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Tissue-Associated "*Candidatus* Mycoplasma corallicola" and Filamentous Bacteria on the Cold-Water Coral *Lophelia pertusa* (Scleractinia)▽†

Sven C. Neulinger,[1]* Andrea Gärtner,[2] Johanna Järnegren,[3] Martin Ludvigsen,[4] Karin Lochte,[1] and Wolf-Christian Dullo[2]

Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany[1]; Leibniz Institute of Marine Sciences (IFM-GEOMAR), Wischhofstrasse 1-3, D-24148 Kiel, Germany[2]; Norwegian Institute for Nature Research, NO-7485 Trondheim, Norway[3]; and Department of Marine Technology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway[4]

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The cold-water coral *Lophelia pertusa* (Scleractinia, Caryophylliidae) is a key species in the formation of cold-water reefs, which are among the most diverse deep-sea ecosystems. It occurs in two color varieties: white and red. Bacterial communities associated with *Lophelia* have been investigated in recent years, but the role of the associated bacteria remains largely obscure. This study uses catalyzed reporter deposition fluorescence in situ hybridization to detect the in situ location of specific bacterial groups on coral specimens from the Trondheimsfjord (Norway). Two tissue-associated groups were identified: (i) bacteria on the host's tentacle ectoderm, "*Candidatus* Mycoplasma corallicola," are flasklike, pointed cells and (ii) endoderm-associated bona fide TM7 bacteria form long filaments in the gastral cavity. These tissue-bound bacteria were found in all coral specimens from the Trondheimsfjord, indicating a closer relationship with the coral compared to bacterial assemblages present in coral mucus and gastric fluid.

Lophelia pertusa (L., 1758) (Scleractinia, Caryophylliidae) is a eurybathic, stenothermal cold-water coral that occurs as white and red color varieties. Its habitat is characterized by high biological production and vigorous hydrodynamic regimes (27), comprising continental slopes, seamounts, and fjords. *L. pertusa* is a key species in the formation of cold-water reefs, which are among the most diverse deep-sea ecosystems. More than 980 invertebrate species are known to be associated with cold-water corals, belonging to a broad range of taxa: Foraminifera, Cnidaria, Nemertini, Polychaeta, Crustacea, Gastropoda, Bivalvia, and Ophiuroidae (Echinodermata) (6). Although most of these organisms are not found exclusively on *Lophelia* banks, many of them are much less common in other habitats (19).

Investigation into the bacterial microbiota of *L. pertusa* has started only recently and is scarce compared to research on the microbiology of tropical corals (11, 31, 35; C. A. Kellogg, unpublished data presented at the 2006 Ocean Sciences Meeting, Honolulu, HI, 20 to 24 February 2006; C. A. Kellogg and R. P. Stone, unpublished data presented at the ASLO/TOS Ocean Research Conference, Honolulu, HI, 15 to 20 February 2004). Most recently, community structure and taxonomy of bacteria on *L. pertusa* samples from the Trondheimsfjord (Norway) were characterized by the culture-independent 16S rRNA gene-based techniques T-restriction fragment length polymorphism and sequence analysis (22): *L. pertusa* shows a high microbial richness, which differs conspicuously from environmental microbiota, and varies both with location and color variety of the coral; these bacteria are very likely to influence nutrition, health, and distribution of *L. pertusa*.

In recent years, fluorescence in situ hybridization (FISH) has proven to be a powerful tool for direct imaging of bacteria associated with corals. This comprised identification and quantification of potential disease agents (7, 15), as well as of biofilm bacteria inducing coral metamorphosis (34). In the present study, the in situ location of bacterial groups on the coral was to be revealed by FISH on coral thin sections, with the aim to provide insights into possible microbe-host interactions. Shallow-water stony corals exhibit a typical strong tissue autofluorescence ascribed to UV protection proteins, so-called "pocilloporins," in their tissue (29). Although cold-water corals are not affected by UV radiation, tissue autofluorescence is also observed in *L. pertusa*, interfering with the signals of fluorescently labeled DNA probes commonly used to detect bacteria in situ. To surmount this impediment, catalyzed reporter deposition FISH (CARD-FISH) (24, 30) was used in the present study, providing signals up to 20-fold brighter relative to conventional monolabeled probes (30). This enables detection of marked cells even against the bright background of coral tissue autofluorescence.

MATERIALS AND METHODS

Sampling and fixation. Sampling was performed as described previously (22). All coral samples were incubated in sterile-filtered $MgCl_2$ solution to anesthetize the coral polyps for 30 min. Anesthesia was regarded as beneficial for FISH in order to prevent the polyps from full retraction, providing an unobscured view on the tentacles (cf. reference 7). The samples were incubated in a sterile-filtered solution of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 8.3) (PBS was composed of 8.0 g of NaCl, 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4, 0.2 g of KCl, and H_2O to 1,000 ml; the pH was adjusted with NaOH) for 5 to 7 h and then transferred into 50% (vol/vol) ethanol in sterile-filtered seawater and frozen at −20°C.

* Corresponding author. Present address: Leibniz Institute of Marine Sciences (IFM-GEOMAR), West Shore Campus, Duesternbrooker Weg 20, D-24105 Kiel, Germany. Phone: 49 431 600-1987. Fax: 49 431 600-4452. E-mail: sneulinger@ifm-geomar.de.

