

# Culture-Independent Characterization of Bacterial Communities Associated with the Cold-Water Coral *Lophelia pertusa* in the Northeastern Gulf of Mexico<sup>∇†</sup>

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**Bacteria are recognized as an important part of the total biology of shallow-water corals. Studies of shallow-water corals suggest that associated bacteria may benefit the corals by cycling carbon, fixing nitrogen, chelating iron, and producing antibiotics that protect the coral from other microbes. Cold-water or deep-sea corals have a fundamentally different ecology due to their adaptation to cold, dark, high-pressure environments and as such have novel microbiota. The goal of this study was to characterize the microbial associates of *Lophelia pertusa* in the northeastern Gulf of Mexico. This is the first study to collect the coral samples in individual insulated containers and to preserve coral samples at depth in an effort to minimize thermal shock and evaluate the effects of environmental gradients on the microbial diversity of samples. Molecular analysis of bacterial diversity showed a marked difference between the two study sites, Viosca Knoll 906/862 (VK906/862) and Viosca Knoll 826 (VK826). The bacterial communities from VK826 were dominated by a variety of unknown mycoplasmal members of the *Tenericutes* and *Bacteroidetes*, whereas the libraries from VK906/862 were dominated by members of the *Proteobacteria*. In addition to novel sequences, the 16S rRNA gene clone libraries revealed many bacterial sequences in common between Gulf of Mexico *Lophelia* corals and Norwegian fjord *Lophelia* corals, as well as shallow-water corals. Two *Lophelia*-specific bacterial groups were identified: a cluster of gammaproteobacteria related to sulfide-oxidizing gill symbionts of seep clams and a group of *Mycoplasma* spp. The presence of these groups in both Gulf and Norwegian *Lophelia* corals indicates that in spite of the geographic heterogeneity observed in *Lophelia*-associated bacterial communities, there are *Lophelia*-specific microbes.**

Cold-water and deep-sea corals have become a topic of interest due to conservation concerns over the impacts of trawling, exploration for oil and gas, and climate change (51, 52). Although the existence of these corals has been known since the 1800s, our knowledge of their distribution, ecology, and biology is limited due to the technical difficulties of studying them. *Lophelia pertusa* is a globally distributed cold-water scleractinian coral (53). In the Gulf of Mexico, *Lophelia* reefs occur primarily along the continental shelf break (300- to 500-m depth), providing an important complex habitat for a wide variety of fishes, crustaceans, and other invertebrates living below the photic zone (48).

The microbial ecology of cold-water corals in deep water is fundamentally different from that of shallow-water corals due to the ambient environmental parameters (e.g., darkness, low temperature, and increased pressure) and the absence of symbiotic zooxanthellae. A few studies have begun to address the microbial associates of deep-sea corals, focusing on octocorals (9, 44) and on *L. pertusa* (27, 41, 42, 57, 72). To date, all the *Lophelia* studies have been conducted on the eastern side of the Atlantic: the Mediterranean basin (72), Mingulay Bay, Scotland (27), and Norwegian fjords (41, 42, 57). These studies

have confirmed that the *Lophelia*-associated bacterial community is distinct from that of the surrounding seawater and sediments (27, 42, 57, 72). A variety of community profile methods (automated rRNA intergenic spacer analysis, terminal restriction fragment length polymorphism, and denaturing gradient gel electrophoresis [DGGE]) were used to demonstrate differences between samples within a geographic area, suggesting that the *Lophelia*-associated microbial community varies depending on regional environmental factors (27, 42, 57). Sequencing of 16S rRNA genes was done in only two studies, and there was no overlap between their data (42, 72). However, different methods of collection, extraction, amplification, and sequencing were employed, so the lack of commonality may be due to methodology rather than biogeography.

Methodology is a concern, particularly the care with which samples need to be collected for microbial ecology studies. Deep-sea coral samples are typically collected by a trawl, net, or dredge or by a submersible/remotely operated vehicle (ROV). With these methods, many corals may be combined in a single container, which is not acceptable for microbiological studies because the microbial community of one coral could contaminate that of the other. Similarly, contact with sediment, other invertebrates, mobile fauna, or water masses between the collection point and the surface could contaminate the coral samples. Unlike the case with the northeastern Atlantic and Norwegian fjords, the temperature and salinity gradients in the Gulf of Mexico during the warm months of the year can be considerable. In the case of the Viosca Knoll sites, the bottom

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TABLE 1. Northern Gulf of Mexico *Lophelia pertusa* samples from which 16S rRNA gene sequences were obtained

Sample	Site	Lat/Long <sup>a</sup>	Depth (m)	Temp (°C)	Salinity (psu)	No. of sequences	FastGroup result (no. of sequences) <sup>b</sup> : Sing, Doub, Trip, cluster
4746K1	VK906/862	29°06.4253' N/88°23.0651' W	311.5	10.7	35.3	74	34, 6, 5, 3
4746K7	VK906/862	29°06.4016' N/88°23.0418' W	310.3	10.7	35.3	43	24, 6, 1, 1
4746K9	VK906/862	29°06.3766' N/88°23.0395' W	319.4	10.5	35.3	52	27, 3, 2, 3
4753K3	VK826	29°10.2439' N/88°00.6962' W	460.6	7.8	35.0	50	47, 2, 0, 0
4753K4 <sup>c</sup>	VK826	29°10.2439' N/88°00.6962' W	460.6	7.8	35.0	106	56, 8, 1, 4
4753K5	VK826	29°10.2254' N/88°00.7295' W	461.2	7.7	35.0	87	55, 7, 6, 0
4753K9	VK826	29°10.1894' N/88°00.7746' W	474.3	7.6	35.0	96	39, 3, 1, 6

<sup>a</sup> Lat/Long, latitude/longitude.

<sup>b</sup> Results of sorting sequences using FastGroup. Sing, unique sequences; Doub, doubletons (two identical sequences), Trip, tripletons (three identical sequences); cluster, cluster of more than three identical sequences.

