Bacteria Associated with Mucus and Tissues of the Coral *Oculina patagonica* in Summer and Winter

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The relative abundance of bacteria in the mucus and crushed tissue of the Mediterranean coral *Oculina patagonica* was determined by analyses of the 16S rRNA genes of isolated colonies and from a 16S rRNA clone library of extracted DNA. By SYBR gold staining, the numbers of bacteria in mucus and tissue samples were 6.2 x 10^7 and 8.3 x 10^9/cm^2 of coral surface, respectively, 99.8% of which failed to produce colonies on Marine Agar. From analysis of mucus DNA, the most-abundant bacterium was *Vibrio splendidus*, representing 68% and 50% of the clones from the winter and summer, respectively. After removal of mucus from coral by centrifugation, analyses of DNA from the crushed tissue revealed a large diversity of bacteria, with *Vibrio* species representing less than 5% of the clones. The most-abundant cultivable bacteria were a *Pseudomonas* sp. (8 to 14%) and two different α-proteobacteria (6 to 18%). Out of a total 1,088 16S rRNA genes sequenced, 400 different operational taxonomic units were identified (>99.5% identity). Of these, 295 were novel (<99% identical to any sequences in the GenBank database). This study provides a comprehensive database for future examinations of changes in the bacterial community during bleaching events.

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Corals consist of symbioses among the coral animal, endosymbiotic algae commonly referred to as zooxanthellae, and a large and diverse community of associated bacteria. In recent years, there have been numerous investigations of the different clones of zooxanthellae present in corals and their possible role in the sensitivity of corals to temperature-induced bleaching (3). Early studies using culturing methods demonstrated the importance of coral-associated bacteria in coral nutrition (24) and in response to stress (8). It was also shown that the bacterial community changes when corals are bleached (19) or exhibit white-band disease (17).

The first culture-independent studies of coral-associated bacteria demonstrated very high diversity, including a majority of novel species, and host specificity; i.e., similar bacterial populations were found on the same coral species that were geographically separated, and different populations were found on different coral species (20, 21). Coral-associated archaea have also been demonstrated by culture-independent methods (28).

Recently, Bourne and Munn (7) used both culture-based and culture-independent techniques to investigate the microbial community of the reef-building coral *Pocillopora damicornis*. They found that the majority of clones obtained from coral tissue slurry libraries were γ-proteobacteria, whereas the coral mucus was dominated by α-proteobacteria. Many of the retrieved clone sequences were conserved between coral colonies, further supporting the hypothesis of specific bacterium-coral associations.

We report here a study of the bacterial community of the Mediterranean coral *Oculina patagonica* during the summer and winter. One of the unusual characteristics of *O. patagonica* is its ability to grow under highly variable conditions of temperature and salinity. Off the coast of Israel, seawater temperatures vary from 31°C (summer maximum) to 16°C (winter minimum). In shallow pools, where coral colonies are found, temperatures can reach 41°C and salinity can fluctuate between 2.8 and 5%. The ease of obtaining genetically identical coral fragments from the same colony and maintaining them in aquaria makes *O. patagonica* an excellent experimental coral model system (10).

The *Vibrio shilonii*-O. *patagonica* model system of bacterial bleaching of corals has been studied extensively (22). Each summer, at least for the last 12 years, approximately 70% of the coral colonies have shown bleaching. The causative agent of this bleaching is *V. shilonii* (13, 14). This pathogen is chemotactic to the coral mucus (4), binds to a β-galactoside-containing receptor in the coral mucus (27), and then penetrates the epidermal layer of the coral, where it multiplies, reaching >10^9 bacteria per cm³ of tissue (6). *V. shilonii* produces a proline-rich toxin (YPYVAPPVPV) which inhibits photosynthesis of the intracellular zooxanthellae (5). In the winter, when seawater temperatures drop below 20°C, *V. shilonii* cannot survive in the coral and the coral recovers (11). Information on the bacterial community of *O. patagonica* should be useful in understanding its sensitivity to infection and its ability to survive bleaching during the summer months and recover during the winter.