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TABLE 1. Oligonucleotide probes used in FISH for identification of bacterial populations[a]

Probe	Target group	Target rRNA	Target site[c]	Sequence (5′→3′)	% Formamide	Source or reference
ALF968	*Alphaproteobacteria*	16S	968–985	GGTAAGGTTCTGCGCGTT	45	21
EUB338 I	Most *Bacteria*	16S	338–355	GCTGCCTCCCGTAGGAGT	55	2
EUB338 II	*Planctomycetes*	16S	338–355	GCAGCCACCCGTAGGTGT	55	8
EUB338 III	*Verrucomicrobia*	16S	338–355	GCTGCCACCCGTAGGTGT	55	8
GAM42a	*Gammaproteobacteria*	23S	1027–1043	GCCTTCCCACATCGTTT	50	16
BET42a[b]	*Betaproteobacteria*	23S	1028–1043	GCCTTCCCACTTCGTTT	50	16
HGC236	*Actinobacteria*	16S	236–253	AACAAGCTGATAGGCCGC	30	9
LGC0355	*Firmicutes*	16S	355–373	GGAAGATTCCCTACTGCTG	45	12
LGC0355b	*Mycoplasmataceae*	16S	355–373	GGAATATTCCCTACTGCTG	35	This study
NON338	None (control)			ACTCCTACGGGAGGCAGC	55	33

[a] Names, targeted taxa, targeted rRNA molecules and sites, sequences of the probes, and the formamide concentrations in the hybridization buffer required for specific in situ hybridization are presented.
[b] Used as unlabeled competitor with the probe GAM42a.
[c] *E. coli* numbering (5).

Bacterial test strains. For evaluation of the CARD-FISH method and the applied oligonucleotide probes, the following bacterial test strains were used: DSM498 (*Gammaproteobacteria*: *Escherichia coli*); DSM347 (*Firmicutes*: *Bacillus subtilis*); environmental isolate, 98% 16S rRNA gene sequence similarity to type strain KMM 3465^T (*Alphaproteobacteria*: *Erythrobacter vulgaris*); and environmental isolate, 99% 16S rRNA gene sequence similarity to type strain ATCC 25495^T (*Actinobacteria*: *Streptomyces sampsonii*). All strains were cultured on agar plates and harvested during exponential growth phase. After fixation with 4% formaldehyde in PBS (pH 8.3) at 4°C overnight, bacterial cells were spread onto polycarbonate Nuclepore membrane filters (0.2-μm pore size, 47 mm in diameter [Whatman]) by vacuum filtration and frozen at −20°C in small petri dishes. Sections of these filters were processed the same way as for the coral thin sections in CARD-FISH (see below).

Decalcification and thin sectioning. Coral branches were decalcified in 20% (wt/vol) EDTA in PBS (pH 8.3). The solution was changed two to three times over 2 days until the coral skeleton was completely dissolved and polyps remained connected only by the coenosarc. The polyps were then singularized and dehydrated in a graded ethanol-xylene series (ethanol at 70, 90, and 95% and twice at 100%; xylene, three times at 100% [vol/vol]) at room temperature for 20 min each step, followed by two infiltrations in paraffin at 60°C for 10 min each time and paraffin embedding in cuboid tin foil molds. Series of four to five sagittal sections (6 to 10 μm) of the embedded polyps were immobilized onto SuperFrost Plus slides (Menzel; two series per slide). They were deparaffinated by heating to 60°C and immediate washing in pure xylene and ethanol for 30 s each step.

Permeabilization and peroxidase inactivation. Coral thin sections were rehydrated in ethanol (100 and 70% [vol/vol]) and H_2O for 30 s each step. To avoid merging of liquids on the slide, thin sections were framed with a hydrophobic border by using a paraffin crayon. Bacterial cell walls were permeabilized by incubation in 100 to 200 μl of lysozyme buffer (1.355 10^6 U of lysozyme [Serva] ml^{-1}, 500 mM EDTA [pH 8.0], 300 mM Tris-HCl [pH 8.0]) at 37°C for 2 h. After being washed with H_2O at room temperature for three times for 1 min each time, some samples were additionally permeabilized with 100 to 200 μl of achromopeptidase buffer (60 U of achromopeptidase [Sigma] ml^{-1}, 10 mM NaCl, 10 mM Tris-HCl [pH 8.0]) at 37°C for 1 h. Slides were again washed three times with H_2O at room temperature for 1 min each time and air dried. For inactivation of endogenous peroxidases, thin sections were incubated in 100 to 200 μl of 3% (vol/vol) H_2O_2 at room temperature for 30 min, followed by three 1-min washes with H_2O at room temperature, air drying, and storage at −20°C until further processing.

Hybridization. Horseradish peroxidase (HRP)-labeled oligonucleotide probes (Table 1) were purchased from biomers.net. The lyophilized probes were rehydrated in H_2O, quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and adjusted to 50 ng μl^{-1} (probe stock solution). The hybridization buffer consisted of 20 to 60% (vol/vol) formamide (depending on the probe; see Table 1), 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% (wt/vol) dextran sulfate (Sigma), 0.01% (wt/vol) sodium dodecyl sulfate, and 10% (vol/vol) blocking solution. The blocking solution (pH 7.5) was made of 10% (wt/vol) blocking reagent for nucleic acid hybridization (Roche), 1.16% (wt/vol) maleic acid (Fluka), and 150 mM NaCl. Portions (100 to 200 μl) of a 1:200 (vol/vol) mixture of probe stock solution and hybridization buffer were spread onto the thin sections. Slides were incubated at 35°C for 3 to 4 h in a vapor-saturated repository and subsequently washed in Falcon tubes containing 50 ml of washing buffer at 37°C for 5 min. The washing buffer consisted of NaCl (with concentrations corresponding to the amount of formamide in the hybridization buffer, see Table S1 in the supplemental material), 20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 0.01% (wt/vol) sodium dodecyl sulfate.

