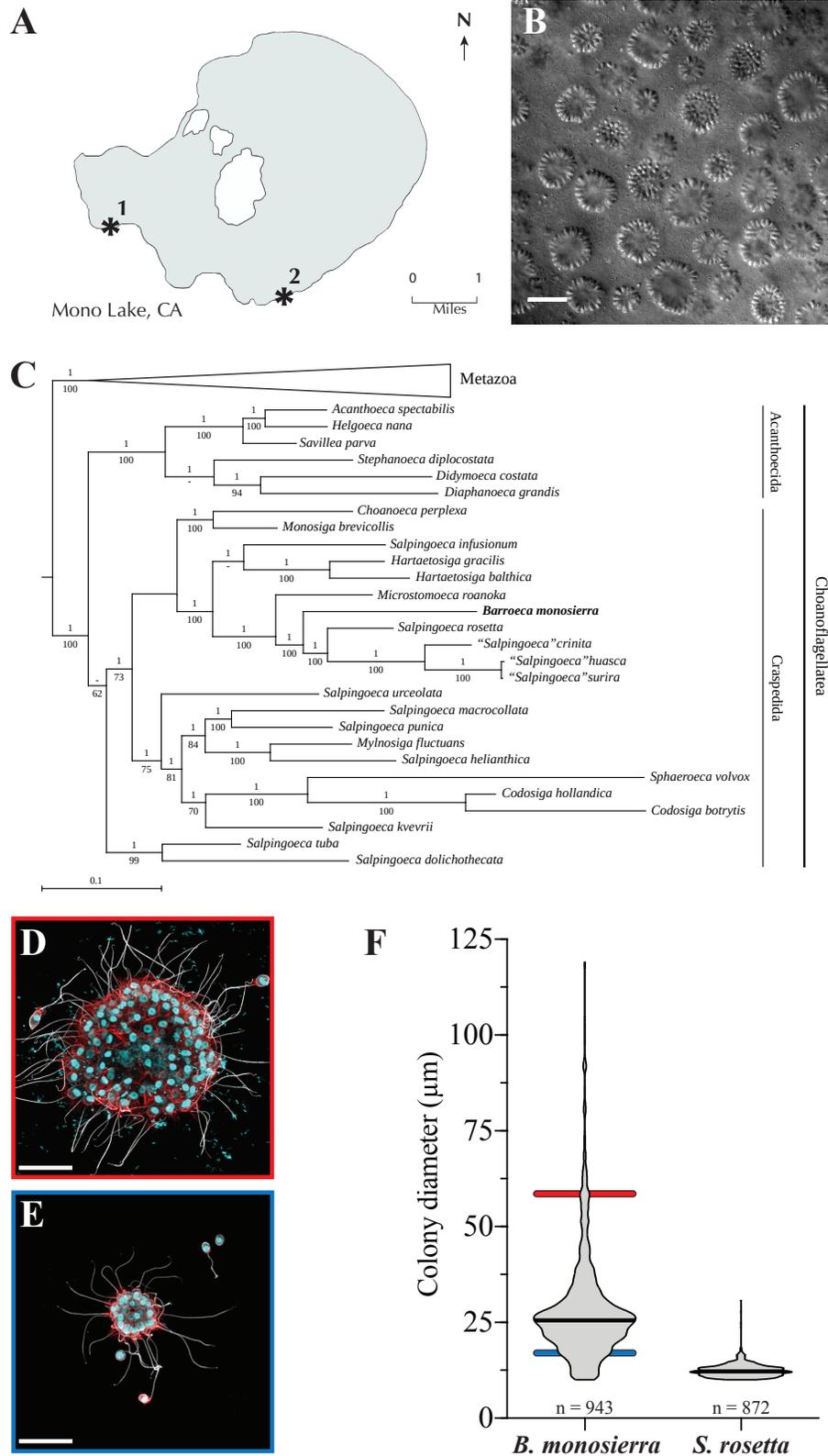
315

Figure 1

Figure 1. A new colony-forming choanoflagellate isolated from Mono Lake.
(A) Choanoflagellates were collected from two sampling sites (asterisks) near the shore of Mono Lake, California. (Modified from map at monolake.org.) (B) *B. monosierra* forms large colonies (DIC image). Scale bar = 50µm. (C) *B. monosierra* (shown in bold) is a craspedid choanoflagellate closely related to *S. rosetta* and *Microstomoeca roanoka.* Phylogeny based on sequences of 3 genes: 18S rDNA, EFL and HSP90. Metazoa (7 species) were collapsed to save space. Bayesian posterior probabilities are indicated above each internal branch, and maximum likelihood bootstrap values below. (A '-' value indicates a bifurcation lacking support or not present in one of the two reconstructions.) (D-E) Two representative