**MATERIALS AND METHODS**

**Sample collection and mucus extraction.** The site sampled in this study was Sedot Yam, in the Mediterranean off the coast of Israel. Three healthy *O. patagonica* fragments were removed from separate colonies during September 2004 (summer, 27°C) and March 2005 (winter, 17°C) with a hammer and chisel. The colonies were located at 1- to 3-m depths and were separated by distances of at least 3 m. The samples were immediately placed in plastic bags underwater and were transported back to the laboratory in a cooler (in <2 h). The corals were then broken into pieces measuring ca. 2 by 2 cm and placed in 50-ml centrifuge tubes. The coral samples were centrifuged for 3 min at 2,675 x g to remove the mucus. After centrifugation, the coral samples were removed and placed in new 50-ml centrifuge tubes, which were allowed to stay for 60 min at...
TABLE 1. Most-abundant bacterial clusters associated with *O. patagonica* in winter 2005a

<table>
<thead>
<tr>
<th>Cluster or parameter</th>
<th>Class</th>
<th>Closest match in Blast (% identity)</th>
<th>No. of clones</th>
<th>Abundance (% of)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>1</td>
<td>γ-Proteobacteria</td>
<td><em>V. splendidus</em> AJ874360 (100)</td>
<td>72</td>
<td>28.5</td>
</tr>
<tr>
<td>2</td>
<td>γ-Proteobacteria</td>
<td><em>V. splendidus</em> biovar II AB038303 (99)</td>
<td>11</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>γ-Proteobacteria</td>
<td>Sulfur-oxidizing bacterium AF170424 (97)</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>γ-Proteobacteria</td>
<td><em>V. splendidus</em> strain B17 AY046955 (100)</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>γ-Proteobacteria</td>
<td>Uncultured γ-proteobacterium AF424056 (98)</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>γ-Proteobacteria</td>
<td><em>V. litoralis</em> DQ097524 (97)</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>7</td>
<td>γ-Proteobacteria</td>
<td><em>V. superbus</em> AF519806 (100)</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>α-Proteobacteria</td>
<td><em>α-Proteobacterium</em> AJ890008 (97)</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>Nitrospira</td>
<td>Uncultured isolate AF93 AJ373422 (96)</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>Unclassified proteobacteria</td>
<td>Uncultured isolate T26-22 AF332308 (90)</td>
<td>3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Total in the 10 clusters: 112

a Based on analysis of 253 16S rRNA clones.
b A cluster is defined here as 16S rRNA clones that have at least 99.5% sequence identity.
c The consensus sequence of the cluster was used in searching the GenBank database.
d Mucus bacteria were separated from tissue bacteria by centrifugation (see Materials and Methods).

4°C. The coral samples were then centrifuged again to remove the newly secreted mucus, at 2,000 × g for 3 min. After the second centrifugation, the coral pieces were crushed in seawater with a mortar and pestle. After allowing the CaCO3 skeleton to settle, the supernatant was removed and is referred to as crushed tissue.

Isolation and enumeration of bacteria. Tenfold serial dilutions of coral mucus and crushed coral pieces in filtered seawater were prepared and plated on Marine Agar 2216 (Difco, Detroit, MI). All plates were incubated at 30°C for a week. Colonies that appeared at the highest dilutions were restreaked and subsequently used for DNA isolation. Total bacterial counts were performed on mucus and crushed coral pieces with SYBR gold (Molecular Probes Inc.) according to the SYBR green protocol (16).

DNA extraction and PCR amplification of 16S rRNA genes. Coral mucus, crushed tissue, and seawater samples were centrifuged at 9,300 × g for 15 min, and the pellets were used for DNA extraction. DNA was extracted from the pellets with the UltraClean Soil DNA kit (MoBio, Carlsbad, CA). For culturable bacteria, colony DNA was extracted with the GenElute bacterial genomic DNA kit (Sigma). Primers 8F and 1492R (15) were used for amplification of the 16S rRNA genes from isolated bacterial genomic DNA and environmentally extracted DNA from seawater, coral mucus, and crushed tissue samples. 16S rRNA genes were amplified in a 50-μl reaction mixture consisting of 5 μl of 10× buffer, 1 μl of a 2.5 mM total deoxynucleoside triphosphate mixture, each primer at 5 μM, 10 ng of template DNA, and 2.5 U of Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan). Amplification conditions for the PCR included an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 10 min. Reaction products were checked for size and purity on 1% agarose gel. PCR products were cleaned with ExoSAP-IT (USB Corp.). DNA sequencing was performed by the chain termination method in an ABI Prism (model 377, version 2.1.1) automated sequencer. Primers used for the sequencing reaction were complementary to the conserved regions of the 16S rRNA genes.