TSA. Cyanine 3 (Cy3) and fluorescein tyramide conjugates from Tyramide signal amplification (TSA) tyramide reagent packs (Perkin-Elmer) were dissolved according to the manufacturer's instructions. The amplification buffer contained 10% (vol/vol) blocking solution (see above), 2 M NaCl, 10% (wt/vol) dextran sulfate, 0.0015% (vol/vol) H_2O_2, and PBS (pH 7.3) to 100% (vol/vol). Thin sections were equilibrated with PBS (pH 7.3) at room temperature for 15 min. Portions (100 to 200 μl) of a 1:500 (vol/vol) mixture of tyramide solution and amplification buffer were spread onto the thin sections. Incubation of the slides at 46°C for 30 min in a dark, vapor-saturated repository was followed by washing with PBS (pH 7.3) at room temperature for 20 min and three washes with H_2O at room temperature for 1 min each time.

Double hybridization. Simultaneous marking of bacteria with two different probes was accomplished as follows. After the first signal amplification with Cy3 tyramide, HRP was inactivated with H_2O_2 as described above. Then, a second hybridization and signal amplification with fluorescein tyramide was carried out. In a parallel treatment, probe NON338 was used for the second hybridization to prove that these signals did not result from persisting HRP activity of the first hybridization.

Mounting. Air-dried thin sections were covered in mountant (80% [vol/vol] Citifluor AF1, 14% [vol/vol] Vectashield mounting medium [Vector Laboratories], 1 μg of DAPI [4′,6′-diamidino-2-phenylindole]; Sigma] ml^{-1}, and PBS [pH 9] to 100% [vol/vol]) and stored at −20°C until microscopic analysis.

Microscopy and image processing. Thin sections were viewed on a Leitz DMRB epifluorescence microscope, equipped with filter sets A, N2.1, and I3 (for fluorescence detection of DAPI, Cy3, and fluorescein, respectively), and ×40 and ×100 PL FLUOTAR objective lenses (all from Leica). Image stacks were taken with a digital still camera by manually advancing the focus layer through the *z*-axis of the whole thin section in steps of ~2 μm and ~1 μm for the ×40 and ×100 objective lenses, respectively. Deconvolved composite images were produced from these stacks with the software Helicon Focus v4.21 (Helicon Soft, Ltd.). Overlay images for the simultaneous display of fluorescence signals of different dyes were produced with Photoshop CS (Adobe). Contrast enhancement was used equally to all images of a series.

Probe specificity assay. To cross-check the specificity of the newly designed probe LGC0355b, a culture of *B. subtilis* DSM347 (which has the probe LGC0355 target site) was hybridized with the probes LGC0355 and LGC0355b, respectively, each with 35% formamide. Signal brightness was compared between the two approaches after TSA with Cy3 tyramide. Since bacterial cultures harvested during their exponential growth phase have a vast number of ribosomes, even suboptimal probe binding results in bright fluorescence signals. Digital image analysis was used to compare signal intensities. Of each hybridization, five digital images from filter sections densely covered with bacteria were obtained with identical camera settings. Cumulative histograms of pixel brightness values were obtained by using ImageJ v1.37 (25). To ensure that results were not biased by dark spaces between the bacterial cells or pixel super saturation, only intermediate brightness values between 80 and 165 (arbitrary units) were taken into account.