<sup>c</sup> Sample 4753K4 is from the same colony as sample 4753K3; however, it was not fixed at depth with a preservative like the other six samples.

temperature was 8 to 11°C, compared to a surface temperature of  $\geq 30^\circ\text{C}$ . Coral samples collected in uninsulated containers in this area have been observed to be affected (e.g., polyps retracted and copious stress mucus production) compared to those in insulated containers. Viosca Knoll is also impacted by the Mississippi River plume. The surface waters at these sites were turbid and green and had a salinity of 30 practical salinity units (psu), but below the plume the waters were clear and had a salinity of 35 psu. With this in mind, we designed a sampling container that would protect the coral samples from dramatic changes in temperature and salinity by sealing them in individual insulated compartments (see Fig. S1 in the supplemental material). However, the question remained whether environmental gradients in light and pressure would have an effect on the microbial diversity of the samples. To address this question, each sample was collected in duplicate: one piece was sealed in a compartment alive, and a replicate piece was sealed in another compartment and preserved at depth with a fixative solution. Both sample types (“live” versus “fixed”) were sealed and insulated, so temperature and salinity gradients did not affect them; live samples were subject to gradients in light and pressure, while fixed samples were not.

The main objective of this study was to characterize the bacterial associates of *Lophelia pertusa* from two sites in the northern Gulf of Mexico. Comparing multiple individual colonies from two geographic locations in the Gulf to each other and to bacterial data from *Lophelia* samples on the eastern side of the Atlantic will clarify whether *Lophelia* has a species-specific bacterial community, as has been described for shallow-water corals (49, 55). The results of this study will also better define the total microbial diversity associated with this cold-water coral. A specialized sampling device (see Fig. S1 in the supplemental material) was designed to minimize contamination and thermal shock and to allow the introduction of preservative at depth to determine if environmental gradients were affecting microbial diversity during sampling.

#### MATERIALS AND METHODS

**Site descriptions.** Samples of *Lophelia pertusa* were collected by using a submersible during a cruise in the northern Gulf of Mexico: USGS-GM-2004-03, 29 July 29 to 5 August 2004 (Table 1). The collection sites were on the upper continental shelf within the Viosca Knoll Lease Block of the Minerals Management Service (Fig. 1). Station Viosca Knoll 826 (VK826) was at a depth horizon of 500 m and featured prolific *Lophelia* thickets (13, 58). The corals had thick, heavily calcified skeletons (“brachycephala” morphotype [12]), and there was

localized seepage of hydrocarbons. The water temperature during collections was 8°C and typically ranged from 7° to 9°C at this site (12). The average oxygen saturation was  $6.65 \pm 0.02$  ml/liter, and the average pH was  $7.79 \pm 0.01$ . Station Viosca Knoll 906/862 (VK906/862) was at a depth horizon of 315 m and contained many small isolated colonies of *Lophelia* (13). At this site, the corals had less-calcified, fragile skeletons (“gracilis” morphotype [12]), and there was no hydrocarbon seepage. Water temperature during collections was 11°C and ranged from 9° to 13°C at this site during 375 continuous days of measurement (12), occasionally exceeding the hypothetical upper thermal limit of 12°C for *Lophelia* (22, 23). The average oxygen saturation was  $6.22 \pm 0.02$  ml/liter, and the average pH was  $7.86 \pm 0.00$ . Salinity was 35 psu at both locations. Although *Lophelia* is known to have both red and white color morphs (42), only the white color morph was observed at Viosca Knoll.

**Sampling device.** None of the standard sample containers available on the *Johnson-Sea-Link* submersible were adequate for microbiological sampling. A custom sampling device was designed and constructed specifically for this study. The “Kellogg sampler” is an insulated container that has 10 separate sample compartments, each with a sliding door and o-ring seal (see Fig. S1 in the supplemental material). Syringes of preservative solution (20% dimethylsulfoxide, 0.25 mM EDTA, saturated salt) (17, 59) are connected to five of the sample compartments by one-way valves. The preservative solution is added at depth by

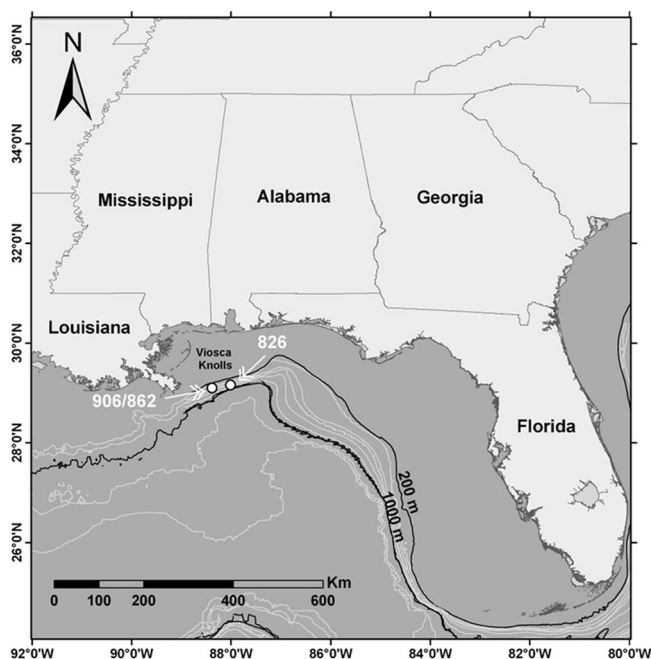


FIG. 1. Locations of the two sampling sites in the northern Gulf of Mexico.

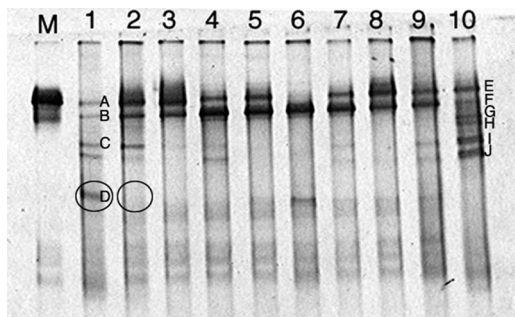


FIG. 2. DG-DGGE gel showing bacterial community DNA "fingerprints" from five *Lophelia* communities, comparing DNA extracted from coral samples preserved at depth (odd numbers) to samples from the same colony brought up alive (even numbers). The gel image has been reversed (i.e., converted to a photo negative) to more clearly show the bands. M, marker; 1, 4753K1; 2, 4753K2; 3, 4753K3; 4, 4753K4; 5, 4753K5; 6, 4753K6; 7, 4753K7; 8, 4753K8; 9, 4753K9; 10, 4753K10. Circles highlight a prominent band in a preserved sample (band D) and its absence in the corresponding unpreserved sample. Band A was cloned and sequenced from lanes 2 to 5 and 7 to 9. Band B was cloned and sequenced from lanes 1, 4, and 6 to 9. Band C was cloned and sequenced from lanes 1 to 4, 7, and 9. Band D was cloned and sequenced from lane 1. Bands E, F, G, H, I, and J were cloned and sequenced from lane 10.