Clonal library construction. Amplified DNA from water, mucus, and crushed coral samples was ligated into the pGEM-T Easy vector by the protocol of the manufacturer (Promega Corp., Madison, WI). The ligated vector and insert were transformed into competent *Escherichia coli* DH5α cells. Each clone was amplified by colony PCR with M13 forward and reverse primers. Amplification conditions for the colony PCR included an initial denaturation step of 95°C for 4.5 min, followed by 30 cycles of 94°C for 0.5 min, 59°C for 0.5 min, and 72°C for 1 min and a final extension step of 72°C for 10 min. Reaction products were checked for size and purity on 1% agarose gel. Purification and sequencing were done according to the protocol described above.

Sequence analysis. Sequences were aligned with ClustalX (26), and a DNA distance matrix was created with BioEdit. Sequences that had >99.5% identity were clustered together with DOTUR (23). Sequences shorter than 350 bp were removed from the alignment. BLASTN (2) (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) was then used to characterize each sequence cluster.

Nucleotide sequence accession numbers. The nucleotide sequence data for all of the clones and colonies reported in this paper will appear in the GenBank nucleotide sequence database under accession numbers DQ416209 to DQ416683.

RESULTS

Bacteria associated with *O. patagonica* during the winter. Three coral fragments were obtained from different *O. patagonica* colonies in the winter of 2005. Before isolation of DNA, the coral mucus from each fragment was separated from the remainder of the coral by centrifugation. Microscopic observations indicated that the coral tissue was not damaged by the centrifugation procedure. The average volume of mucus ± the standard error was 0.21 ± 0.02 ml/cm² of coral surface. Thus, the average thickness of the mucus was 1 mm. Following SYBR gold staining, the average numbers of bacteria in the coral mucus and tissue (coral minus mucus) were (6.2 ± 4.2) × 10⁷ and (8.3 ± 1.6) × 10⁷/cm², respectively. Thus, the concentration of bacteria in the mucus was 3.0 × 10⁹/ml.

Table 1 summarizes the data on the most-abundant 16S rRNA gene sequences obtained from the clone libraries of *O. patagonica* winter DNA. Clones which matched known *Vibrio* species dominated the mucus, representing more than 68% of the clones sequenced. Clusters 1, 2, and 4, which closely matched *Vibrio splendida* strains, made up 63% of the mucus clones. Despite their higher abundance in the mucus, *Vibrio* species made up only a small percentage of the tissue bacteria, less than 5%. In general, the tissue bacteria showed much greater diversity than the mucus bacteria. Of the 253 sequences analyzed, 112 or 44% appeared in 1 of the 10 clusters shown in Table 1. The remaining sequences appeared only once (127 times) or twice (7 times). Thus, the number of different bacterial operational taxonomic units (OTUs) sequenced was 144 (by using >99.5% identity to define an OTU). Of these 144, 129 were novel (by using <99% identity to sequences in the GenBank database to define a novel OTU).

The same winter coral mucus and tissue fractions that were used to prepare the clone libraries were also used to determine culturable bacteria (Table 2). The viable counts of the mucus and tissue fractions were (1.6 ± 0.8) × 10⁷ and (9.6 ± 4.2) × 10⁷/ml. 

Downloaded from www.halifax.org at Albert R. Mann Library on April 13, 2010.
10^7 CFU/cm² of coral surface, respectively. These values are ca. 0.2% of the total count. The 20 most-abundant clusters made up 76% of the total culturable bacteria (Table 2). The most-abundant culturable bacteria were very different from the nonculturable bacteria from the same source. For example, none of the eight most-abundant clusters shown in Table 2 were found even once in the 253 clones sequenced from the coral mucus. The colonies not shown in Table 2 consisted of 39 sequences that appeared only once and 12 sequences that appeared twice. Thus, the total number of different culturable clusters was 71 out of the 263 colonies analyzed. Of the 71, 39 were novel species (>99% identity to bacterial 16S rRNA gene sequences in the GenBank database).