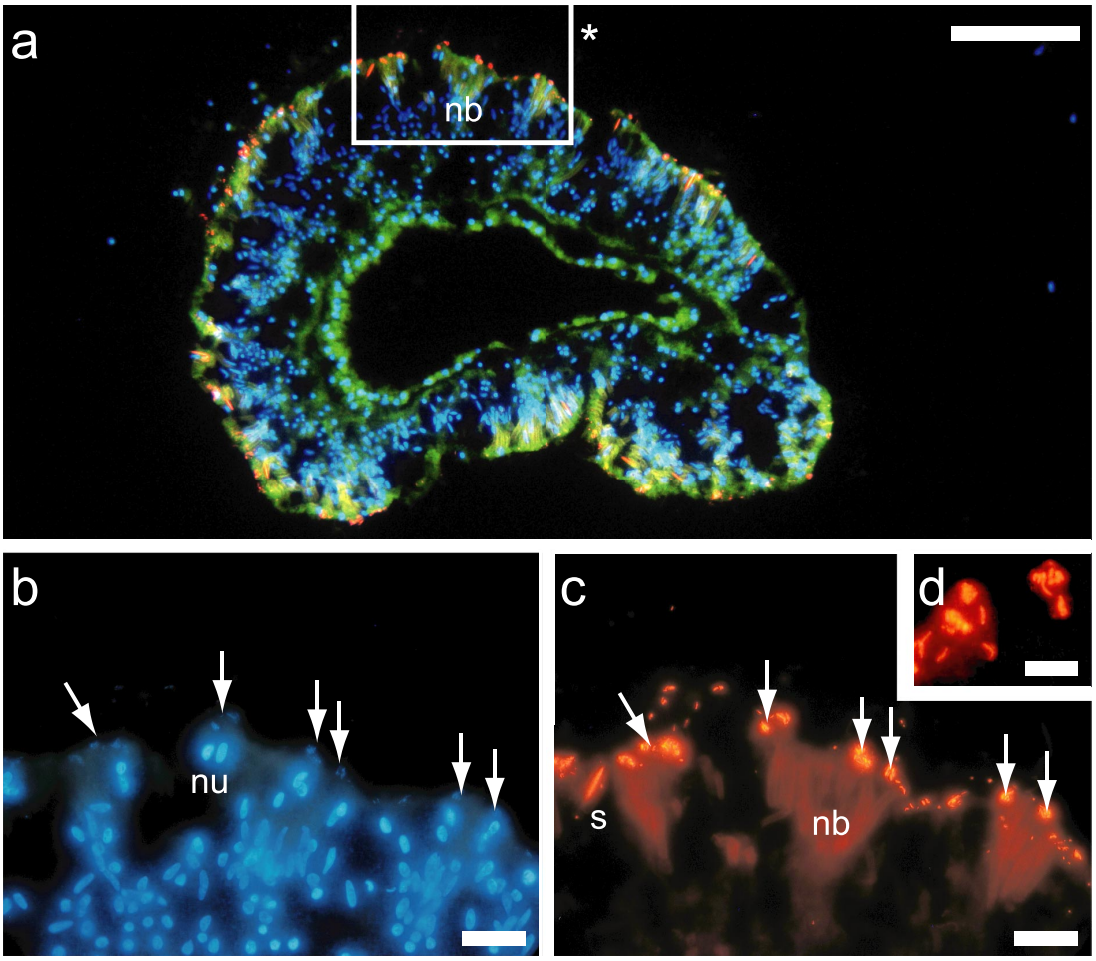

FIG. 1. Epifluorescence micrographs of bacteria on a thin section in the tentacle region of a *L. pertusa* polyp (red coral color variety from station 3). (a) Overview of a cross-sectioned tentacle. The image is an overlay of coral tissue autofluorescence (green), DAPI signals of coral cell nuclei (blue), and hybridization signals of probes EUB338 I to III with Cy3 TSA (red). (b and c) Micrograph detail showing DAPI (b) and Cy3 (c) signals of bacterial aggregates on the nematocyst batteries of the tentacle ectoderm. (d) Single flask-shaped bacterial cells. *, frame of micrograph detail (panels b and c); nb, nematocyst battery; nu, coral cell nuclei; s, nematocyst stylet. Arrows in panels b and c denote corresponding DAPI and Cy3 signals of selected bacterial aggregates. Scale bars: 100 μm (a), 20 μm (b and c), and 5 μm (d).

RESULTS

Bacteria associated with coral ectoderm. For testing of the CARD-FISH method, freshly gathered polyps of aquarium-reared *L. pertusa* were hybridized with a mixture of probes EUB338 I to III. This probe combination targets the majority of all known bacteria (8). Bright fluorescence signals were obtained from remains of mucus attached to the thin-sectioned coral tissue (see Fig. S1 in the supplemental material), indicating that the coral mucus was densely inhabited by microbes. In contrast, thin sections of coral samples from the Trondheimsfjord hybridized with probes EUB338 I to III did not feature mucus-colonizing bacteria like those in aquarium-reared *L. pertusa*. Instead, fluorescence signals were observed in the peripheral ectoderm of the tentacles (Fig. 1 a) and were not as abundant and dense as the signals seen in Fig. S1 in the supplemental material. The brightest fluorescence was caused by aggregates of bacterial cells situated on the distal ends of nematocysts of the spirocyst type arranged in nematocyst batteries (Fig. 1a and c). The fluorescing bacterial cells had a flasklike shape (about 1.6 μm long and 0.5 μm wide) with a tip at one end (Fig. 1d). DAPI counterstaining confirmed that the bacterial cells highlighted by CARD-FISH contained DNA and were thus not artifacts (Fig. 1b). Documentation of DAPI signals was hampered, however, by coral tissue autofluorescence and the much brighter signals of the coral cell nuclei underneath or in the direct vicinity of the prokaryote aggregates. Beside bacterial cells, also stylets of nematocysts belonging to the stenotele type and other nematocyst parts were marked by the fluorescent dyes (Fig. 1c). However, from their sizes and shapes, these structures could be clearly distinguished from bacterial cells. No nonspecific marking of bacterial cells was observed after hybridization with control probe NON338 (data not shown). The flasklike bacteria described above prevailed on the nematocyst batteries. Sporadic cells of the same morphotype were only observed on the coral coenosarc (data not shown) and not on the endoderm.

To identify the prokaryotes on a subdomain level, probes targeting all abundant *L. pertusa*-associated bacterial groups in