pressing down on a plunger with the submersible's manipulator arm. The Kellogg sampler was mounted on the front of the *Johnson-Sea-Link* submersible and filled using the manipulator arm or suction tube. The sealed separate compartments keep the individual coral samples from becoming contaminated by contact with other coral samples, sediment, or water during the return to the surface. The insulated container keeps the corals at near collection temperature. The ability to add fixative to half the samples at depth makes it possible to test the hypothesis that coral-associated microbial communities shift during sample retrieval in response to changes in light, pressure, or other uncontrolled factors.

**Sample collection.** Branches were removed from *Lophelia* colonies using either the submersible's manipulator claw or suction tube (with a screen to prevent the coral from being sucked into the hose) and then placed in individual sample compartments in the Kellogg sampler. Coral samples were collected in duplicate from each colony; one sample was brought to the surface alive, and the other was fixed at depth by the addition of a preservative solution. Metadata including time of collection, location, depth, temperature, and salinity were recorded (Table 1). The amount of time between collection of a coral sample and arrival on the deck of the ship ranged from 45 min to 3 h. Within 15 min of arriving on deck, *Lophelia* samples were aseptically transferred from the Kellogg sampler compartments into sterile containers and brought into the ship's lab for immediate processing. The corals remained in 7 to 10°C water or fixative until processed.

Previous studies of both shallow-water and deep-sea corals have found that most of the coral-associated bacteria were novel species not present in the surrounding seawater (6, 24, 27, 42, 43, 50, 54, 55, 72). Based on this knowledge, we have chosen to concentrate our efforts solely on characterizing the *Lophelia*-associated bacterial communities.

**DNA extraction.** Flame-sterilized needle-nose pliers were used to snip off small pieces of all *Lophelia* samples (live and preserved at depth) and place them in sterile aluminum weigh dishes. Each piece was crushed using a sterile hammer, and the mixture of coral skeleton fragments, polyp tissue, and mucus was transferred to a 2-ml microcentrifuge tube. Microbial community DNA was extracted from the *Lophelia* samples using the PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Solana Beach, CA), following the manufacturer's protocol. All samples were processed in triplicate. DNA extracts were frozen (−20°C) for transport back to the U.S. Geological Survey microbiology laboratory in St. Petersburg, FL.

**DG-DGGE.** Ten samples from site VK826 were compared using double-gradient DGGE (DG-DGGE) (Fig. 2). These 10 samples were actually pairs of samples from five *Lophelia* colonies: 4753K1/K2, K3/K4, K5/K6, K7/K8, and K9/K10. Odd-number samples were preserved at depth, and even-number samples were recovered without preservation. PCRs (50- $\mu$ l total volume) were composed of 25  $\mu$ l HotStarTaq master mix (Qiagen, Valencia, CA), 1  $\mu$ l (10 pmol) 1070F primer, 2  $\mu$ l (20 pmol) 1392R-gc clamp primer, 21  $\mu$ l deionized water, and

1  $\mu$ l template (a 1:1,000 dilution of a 16S rRNA gene amplicon from 8F/1492R PCR). PCR products were resolved on a D-Code universal mutation detection system (Bio-Rad, Hercules, CA) running a 6- to 12%-gradient polyacrylamide gel containing a 40 to 80% denaturing gradient for 4 h at 60°C and 109 V (14). The gel was stained with SYBR gold (Invitrogen, Carlsbad, CA) and visualized using a UV transilluminator. DNA bands were cut out of the gel using sterile razor blades and extracted from the polyacrylamide using Qiaquick gel extraction kit (Qiagen, Valencia, CA). The primers 1070F and 1392R were used to reamplify the DNA, which was then cloned as described above. Plasmid preps were conducted using a MacConnell Mini-Prep 96 system (MacConnell Research, San Diego, CA). Cluster analysis was conducted using the BioNumerics software program (version 5.0; Applied Maths, Austin, TX).

**Clone libraries.** Libraries designated 4746K were from samples collected at site VK906/862, and libraries designated 4753K were from samples collected at site VK826 (Table 1). The primers 8F (5'-AGAGTTTGATCCTGGCTCAG) (19) and 1492R (5'-GGTTACCTGTTCAGACTT) (62) were used to amplify bacterial-community DNA from the *Lophelia* samples. PCRs (50- $\mu$ l total volume) were composed of 25  $\mu$ l HotStarTaq master mix (Qiagen, Valencia, CA), 1  $\mu$ l (10 pmol) of each primer, 13  $\mu$ l of DI water, and 10  $\mu$ l template. PCR products were cloned into vectors using a Topo TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid preps of transformed *E. coli* were conducted using the Wizard Preps system (Promega, Madison, WI).

**Sequencing.** Sequencing of PCR products from clones containing the correct-size inserts was done by using Northwoods DNA (Bemidji, MN). Partial sequences (500 to 755 bp) were obtained from the 5' end of the clone library sequences using the primer 8F. Clone library sequences of less than 500 bp were discarded. Complete sequences of the DGGE bands were obtained using the primer 1070F (260 to 313 bp).

**Phylogenetic analysis.** The following software programs were used to process the raw sequence data: Phred (20) to base call and add quality scores; GreenGenes (18) to trim poor-quality sections, and BLAST (1) to compare the sequences against the GenBank database. The sequences were dereplicated using the FastGroup II software program (73) and chimera checked with the Bellerophon program (30). Percent similarity was interpreted as follows: a 97 to 100% match to a GenBank entry was considered to be within the same species, a 93 to 96% match was considered to be within the same genus, and an 86 to 92% match was considered to be a related organism (61). However, note that there can be significant genetic and physiological differences between two bacteria that have 99% similarity in their 16S rRNA genes (31), so all estimates of diversity are actually minimum approximations. The Ribosomal Database Project (release 10, update 3) Classifier was employed to assign the 16S rRNA genes to appropriate taxonomic groups (67). Rarefaction analyses were conducted using FastGroup II (73). Alignments were constructed using ClustalX (66). Maximum-likelihood trees were created and bootstrapped using the PAUP software package (64).