**Bacteria associated with O. patagonica during the summer.**

Three coral fragments were obtained from different O. patagonica colonies in the summer of 2004. As described above, the coral mucus was separated from the coral tissue before analyses. The volume of mucus was 0.19 ± 0.01 ml/cm². Thus, the

### Table 2. Most-abundant cultured bacterial species associated with O. patagonica in winter 2005^a^

<table>
<thead>
<tr>
<th>Cluster^b or parameter</th>
<th>Class</th>
<th>Closest match in Blast (% identity)^c</th>
<th>No. of colonies</th>
<th>Abundance (% of)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
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<tr>
<td>1</td>
<td>γ-Proteobacteria</td>
<td>Pseudomonas sp. AF500211 (99)</td>
<td>21</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>γ-Proteobacteria</td>
<td>V. splendidus AY246955 (99)</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>γ-Proteobacteria</td>
<td>V. litoralis DQ97524 (99)</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>γ-Proteobacteria</td>
<td>V. superstes AF519806 (99)</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>γ-Proteobacteria</td>
<td>V. splendidus biowar II AB038030 (99)</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>γ-Proteobacteria</td>
<td>Vibrio sp. AF546335 (100)</td>
<td>3</td>
<td>1.1</td>
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<tr>
<td>7</td>
<td>γ-Proteobacteria</td>
<td>Uncultured isolate PDA-OTU4 AY700602 (99)</td>
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<td>1.1</td>
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<tr>
<td>8</td>
<td>γ-Proteobacteria</td>
<td>Photobacterium sp. AY576761 (99)</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AB026194 (99)</td>
<td>48</td>
<td>18.3</td>
</tr>
<tr>
<td>10</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AY584527 (99)</td>
<td>33</td>
<td>12.5</td>
</tr>
<tr>
<td>11</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AY562560 (100)</td>
<td>21</td>
<td>8.0</td>
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<td>12</td>
<td>α-Proteobacteria</td>
<td>Vibrio sp. DQ146982 (99)</td>
<td>19</td>
<td>7.2</td>
</tr>
<tr>
<td>13</td>
<td>α-Proteobacteria</td>
<td>Pseudovibrio sp. AB112827 (99)</td>
<td>7</td>
<td>2.7</td>
</tr>
<tr>
<td>14</td>
<td>α-Proteobacteria</td>
<td>Uncultured isolate 20BSU27 AJ863180 (99)</td>
<td>6</td>
<td>2.3</td>
</tr>
<tr>
<td>15</td>
<td>α-Proteobacteria</td>
<td>Roseobacter sp. AY394680 (99)</td>
<td>5</td>
<td>1.9</td>
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<tr>
<td>16</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AY576758 (98)</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>17</td>
<td>α-Proteobacteria</td>
<td>Uncultured isolate 4^C^-N-26d-5 AF432337 (99)</td>
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<td>α-Proteobacteria</td>
<td>α-Proteobacterium AB015896 (99)</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>19</td>
<td>Flavobacteria</td>
<td>Muricauda aquimarina AY445076 (97)</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>Sphingobacteria</td>
<td>Bacterial isolate DG1129 AY258133 (97)</td>
<td>4</td>
<td>1.5</td>
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</table>

Total in 20 clusters: 200

<table>
<thead>
<tr>
<th>Abundance (% of)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
</tr>
<tr>
<td>76</td>
</tr>
</tbody>
</table>

---

^a Based on analysis of 263 colonies.
^b A cluster is defined here as colonies that have at least 99.5% sequence identity.
^c The consensus sequence of the cluster was used in searching the GenBank database.
^d Mucus bacteria were separated from tissue bacteria by centrifugation (see Materials and Methods).
average thickness of the mucus was 0.95 mm. The total number of bacteria in the mucus was $(4.9 \pm 2.1) \times 10^7/\text{cm}^2$, corresponding to a concentration of bacteria in the mucus of $2.5 \times 10^9/\text{ml}$.