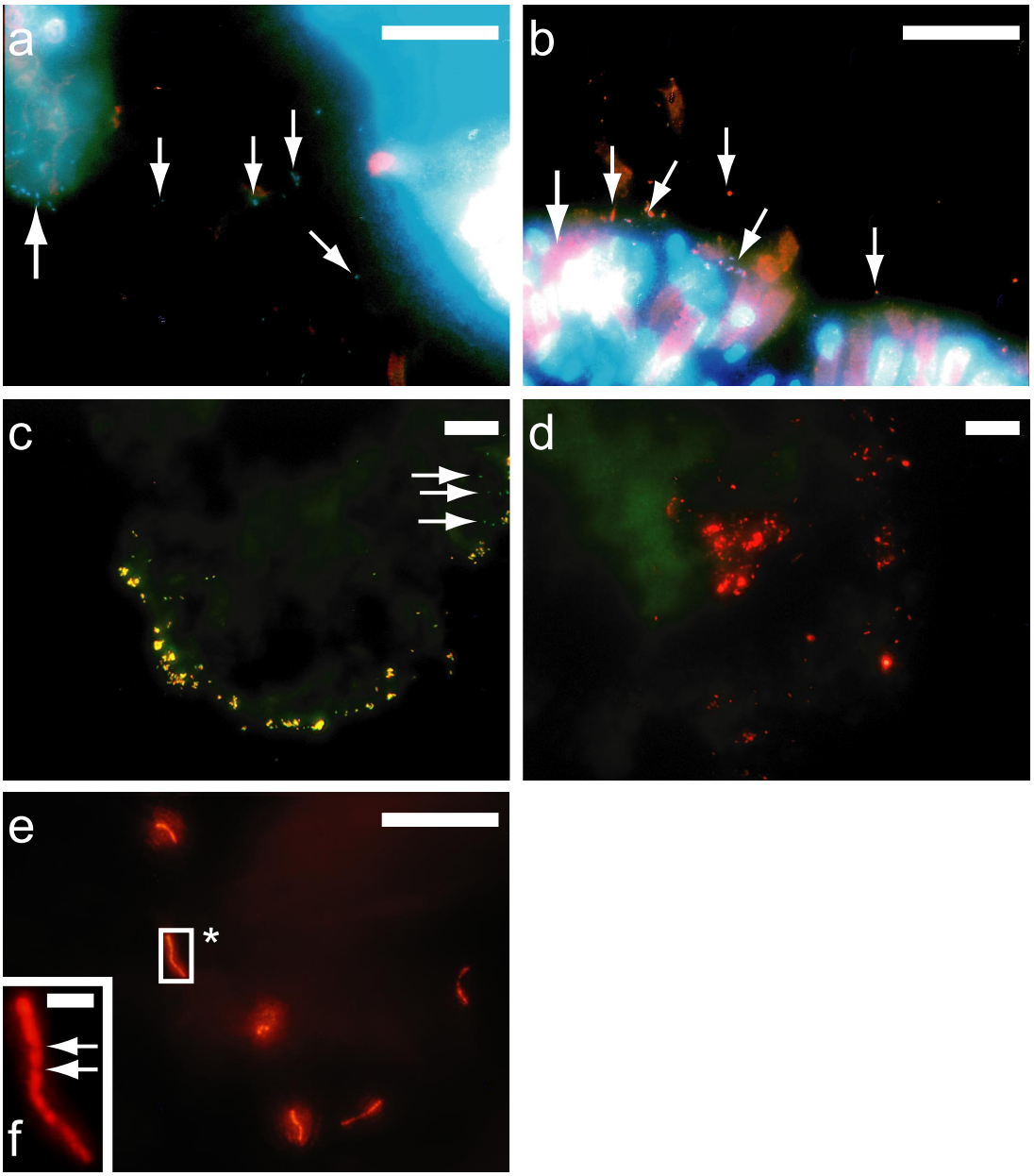

FIG. 2. Epifluorescence micrographs of bacteria on thin sections of *L. pertusa* polyps. (a and b) Tentacle region of red coral color variety from station 1. Overlays of DAPI (blue) and Cy3 signals (red) are shown. Thin sections were hybridized with probes LGC0355 (a) and LGC0355b (b), respectively, under the same conditions (35% formamide in hybridization buffer). (c and d) Tentacle region of red coral color variety from station 2. (c) Double hybridization with probes LGC0355b (+Cy3) and EUB338 I (+fluorescein) resulted in a yellowish overlay signal. (d) Double hybridization with probes LGC0355b (+Cy3) and NON338 (+fluorescein) resulted in a red overlay signal, since no hybridization signal was obtained with control probe NON338. (e) Gastral cavity of white coral color variety from station 3. Filamentous bacteria hybridized with probe LGC0355 under low-stringency conditions (20% formamide in hybridization buffer), followed by Cy3 TSA. (f) Single bacterial filament (micrograph detail of panel e). The arrows in panels a and b mark DAPI and Cy3 signals of selected bacterial cells. The arrows in panel c mark exemplary bacterial cells not stained by probe LGC0355b. *, frame of micrograph detail (panel e). The arrows in panel f mark the gaps between a single cell and adjacent cells in the filament. Scale bars: 20 μm (a through e) and 2 μm (f).

the sequence library (22) (Table 1) were used. In all cases, hybridization under stringent conditions failed to mark the majority of ectoderm-associated bacteria. Cells hybridized with *Alphaproteobacteria*- and *Gammaproteobacteria*-specific probes were observed only few and far between on the ectoderm (data not shown). Additional application of achromopeptidase to support cell wall permeabilization had no effect. Subsequently, hybridization was repeated with only 20% formamide in the hybridization buffer allowing the probes also to bind to not strictly complementary target sites. This procedure proved successful in case of the *Firmicutes*-specific probe LGC0355. Comparison with the sequence library (22) revealed two clusters with a single-nucleotide deviation from the probe's target sequence (5′-CAGCAGTAGGGAATCTTCC-3′): the *My-*

coplasma cluster with reference sequence D11_CW02_full (EMBL accession no. AM911412; target site, 5′-CAGCAG TAGGGAAT**A**TTCC-3′) and the TM7 cluster with reference sequence F05_CW03 (EMBL accession no. AM911417; target site, 5′-CAGCAGTAGGGAAT**T**TTCC-3′). No mismatches were observed for the other (16S rRNA-targeting) FISH probes with their respective binding sites in sequences originating from Norwegian *L. pertusa*.

For the larger *Mycoplasma* cluster, a modified probe LGC0355b (Table 1) was designed that compensated for the sequence deviation in the target site of the original probe LGC0355. If the bacteria on the coral ectoderm had the same target site as the *Mycoplasma* sequence D11_CW02_full (22), the binding efficiency of the modified probe would be higher than that of the original probe. Accordingly, there should be a stringency condition (formamide concentration) in hybridization that allowed for specific binding of probe LGC0355b but not of probe LGC0355. Hybridization with 35% formamide gave a negative result for the original probe LGC0355 (Fig. 2a) but yielded conspicuous specific signals for the modified probe LGC0355b (Fig. 2b).

The specificity cross check for probes LGC0355 and LGC0355b indicated a lower binding efficiency of probe LGC0355b with *B. subtilis* compared to that of probe LGC0355 (see Fig. S2 in the supplemental material). For hybridization of *B. subtilis* (with the binding site of probe LGC0355), the main fluorescence peak of the newly designed probe LGC0355b was less pronounced and shifted toward lower brightness values compared to the original probe LGC0355. In addition, probe LGC0355b exhibited more pixels with low brightness values than probe LGC0355.