colonies reveal the extremes of the *B. monosierra* colony size range (D, 58 µm diameter; E, 19 µm diameter; scale bar = 20 µm). In *B. monosierra* colonies, each cell is oriented with its apical flagellum (white; labeled with anti-tubulin antibody) and the apical collar of microvilli (red; stained with phalloidin) pointing out. Nuclei (cyan) were visualized with the DNA-stain Hoechst 33342. (F) Colonies of *B. monosierra* span from 10 µm in diameter, a size comparable to that of small *S. rosetta* colonies, to 120 µm, over an order of magnitude larger. Diameters of *B. monosierra* and *S. rosetta* colonies were plotted as a violin plot; median indicated as thick black line. Diameters of representative colonies indicated as colored bars behind violin plot (D, red bar; E, blue bar).
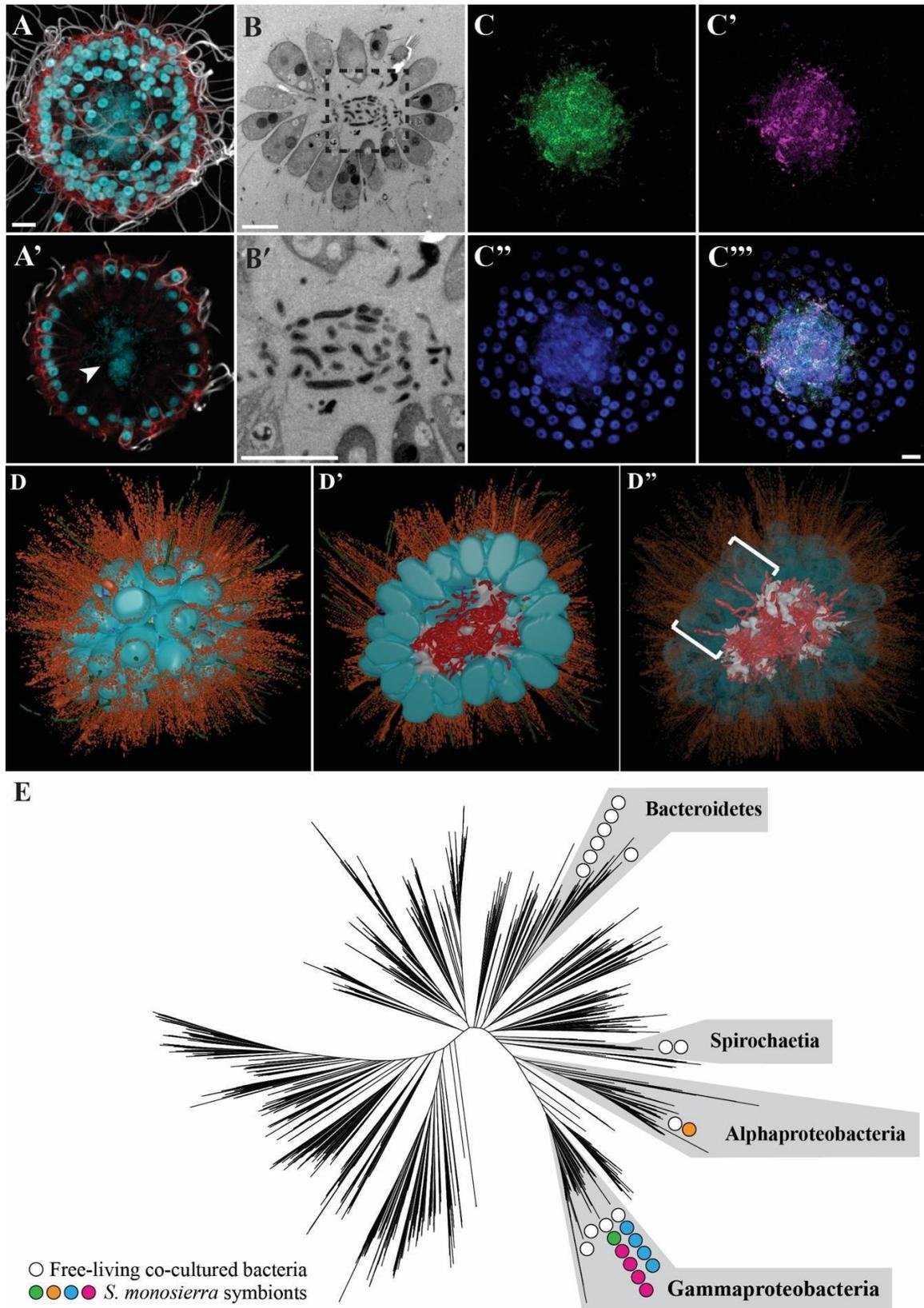
340   **Figure 2**