**Nucleotide sequence accession numbers.** The *Lophelia* clone library sequences are archived in GenBank under accession numbers FJ041383 to FJ041890. Bands A to J from the DGGE analyses are archived under accession numbers FJ196875 to FJ196884.

## RESULTS

Five *Lophelia pertusa* colonies (each) were sampled at VK906/862 and VK826 (Fig. 1 and Table 1). Microbial communities from samples preserved at depth were compared by DG-DGGE to those from the same coral colony brought up alive to determine if environmental factors (e.g., light and pressure) were affecting the microbial diversity of the samples. Clone libraries were prepared from six individual *Lophelia* colonies and used to describe the bacterial diversity associated with this coral at both these Gulf of Mexico sites.

**Preserving samples at depth.** Two samples each (one preserved at depth and one retrieved alive) from five *Lophelia* colonies at site VK826 were compared by DG-DGGE (Fig. 2). This method tends to detect the most abundant members of a bacterial community (40) and therefore was used to screen for major changes resulting from the type of sampling. While the overall patterns are similar, there are a few obvious differences between samples preserved at depth and those brought up

without preservation (i.e., samples that spent up to 3 h under conditions of changing light and pressure) (Fig. 2). For example, band D is present in a fixed sample (Fig. 2, lane 1) but is absent in the companion live sample (Fig. 2, lane 2), as indicated in the figure. Lane 9 (fixed) looks similar to the other samples, but lane 10 (live sample from the same coral colony) has many additional bands (Fig. 2, bands E to J). The band patterns were examined by cluster analysis (see Fig. S2 in the supplemental material). In all but one case (lanes 9 and 10), the samples clustered in pairs based on the colony sampled rather than grouping by treatment (live versus fixed at depth).

**Bacterial diversity.** Several of the prominent bands on the DG-DGGE gel (Fig. 2) were excised, the DNA extracted, and sequences obtained. The top band in the first doublet (Fig. 2, band A) was consistent across lanes 1 to 5 and 7 to 9 and was 100% identical to a gammaproteobacterium clone (G02\_CR02\_full; GenBank accession no. AM911391) from the red color morph of *Lophelia* from a Norwegian fjord (42). The lower band in the first doublet (Fig. 2, band B) also was consistent across lanes 1 to 9 and was also 100% identical to a mycoplasmal sequence (D11\_CW02\_full; also known as “*Candidatus Mycoplasma corallicola*”; GenBank accession no. AM911412) from a white Norwegian *Lophelia* coral (41, 42). The next-closest similarity (89%) was to *Mycoplasma phocirhinis* (35). All the bands in lane 10 (Fig. 2, bands E to J) and one band in several other lanes (Fig. 2, band C) were nearly identical to each other and represented alphaproteobacteria in the family *Rhodobacteraceae*. These bands were equally similar (95 to 96%) to that of clone 2WB\_40 (GenBank accession no. EU574666), which was recently isolated from a microbial mat at a hydrothermal vent site (16) and to clone SGUS450 (GenBank accession no. FJ202844) from a piece of the coral *Montastraea faveolata* that had been kept in an aquarium for 23 days (63). The conspicuous single band in lane 1 (Fig. 2, band D) that is lost in the corresponding live sample belonged to a gammaproteobacterium with 99% sequence similarity to a bacterial sequence (clone s1uc48; GenBank accession no. DQ416306) associated with the shallow-water coral *Oculina patagonica* in the Mediterranean (36).

Seven clone libraries were screened from six individual coral colonies (six samples preserved at depth and one of the coordinating live samples from that set). Three corals were from site VK906/862 (library prefix 4746) and three from VK826 (library prefix 4753). Sequences of less than 500 bp were discarded, resulting in the analysis of 508 total clones, comprised of 273 unique operational taxonomic units (OTUs) (based on a 97%-similarity cutoff). Many of these 273 OTUs were combined into groups when the alignments were trimmed for consistency, so the number of OTUs visible on the phylogenetic trees is reduced. Fifteen percent of the bacterial 16S clones were novel, having less than 97% similarity to previously described sequences. Fifty-two percent of the clones were unknown mycoplasmal members of the *Tenericutes*, 33% were members of the *Gammaproteobacteria*, 9% were members of the *Alphaproteobacteria*, and 3% were members of the *Bacteroidetes* (Fig. 3) (see Table S1 in the supplemental material). Interestingly, BLAST searches resulted in all 16 sequences classified as *Bacteroidetes* being most similar to species of mycoplasma. The remaining 3% consisted of members of the *Betaproteobacteria* (two clones), *Deltaproteobacteria* (one clone), *Epsilonproteobacteria* (two clones), *Cyanobacteria* (one

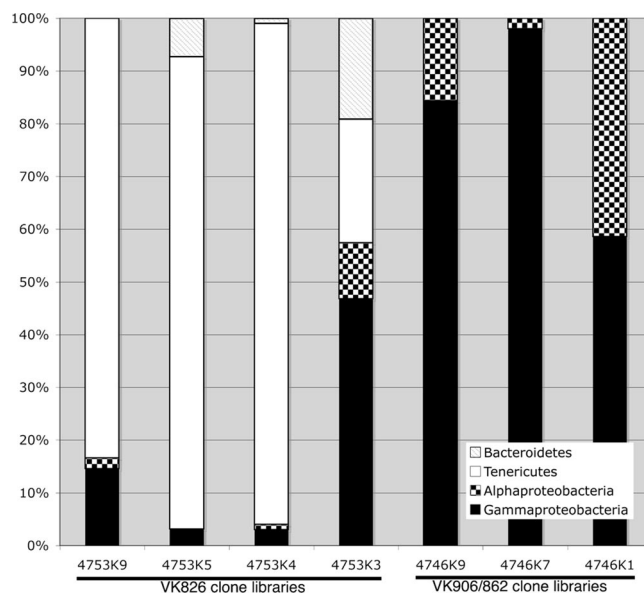


FIG. 3. Composition of the clone libraries showing only taxa comprising at least 15% of sequences in at least one clone library. Libraries 4746K1 (74 clones), 4746K7 (43 clones), 4746K9 (52 clones), 4753K3 (50 clones), 4753K5 (87 clones), and 4753K9 (96 clones) are from samples preserved at depth. Library 4753K4 (106 clones) is from a nonpreserved sample of the same coral as library 4753K3.

clone), *Planctomycetes* (one clone), *Firmicutes* (one clone), *Lentisphaerae* (three clones), and *Spirochaetes* (one clone) (see Table S1 in the supplemental material). The three clones classified as *Lentisphaerae* were 91 to 94% similar to *Planctomycetes* by BLAST search and clustered with *Planctomycete* sequences (Fig. 4).