An additional assay was conducted to test whether cell wall permeabilization was crucial for hybridization with HRP-coupled oligonucleotide probes (see Fig. S3 in the supplemental material). Permeabilization by lysozyme was necessary for both gram-negative (see Fig. S3a to d in the supplemental material) and gram-positive bacteria (see Fig. S3e and f in the supplemental material). For the bacteria on *L. pertusa* tentacles, however, omission of lysozyme treatment resulted in no apparent loss in abundance and brightness of hybridization signals (see Fig. S3g to k in the supplemental material).

Double hybridization with probes LGC0355b and EUB338 I demonstrated that the vast majority of detectable bacteria on *L. pertusa* thin sections could also be hybridized with the *Mycoplasma*-specific probe (Fig. 2c), save for a few cells (arrows in Fig. 2c). Lack of signals from control probe NON338 confirmed specificity of the double hybridization (Fig. 2d). No evident discrepancies in bacterial abundances were observed between coral samples from different stations or of different color.

Bacteria associated with coral endoderm. Corals from all stations and of both color varieties harbored thin (0.6-μm) filamentous structures of up to 20 μm in their endoderm (Fig. 2e and f). These structures consisted of short rod-shaped cells about 1 μm in length. Filaments were scattered on the septal tissue of the gastral cavity. The structures exhibited bright fluorescence upon hybridization with probe EUB338 I (see Fig. S4a in the supplemental material). Although the cells lacked a conspicuous DAPI signal, negative results with control probe NON338 on parallel thin sections (data not shown) confirmed the specificity of this hybridization.

Identification on the subdomain level was approached as described above. As with the tentacle-associated microbes, only hybridization with the *Firmicutes*-specific probe LGC0355 under low-stringency conditions (20% formamide in the hybridization buffer) resulted in positive FISH signals (Fig. 2e and f). Unlike the ectoderm-associated bacteria, the filaments did not hybridize with probe LGC0355b under stringent conditions (data not shown). Omitting the cell wall permeabilization step in the CARD-FISH protocol resulted in a visible weakening, though not complete loss of the hybridization signal (see Fig. S4b in the supplemental material) compared to permeabilized cells (see Fig. S4a in the supplemental material). Abundances of filamentous structures did not diverge conspicuously between coral samples from different stations or of different color.

DISCUSSION

This study reports the first in situ imaging of tissue-associated bacteria of the cold-water coral *L. pertusa*. The majority of bacteria situated on the host's tentacle ectoderm are flasklike, pointed cells, while bacteria found on the endodermal tissue of the gastral cavity form thin, long filaments.

Bacteria associated with coral ectoderm. By far, the most bacteria were detected on the nematocyst batteries of *L. pertusa* (Fig. 1). The bacteria could be successfully hybridized with a modified *Firmicutes*-specific oligonucleotide probe LGC0355b (Table 1) targeting the 16S rRNA of *Mycoplasma* present in the sequence library of coral specimens from the Trondheimsfjord (22). Hybridization at stringent conditions (Fig. 2a and b) and specificity cross check (see Fig. S2 in the supplemental material) demonstrated that the modified probe LGC0355b specifically detects the single-nucleotide deviation from the target sequence of the original probe LGC0355. Like most oligonucleotide probes, LGC0355b is not exclusively specific to *Mycoplasma* but also targets other taxa, predominantly of the class *Bacilli* in the common phylum *Firmicutes*. Morphology of the latter is quite similar to that of the observed microbes. However, *Mollicutes* have lost their cell wall during evolution from common ancestors within the *Firmicutes*. With the CARD-FISH protocol used in the present study, cell wall permeabilization was crucial to obtain brightest fluorescence after TSA. The strong hybridization signal obtained even without lysozyme treatment confirmed the lack of a cell wall in the ectoderm-associated bacteria. These results suggest that the microbes colonizing the coral's nematocyst batteries are representatives of the *Mycoplasma* species previously identified on Norwegian *L. pertusa* (22). Their cell morphology (Fig. 1d) resembled the flask-shaped and elongated forms with unipolar tip structures of the closest characterized relative *M. sphenisci* (10, 22). Such a flasklike or clublike cell shape is characteristic for many pathogenic mycoplasmas, the protruding tip serving as an organelle for cytadhesion to epithelial linings of their host (26). Double hybridization with probes LGC0355b and EUB338 I (Fig. 2c and d) showed that for all coral samples, regardless of their provenience or color variety, the bacterial clusters situated on their nematocyst batteries consisted almost entirely of mycoplasmas. Only a small fraction of microbial

cells (most likely *Alpha-* and *Gammaproteobacteria*) did not hybridize with probe LGC0355b (Fig. 2c).

The closest relatives of the newly discovered mycoplasmas are common on octocorals (Isididae and *Muricea elongata*); together, these sequences form a monophyletic cluster within the *Mycoplasma hominis* group (22). It is therefore reasoned that these organisms are members of a novel phylogenetic lineage currently consisting of three apparently coral-specific *Mycoplasma* species. Its representative from *L. pertusa* is proposed as a novel candidate species, "*Candidatus* Mycoplasma corallicola," which is formally described in the following section.

Proposal of "*Candidatus* Mycoplasma corallicola." As suggested by Murray and Stackebrandt (20), the category "*Candidatus*" should be used for a description of prokaryotic entities that is based on more information than just a DNA sequence but lacks characteristics required according to the International Code of Nomenclature of Bacteria. The *Mycoplasma* described here represents a novel species of *Mollicutes*. The species should be designated "*Candidatus* Mycoplasma corallicola" (co.ral.li.co′la. L. n. *corallum*, coral; L. suff. *-cola*, inhabitant, dweller; N.L. n. *corallicola*, coral-dweller, referring to the host of the microorganism). The phylotype inhabiting the cold-water coral *L. pertusa* is currently the only representative of this candidate species. Assignment to "*Candidatus* Mycoplasma corallicola" is based on (i) the associated 16S rRNA gene sequence (D11_CW02_full; EMBL accession no. AM911412) and its low similarity of 91% or less to sequences of next relatives (22), (ii) a flask-shaped cell morphology of 0.5 by 1.6 μm, (iii) the absence of a cell wall, (iv) preferential attachment to the spirocysts on the tentacle ectoderm of *L. pertusa*, and (v) positive hybridization with the 16S rRNA targeting oligonucleotide probe LGC0355b (5′-GGAATATTCC CTACTGCTG-3′).