Figure 2. *B. monosierra* colonies are filled with bacteria.

(A, A') The center of a representative *B. monosierra* colony, shown as a maximum
345   intensity projection (A) and optical z-section (A'), contains DNA (revealed by Hoechst
33342 staining; cyan). Apical flagella were labeled with anti-tubulin antibody (white);
microvilli were stained with phalloidin (red). Hoechst 33342 staining (cyan) revealed the
toroidal choanoflagellate nuclei along the colony perimeter and an amorphous cloud of
DNA sitting within the central cavity formed by the monolayer of choanoflagellate cells.
350   (B-B') Thin section through a representative *B. monosierra* colony, imaged by
transmission electron microscopy (TEM), revealed the presence of small cells in the
central cavity. (B') Inset (box; panel B') reveals that the interior cells are each
surrounded by a cell wall. (C-C''') The small cells inside *B. monosierra* colonies are
bacteria, as revealed by hybridization with a broad spectrum 16S rRNA probe (C,
355   green) and a probe targeting Gammaproteobacteria (C', red). Choanoflagellate nuclei
and bacterial nucleoids were revealed by staining with Hoechst (C'', cyan). (C''') Merge
of panels C – C''. Scale bar for all = 5 μm. (D-D'') 3D reconstruction of a 70-cell *B.
monosierra* choanoflagellate colony from transmission electron micrographs of serial
ultrathin sections revealed that the bacteria are closely associated with and wrapped
360   around the ECM inside the colony. (D) Whole colony view. (D') Cut-away view of colony
center. Color code: cell bodies (cyan); microvilli (orange); flagella (green); bacteria (red);
ECM (white); intercellular bridges (yellow, see also Fig. S5); filopodia (purple). (D'')
Reducing the opacity of the choanoflagellate cell renderings revealed the presence of
bacteria positioned between the lateral surfaces of choanoflagellate cells (brackets, see
365   also Fig. S7). (E) Unrooted phylogenetic tree based on 16 concatenated ribosomal
protein sequences representing bacterial diversity modified from [42], illustrated to
indicate the phylogenetic placement of bacteria co-cultured from Mono Lake with *B.
monosierra.* The bacteria belonged to four major classes: Spirochaetia,
Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes, however the bacteria
370   found associated with *B. monosierra* colonies came only from Alphaproteobacteria and
Gammaproteobacteria. Circles represent the phylogenetic placement of non-symbionts
(white) and symbionts (*Oceanospirillaceae sp.,* magenta*; Saccharospirillaceae sp.,*
green*; Ectothiorhodospiraceae sp.,* blue*; Roseinatronobacter sp.,* orange). See also
Figs. S10 and S11.

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