Many of the clones had high similarity to previously described sequences. Eighty-six percent of the clones had highest BLAST similarity matches to other coral-associated bacteria. Of those, 79% were most similar to one of two sequences from Norwegian *Lophelia* (42): either the thiotrophic symbiont sequence (G02\_CR02\_full; GenBank accession no. AM911391) (Fig. 4) or the mycoplasmal sequence (D11\_CW02\_full; also known as “*Candidatus Mycoplasma corallicola*”; GenBank accession no. AM911412) (Fig. 5). The “thiotrophic cluster” (Fig. 4) also includes another clone exclusive to red *Lophelia* corals from Norway (E12\_CR02\_full; GenBank accession no. AM911378) and closely related shallow-water coral sequences from *Turbinaria mesenterina* (S. E. Godwin, J. Borneman, E. Bent and L. Pereg-Gerk, unpublished). This coral-associated group is similar to sulfur-oxidizing bacterial symbionts of methane seep clam species (e.g., *Calyptogena* spp. and *Bathymodiolus* species) (Fig. 4). These sulfur oxidizers were the numerically dominant clone type in two libraries (4746K7 and 4746K9) and were present in all the *Lophelia* bacterial 16S rRNA gene libraries. Of the remaining 7%, 32 clones were 92 to 95% similar to clone FungiaD34clE09 from the Philippines (26), two clones were 95% similar to clone 1HP1-A14, which was associated with *Turbinaria mesenterina* (Godwin et al., unpublished), and two clones showed similarity to sequences derived from *Montastraea faveolata* (clones SHFS395 and SHFH617) (63). Although not included as “coral associated”

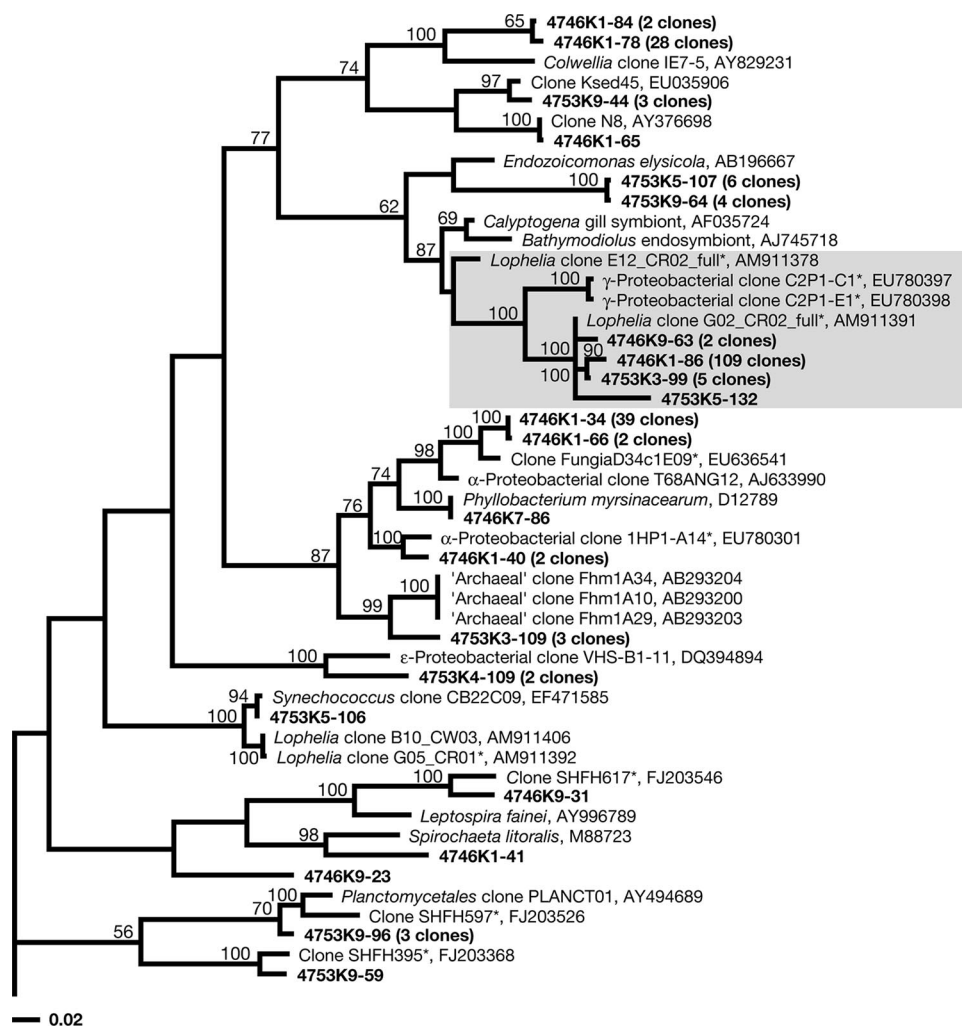


FIG. 4. Maximum-likelihood tree of *Lophelia*-associated sequences (excluding the mycoplasma sequences) created with PAUP using the general time-reversible model with estimated base frequencies site specific by codon position distribution. Percentages of 1,000 bootstrap resamplings are shown at the nodes (values  $\geq 50\%$  only). The tree was rooted using *Methanococcus voltae* U38488 as an outgroup (not shown). The scale bar denotes 0.02 nucleotide substitutions per alignment position. The gray box indicates coral-associated sequences most similar to those of thiotrophic symbionts. Anchor sequences labeled with an asterisk are coral associated. The tree is based on an alignment made with ClustalX, containing 534 characters per sequence.

in the strictest sense, there were also three clones that were 97% similar to Ksed45, a sequence from sediments near a deep-water coral reef in Norway (S. Jensen, J. D. Neufeld, N.-K. Birkeland, M. Hovland, and J. C. Murrell, unpublished data).