Bacteria associated with coral endoderm. Long and thin filamentous structures were found in the gastral cavity of red and white coral phenotypes from all stations in the Trondheimsfjord (Fig. 2e and f). Although DAPI staining failed to identify them as bacteria, specificity of the hybridization was proven by means of the control probe NON338. Poor DNA staining with DAPI was previously reported for other microbes (3, 32). Similar filaments were also observed with FISH in *L. pertusa* samples from other Norwegian locations (S. Schöttner, unpublished data). Other filamentous bacteria were described to occur endolithically (i.e., in the skeleton) in the Mediterranean coral *O. patagonica* but, due to their autofluorescence typical for chlorophyll, they were most likely cyanobacteria (1). The phylum *Actinobacteria*, abundantly represented in Norwegian *L. pertusa* by 16S rRNA gene sequences (22), is also rich in filamentous morphotypes. However, the *Actinobacteria*-specific probe HGC236, like all subphylum level probes used in this survey, failed to hybridize the filamentous bacteria under stringent conditions. They could only hybridize with the *Firmicutes*-specific probe LGC0355 with low stringency, just like the ectoderm-associated mycoplasmas. The only *L. pertusa*-associated sequences, except for those of "*Candidatus* Mycoplasma corallicola," which permitted such unspecific probe binding, were that of candidate division TM7 (reference sequence F05_CW03; EMBL accession no. AM911417) (22). The sequence of probe LGC0355 is 5′-GGAA**G**ATTCCCTACTGC TG-3′, and its original (reverse complement) target site on the bacterial ribosome is 5′-CAGCAGUAGGGAAU**C**UUCC-3′. TM7 sequences show only one mismatch compared to this site (5′-CAGCAGUAGGGAAU**U**UUCC-3′), which is at the same position as with "*Candidatus* Mycoplasma corallicola" (5′-CAGCAGUAGGGAAU**A**UUCC-3′) but has a uridine instead of an adenosine. If the filaments belonged to the previously sequenced TM7, this could explain why their hybridization with the *Mycoplasma*-specific probe LGC0355b (5′-GGAA**T**ATTCCCTACTGCTG-3′) failed under stringent conditions: the energy yield of the target-probe hybrid formation is lower for the U-T mismatch between TM7 and LGC0355b than it is for the matching pair A-T in "*Candidatus* Mycoplasma corallicola" under the same hybridization conditions. The filaments found in the present study also resembled morphotypes of TM7 bacteria from the human oral cavity (4, 23) and from a laboratory scale bioreactor (14). The latter study reported members of candidate division TM7 to be gram positive. Coral endoderm-associated bacteria showed visible fluorescence attenuation when cell wall permeabilization was omitted (unlike "*Candidatus* Mycoplasma corallicola"). This indicates that they have a cell wall. It appears, however, to be a priori more permeable to the large CARD-FISH probe molecules than the cell walls of other, namely, gram-positive bacteria (cf. Fig. S3a through f in the supplemental material). For the time being, the identity of the endoderm-associated filaments found in *L. pertusa* cannot be determined with sufficient confidence. Nevertheless, the indirect evidence presented here permits addressing these bacteria as bona fide TM7.

Comparison of sequence frequencies and bacterial in situ abundances. The CARD-FISH results are in marked contrast to the previously depicted high microbial diversity of 27 and 54 operational taxonomic units (based on the 97% similarity criterion) determined by 16S rRNA gene analysis in white and red corals, respectively (22). Irrespective of some sporadic *Alpha-* and *Gammaproteobacteria*, only "*Candidatus* Mycoplasma corallicola" and bona fide TM7 were abundant and in direct association with the coral tissue. Test hybridizations with aquarium-reared *L. pertusa* polyps (see Fig. S1 in the supplemental material) indicated that the mucus of this coral was densely populated by bacteria, which were apparently far more abundant than bacteria on coral tissue thin sections from the Trondheimsfjord. These findings reveal that the major bacterial diversity—and most likely the numerical majority of bacteria—is located in the mucus and probably also in the gastric fluid of *L. pertusa*.

The reason why bacteria associated with coral mucus and gastric fluid were observed by 16S rRNA gene analysis but not CARD-FISH is given by the different preparation procedures of the two techniques. For DNA extraction, mucus and gastric fluid were never completely removed from coral specimens (22). In contrast to that, decalcification, paraffin embedding and deparaffination, and the multiple washing steps of the thin sections during CARD-FISH washed away mucus, as well as gastric fluid, from the polyps. Loose bacteria living therein were thus not preserved on the slides except for very few cells of *Alpha-* and *Gammaproteobacteria*, the two largest groups on *L. pertusa* (22). Even in the aquarium-reared polyps that were immediately decalcified after fixation without additional stor-

age time, only wisps of mucus remained attached to the tissue thin sections.