The 14% of clones without sequence similarity to other coral-associated sequences were an eclectic mix. There was a single clone with 99% similarity to a bacterial symbiont of the pink sugarcane mealybug (21) and another clone with 99% similarity to four *Synechococcus* clones from Chesapeake Bay (15). There were 30 clones with 97 to 98% similarity to uncultured *Colwellia* strains (68, 69). A small number of clones were similar to other metazoan-associated bacteria. Nine alphaproteobacterial clones were 90 to 92% similar to a squid symbiont (45), two epsilonproteobacterial clones were 92 to 93% similar to a bacterial sequence from deep-sea shrimp guts (74), one gammaproteobacterial clone was 84% similar to a clone from a sponge (D. Sipkema, unpublished), and two gammapro-

teobacterial clones were 83% similar to a clone from a colonial ascidian in the Mediterranean (39). One *Lophelia*-associated sequence belongs to the *Spirochaetes*, but it is less than 90% similar to anything in the current genetic database. Members of the *Spirochaetes* have been detected previously in deep-sea bamboo corals (44), but this is the first description of one associated with *Lophelia*. Two clones showed most sequence similarity to three putative archaeal sequences derived from a hydrothermal vent chimney (H. Oba, K. Inoue, S. Kato, and A. Yamagishi, unpublished). However, phylogenetic analyses place this entire group of sequences firmly in the *Alphaproteobacteria* (Fig. 4).

The rarefaction curve generated from the 508 bacterial clones derived from all Gulf of Mexico *Lophelia pertusa* samples was compared to a rarefaction curve based on 534 clones from deep-sea bamboo corals (44) and was nearly identical (see Fig. S3 in the supplemental material). Compared to rarefaction curves of the red and white color morphs of *Lophelia*

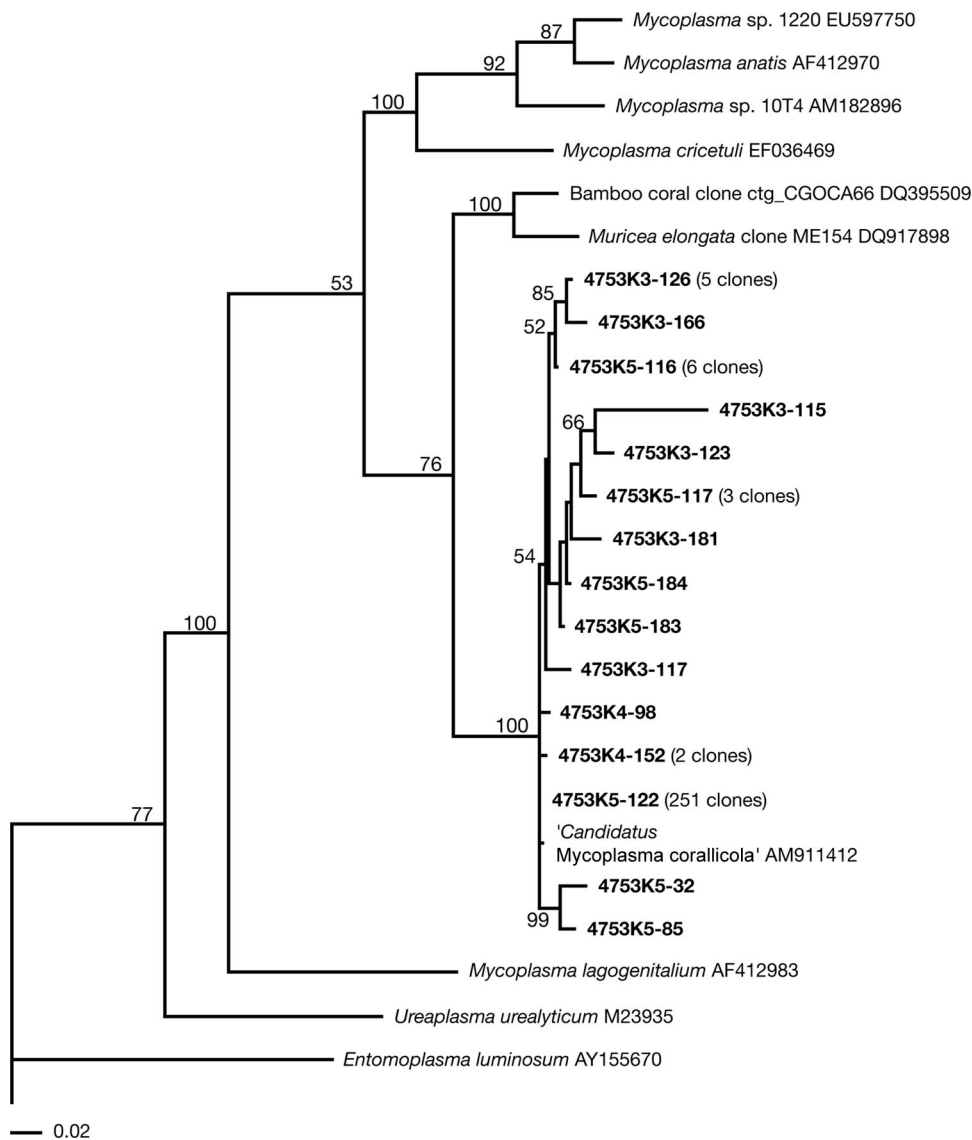


FIG. 5. Maximum-likelihood tree of the *Lophelia*-associated *Mycoplasma* sequences created with PAUP using the general time-reversible model with estimated base frequencies site specific by codon position distribution. Percentages of 1,000 bootstrap resamplings are shown at the nodes (values  $\geq 50\%$  only). The tree was rooted using *Clostridium innocuum* M23732 as an outgroup (not shown). The scale bar denotes 0.02 nucleotide substitutions per alignment position. The tree is based on an alignment made with ClustalX, containing 533 characters per sequence.

corals sampled in Norway (42), Gulf of Mexico *Lophelia* displays greater diversity: 79 ribotypes in the first 100 clones versus ~25 (Norwegian white) and ~40 (Norwegian red).