Both coral color varieties hosted "*Candidatus* Mycoplasma corallicola" and bona fide TM7 without discernible differences in abundance. This finding disagrees with phylogenetic analysis (22), according to which both groups of bacteria should live exclusively on the white *L. pertusa* color variety. This inconsistency can be explained by statistical effects and PCR interference: the probability for a distinct PCR product to become cloned and sequenced is a function of its relative proportion in the overall mixture of amplified gene fragments. This probability is particularly low for rare fragments in a diverse mixture. Such conditions prevail in red *L. pertusa* with 54 operational taxonomic units compared to 27 in white *L. pertusa* (22). Since the two epithelium-bound bacterial groups most probably constituted minor fractions of the total bacterial quantum on the coral polyps (22), they are less likely to be detected in red corals by cloning than in white corals. Furthermore, obstruction of primer annealing or elongation could have occurred: primer-template mismatch can never be ruled out with primers targeting a broad range of phylogenetic groups and might occur in both *Mycoplasma* and TM7. In many mycoplasmas, the adenine residues at GATC sites are methylated, while in others the cytosine residues are methylated (26). Because this motif was part of the forward primer 27f sequence (5′-AGAG TTT**GATC**MTGGCTCAG-3′) (22) and its reverse complement reads the same, binding of the primer is likely to be restricted in *Mycoplasma* (13). Methylation may also inhibit elongation (28). These factors could have further reduced the yield of respective PCR products in white *L. pertusa*, leading to the observed underrepresentation of *Mycoplasma* and TM7 sequences. In red *L. pertusa* the effects of low primer binding probability (see above) and obstruction of primer annealing or elongation would be combined. Consequently, the probability of *Mycoplasma* and TM7 sequences to become retrieved from red corals would be further impaired, making detection of these groups unlikely in that coral color variety.

Partitioning and specificity of the bacterial community. Data from other studies obtained with the fingerprinting methods DGGE (11) and ARISA (31) indicate a partitioning of bacterial groups in *L. pertusa* similar to that described above between coral tissues on the one hand and mucus and gastric fluid on the other. To call the observed coral-microbial association truly "specific," it has to be constant over time and space. Although temporal variations still need to be investigated, the results of the present study gave evidence for a spatially constant fraction of tissue-associated bacteria in *L. pertusa*. Yet, this was not mirrored on a wider geographic level, since Grosskurth (11) reported spatial variations of both mucus- and tissue-associated bacteria on *L. pertusa* from offshore locations in the northeast Atlantic. Also, Yakimov et al. (35) did not find *Mycoplasma* or TM7 sequences on Mediterranean *L. pertusa*. Then again, there is the possibility that these bacterial groups had simply been overlooked in the former studies. Unpublished FISH data suggest the presence of filamentous bacteria (probably also bona fide TM7) on corals from other Norwegian locations (Schöttner, unpublished). There is a good chance that the use of CARD-FISH or other bioimaging techniques in future studies will reveal these tissue-associated microbes on *L. pertusa* from other geographic regions.

In summary, the bacterial community of *L. pertusa* from the Trondheimsfjord can be divided into two subcommunities differing in their location on the coral: (i) a tissue-bound bacterial fraction of "*Candidatus* Mycoplasma corallicola" and bona fide TM7 on the ecto- and endoderm, respectively, that is spatially constant at least on a regional scale and (ii) a "liquid-associated" bacterial fraction in the mucus and gastric fluid varying with location and color variety of its host.

Ecological roles of tissue-associated bona fide TM7 and "*Candidatus* Mycoplasma corallicola." With high probability, the previously reported sequences of candidate division TM7 (22) belong to the filamentous bacteria observed in the gastrocoel of *L. pertusa*. The general physiology and ecology of candidate division TM7 remain subject to speculation until additional knowledge from isolation assays becomes available. Considering the prevalence of hypoxia in coral polyps (22), the location of these bona fide TM7 filaments indicates an anaerobic metabolism. Their obviously tight attachment to the endodermal tissue suggests an interaction with the gastromuscular cells, probably involving the exchange of metabolites with their host.

Mycoplasmas commonly live either attached to interior epithelia or inside their hosts' cells. To our knowledge, this is the first reported incidence of a *Mycoplasma* representative residing on exterior parts of its host, directly facing the environment. Since the cell membrane is their only barrier, mycoplasmas are osmotically much more sensitive than other bacteria; however, the constant milieu of their host offers protection (26). Given this osmotic sensivity, protection of "*Candidatus* Mycoplasma corallicola" at this exposed location could only be ascribed to the buffer properties of the coral's mucopolysaccharide layer.

Most mollicutes live as commensals but are also widespread in nature as parasites of a variety of eukaryotes (26). Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and parasitic mode of life (26). They underwent a distinct reductive way of evolution characterized by "genetic economization." Surveys have shown a particular scarcity of genes in mycoplasmas coding for energy and intermediary metabolism (26). Lacking the ability of de novo synthesis of amino and fatty acids makes them totally reliant on external sources of these vital compounds (26). This general *Mycoplasma* biology, together with the distinct boundedness of the observed "*Candidatus* Mycoplasma corallicola" to *L. pertusa*'s spirocysts, leads to the following theory of their mode of life: the capturing procedure in corals involves discharge of stenoteles and other penetrant cnidocysts that perforate the prey and inject neurotoxins. Spirocyst threads bear hollow tubules that solubilize upon discharge and contact with seawater and adhere to the prey (17). Thus, the prey is kept affixed to the tentacles while the paralyzing effect of the neurotoxins evolves. Perforation of the prey will lead to leakage of hemolymph. This process is comparable to the release of dissolved organic carbon from planktonic algae by "sloppy feeding" in zooplankton (e.g., see reference 18). Their location on the distal ends of the spirocysts allows "*Candidatus* Mycoplasma corallicola" to directly assimilate from the leaking hemolymph amino and fatty acids

that they cannot synthesize themselves. The bacteria prevent themselves from getting removed during the coral's feeding process by adhesion to the host tissue via their tip structure. The available evidence suggests that these *L. pertusa*-associated mycoplasmas live as commensals, in contrast to many of their parasitic relatives. They most likely profit from the prey capture activity but are neither advantageous nor detrimental to their coral host.

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