The clone libraries show a definite difference in coral-associated bacterial communities between sites VK906/862 and VK826. The three libraries from VK906/862 (4746K1, 4746K7, and 4746K9) are dominated by the *Proteobacteria*, whereas the four libraries from VK826 (4753K3, 4753K4, 4753K5, and 4753K9) are shifted to the mycoplasmal *Tenericutes* (Fig. 3, Fig. 5).

### DISCUSSION

Unlike collection of shallow-water corals, a situation in which there is only a minimal time lag or temperature differ-

ence between collection and preservation, sampling of cold-water corals in the Gulf of Mexico presents some challenges. There was an approximately 20°C difference between bottom and surface waters, and the Mississippi River plume affects these sites, generating a lens of lower-salinity water at the surface. These factors were taken into consideration, and sampling gear was designed to protect the samples from these gradients. The question was still raised as to whether other environmental gradients, such as light and pressure, could affect the coral-associated microbial community during the approximately 4 h of a submersible dive. The visual differences between DG-DGGE patterns of samples from the same coral colony (one brought up alive and one preserved at depth immediately after collection) (Fig. 2) suggested that there might be some effect; however, cluster analysis (see Fig. S2 in the

supplemental material) indicated that in all but one case (samples 4753K9 and 4753K10), the colony of origin was a more important factor than preservation. This indicates that in most cases, the bacterial communities are not being dramatically altered by exposure to gradients in light and pressure. This is corroborated by Neulinger et al.'s terminal restriction fragment length polymorphism data (42); they collected samples with nets and found statistically significant differences between community patterns at stations 1 (depth, 54 m) and 2 (depth, 264 m) but not between stations 1 and 3 (depth, 240 m), indicating that the differences were not due to depth (or the effects of collecting samples at different depths). This is the first study of deep-sea coral microbial ecology that has preserved samples at depth to investigate this issue. Additionally, this is the only deep-sea coral study to use insulated, individually sealed containers to minimize cross-contamination and thermal shock. Previous submersible and ROV studies that have examined bacterial diversity associated with deep-sea corals in general and *Lophelia* in particular did not take these precautions (27, 42, 44, 72).

With only two geographic sites, it is impossible to determine if the bacterial community at one site is "normal" and the other site "altered." Recent studies have revealed conserved microbial communities within species of shallow-water corals (54, 55) and sponges (28). However, *Lophelia pertusa* may have a more dynamic bacterial community across its wide geographic range. The similarity of "fingerprint" patterns among the five Gulf of Mexico *Lophelia* samples analyzed by DGGE (Fig. 2) and the similarity of dominant clone types in the clone libraries at VK906/862 versus VK826 (Fig. 3) indicate some conservation of microbial communities within geographic sites. This corroborates findings from *Lophelia* corals sampled on the Mingulay reefs off Scotland. DGGE patterns of coral-associated bacterial communities were different at different geographic sites but were more similar between proximal sites (27). However, there was concern that sampling techniques may have influenced the degree of geographic variability detected (27). There was no overlap between the 12 bacterial 16S rRNA gene sequences from Mediterranean *Lophelia* (72) and the bacterial sequences from this study (Fig. 4 and 5) or a recent study of *Lophelia* in a Norwegian fjord (42). While this is most likely due to methodological differences (e.g., different extraction, amplification, and sequencing protocols), it could indicate biogeographical differences in *Lophelia*-associated bacterial communities. Ultimately, it will be valuable to have comparable *Lophelia* samples from other areas in the Gulf of Mexico, such as Green Canyon off the coast of Louisiana (58) and the West Florida slope (48), to determine more clearly what constitutes the "typical" bacterial community of *Lophelia* in the Gulf of Mexico and how much of an effect regional geography has on the structure of those communities.

There were definite similarities between the bacterial communities of Gulf of Mexico *Lophelia* and Norwegian *Lophelia* corals (42). Both were dominated by *Proteobacteria* (with strong representation of the family *Rhodobacteraceae*) and included mycoplasmas (*Tenericutes*), *Bacteroidetes*, and *Planctomycetes* and a small number of cyanobacterial clones. However, the Norwegian samples had a greater representation of alphaproteobacteria whereas the Gulf samples had more gammaproteobacteria. The Norwegian bacterial communities also

included *Actinobacteria*, *Verrucomicrobia*, and TM7, which were not detected in Gulf of Mexico samples. Interestingly, the Norwegian samples contained two color morphs of *Lophelia*, red and white, which had different bacterial communities (42). The bacterial diversity of Gulf of Mexico *Lophelia* (only the white color morph was encountered at the Viosca Knoll sites) is more like that of the Norwegian red color morph, which exhibited greater diversity than the white.

One key commonality is the abundance of members of the *Tenericutes* associated with *Lophelia* corals at site VK826 (Fig. 5). *Mycoplasma* spp. in the class *Mollicutes* have traditionally been listed under the phylum *Firmicutes*, but the division *Tenericutes* (8) has been raised to a phylum to separate the wall-less *Mollicutes* from the gram-positive genera *Bacillus*, *Clostridium*, and *Staphylococcus* within the *Firmicutes* (2). Many mycoplasmas are pathogens that parasitize animal host cells for nutrients they lack due to their small genome size and limited biosynthetic capabilities (47, 56). Mycoplasmas have been detected in both bleached and healthy *Muricea elongata*, a shallow-water gorgonian (L. K. Ranzer, P. F. Restrepo, and R. G. Kerr, unpublished). At least one novel *Mycoplasma* sp. was associated with deep-sea bamboo corals (44). No *Tenericutes* were found in association with Mediterranean *Lophelia* (72), but "*Candidatus* *Mycoplasma corallicola*" has been found in Norwegian *Lophelia* (41, 42). Fluorescent in situ hybridization was used to determine that "*Candidatus* *Mycoplasma corallicola*" in Norwegian *Lophelia* positions itself exclusively around nematocyst batteries, oriented toward the outside of the coral (41). The positioning of the mycoplasmas suggested that they were benefiting from "sloppy feeding" on the coral's part and that they were likely to be harmless commensals. Phylogenetic comparison of all coral-associated mycoplasmas demonstrates that they cluster together (Fig. 5) and that "*Candidatus* *Mycoplasma corallicola*" shares 99% similarity with the largest group of mycoplasma clones from Gulf of Mexico *Lophelia* (Fig. 5). The presence of "*Candidatus* *Mycoplasma corallicola*" in coral colonies on both sides of the Atlantic indicates that this bacterium is a *Lophelia*-specific symbiont.

The question then becomes, why are there not also mycoplasmas at VK906/862? Their absence at VK906/862 might be linked to a physical factor, e.g., temperature variability. Bacterial communities have been shown to differ between diseased/stressed corals and healthy specimens (43), making it possible that the dramatic difference between the *Lophelia*-associated bacterial communities at VK906/862 and VK826 (Fig. 3) was due to thermal stress at the shallower site. Perhaps the difference in bacterial communities between the two sites was linked to a factor intrinsic to *Lophelia*, e.g., the corals at VK906/862 were more genetically differentiated than other populations sampled in the Gulf, including VK826 (C. L. Morrison, personal communication), based on analysis of microsatellite markers. Differences in genetics, nutrition, or both could lead to biochemical or metabolic differences which would select for different microbial biota. *Lophelia* colonies at VK826 were more heavily calcified than those at VK906/862, and based on sediment trap experiments, VK826 had higher numbers of plankton (12). The differences may also be influenced by the limitations of clone libraries; Neulinger et al. found mycoplasma sequences in the clone libraries for white *Lophelia* only (42) but showed by fluorescent in situ hybridiza-

tion that mycoplasmas were present in both white and red color morphs (41).

Molecular analyses of shallow-water corals have uncovered individual species-specific associations between corals and bacteria: an alphaproteobacterium (PA1) has been found consistently associated with *Porites astreoides*, a closely related bacterium, PF1, with *Porites furcata* (55), and a *Silicibacter* sp. with *Montastraea franksi* (54). Bacteria such as these are inferred to have a particular symbiotic association with the coral. In addition to showing a mycoplasmal associate 99% similar to the one described for Norwegian *Lophelia* (Fig. 5), this study has also detected a cluster of closely related gammaproteobacteria (Fig. 4) in all six clone libraries, dominant in two. This cluster is also closely related (95 to 98% similarity) to a sequence (G02\_CR02\_full; GenBank accession no. AM911391) found in association with the red color morph of *Lophelia* in Norway (42), again indicating a specific association that transcends geographic distance. Nearly one-quarter of the 16S rRNA gene clones associated with Gulf *Lophelia* belong to this cluster, with similarity to thiotrophic symbionts of seep-clam species (Fig. 4).

*Lophelia*-associated bacterial sequences also showed similarity to other types of symbionts, including bacterial associates of an insect, squid, sponge, deep-sea shrimp, and sea squirt (ascidian). One of the bands excised from the DGGE (Fig. 2, band D) produced a sequence that was 99% similar to a bacterial sequence that had been extracted from a shallow-water coral, *Oculina patagonica*, in the Mediterranean (36). Clone matches to *Oculina patagonica* have also been found in *Lophelia* from Norwegian fjords (42). Coupled with the 7% of the clone libraries that was most similar to tropical coral-associated sequences, this provides evidence that there may be scleractinian coral-specific bacterial associations, regardless of depth, corroborating similar findings by Neulinger et al. (42).

It seems counterintuitive to find photosynthetic autotrophs like cyanobacteria associated with corals that live in total darkness. *Synechococcus* sequences were found associated with both white and red color morphs of Norwegian *Lophelia* (42), and this study detected a *Synechococcus* sequence associated with *Lophelia* at VK826 (Fig. 5). Cyanobacteria are known to be associated with photosynthetic corals and are believed to benefit the corals through nitrogen fixation (37, 38, 60, 71). A group of globally distributed oceanic cyanobacteria has been shown to lack the genes for oxygenic photosynthesis and carbon fixation (75). Therefore, it is possible that facultatively heterotrophic species are associated with deep-sea corals in spite of the dark environment.

Three *Lophelia*-associated clones were classified into a recently described phylum of marine bacteria, *Lentisphaerae* (10). Taxonomically, this group is regarded as a sister phylum to *Verrucomicrobia* (46). Recent studies have discovered *Lentisphaerae* phylum clones associated with marine sponges (33, 65). This study is the first report of this bacterial phylum being associated with a coral. However, the three clones phylogenetically cluster with *Planctomycetes* (Fig. 4) and were most similar to a planctomycete clone derived from salmon gills (7) and several coral-associated sequences from healthy *Montastraea faveolata* (63).

Whereas spirochetes have been isolated from seawater and

sediments in the vicinity of *Lophelia* (42), this is the first report of spirochete-like bacteria associated with the coral. The single clone (4746K1-41; GenBank accession no. FJ041427) is 87% similar to uncultured *Spirochete* clones but does cluster with a defined spirochete species (Fig. 4). The *Spirochetes* are typically free-living anaerobes but are known to be symbionts of gutless worms commonly found in coral reef sediments (5). Further, these gutless worms host multiple symbionts, mixing the *Spirochetes* with the thiotrophic *Gammaproteobacteria* (5), as is the case with *Lophelia*.

Although archaea are known to be associated with shallow-water tropical corals (4, 32, 70), deep and cold waters (3, 11, 25, 29), and marine sediment (34, 72), no archaea have yet been found associated with *Lophelia pertusa* (72). Two clones from Gulf of Mexico *Lophelia* were most similar to three sequences claimed to be archaeal clones from a hydrothermal vent. However, there is no associated publication to clarify why the submitters were convinced the sequences were archaeal. When included in a phylogenetic tree, both the *Lophelia* sequences and the putative archaeal sequences fall within the *Alphaproteobacteria* (Fig. 4).

The majority of known coral species occur in deep water (52); however, there are very few publications about bacteria associated with deep-sea corals. This paper documents the first survey of *Lophelia*-associated microbes in the Gulf of Mexico and is the first deep-water study in which specimens were collected in insulated, individual containers to minimize sample contamination and thermal stress. The results revealed several novel bacterial associates and two *Lophelia*-specific groups: a group of *Mycoplasma* spp. and a cluster of symbiont-like thiotrophs.

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Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. government.

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