Table 3 summarizes the data on the bacterial clusters obtained from summer coral mucus and tissue DNAs. Cluster 1, which yielded a consensus sequence identical to that of cluster 1 in the winter analysis (Table 1) and the *V. splendidus* sequence with accession no. AJ874360 in the GenBank database, represented 35% of the 432 clones sequenced. Again, the *Vibrio* bacteria were located primarily in the mucus (54% of the clones) and not in the tissue (2.9% of the clones). The 10 most-abundant clusters shown in Table 3 represented 64% of the total number of clones sequenced, 81% of the clones obtained from mucus, and 30% of the clones obtained from tissue. The sequenced clones that do not appear in Table 3 consist of 5 clusters of 4 clones each, 5 clusters of 3 clones, 16 clusters of 2 clones, and 87 clones which appear only once. Thus, the total number of different culturable species was 77 out of the 147 colonies analyzed. Of these 77, 35 were novel species (<99% identity to bacterial 16S rRNA gene sequences in the GenBank database).

**Seawater controls.** Seawater surrounding the coral samples yielded a total count of $(5.2 \pm 2.1) \times 10^9/\text{ml}$ and a viable count of $(2.2 \pm 0.9) \times 10^9/\text{ml}$. These values are approximately 1% of the concentration of bacteria in the mucus samples. The most-abundant and culturable species belonged to the genera *Vibrio* (30.3%) and *Alteromonas* (10.9%). The eight most-abundant cultivable seawater bacteria represented less than 1% of the bacteria in any of the coral samples. The most-abundant bac-

**TABLE 4. Most-abundant cultured clusters associated with* O. patagonica* in summer 2004**

<table>
<thead>
<tr>
<th>Cluster or parameter</th>
<th>Class</th>
<th>Closest match in Blast (% identity)</th>
<th>No. of colonies</th>
<th>Abundance (% of)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>1</td>
<td>γ-Proteobacteria</td>
<td><em>Pseudomonas</em> sp. AF500211 (99)</td>
<td>21</td>
<td>14.3</td>
</tr>
<tr>
<td>2</td>
<td>γ-Proteobacteria</td>
<td><em>Aegirinovorans albus</em> AB76560 (99)</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>γ-Proteobacteria</td>
<td><em>V. splendidus</em> 620972 (100)</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>γ-Proteobacteria</td>
<td><em>Thalassomonas viridis</em> AJ294747 (99)</td>
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<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>γ-Proteobacteria</td>
<td><em>Photobacterium ganghvense</em> 606487 (97)</td>
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<td>1.4</td>
</tr>
<tr>
<td>6</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AB026194 (99)</td>
<td>18</td>
<td>12.2</td>
</tr>
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<td>7</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium 562560 (100)</td>
<td>8</td>
<td>5.4</td>
</tr>
<tr>
<td>8</td>
<td>α-Proteobacteria</td>
<td>Uncultured isolate 711116 (93)</td>
<td>7</td>
<td>4.8</td>
</tr>
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<td>9</td>
<td>α-Proteobacteria</td>
<td><em>Vibrio</em> sp. DQ14982 (99)</td>
<td>4</td>
<td>2.7</td>
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<td>10</td>
<td>α-Proteobacteria</td>
<td><em>Pseudovibrio</em> sp. AB112827 (99)</td>
<td>4</td>
<td>2.7</td>
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<tr>
<td>11</td>
<td>α-Proteobacteria</td>
<td><em>Rhodobacteraceae</em> sp. 442178 (95)</td>
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<td>α-Proteobacteria</td>
<td>Uncultured isolate AJ633968 (100)</td>
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<td>1.4</td>
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<td>13</td>
<td>α-Proteobacteria</td>
<td>Uncultured isolate AM162572 (90)</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td>14</td>
<td>α-Proteobacteria</td>
<td><em>Sphingomonas</em> family member 671615 (96)</td>
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<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>α-Proteobacteria</td>
<td>Marine bacterium Y41 AF388307 (98)</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td>16</td>
<td>α-Proteobacteria</td>
<td>α-Proteobacterium AJ24796 (97)</td>
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<td>17</td>
<td>α-Proteobacteria</td>
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<td>1.4</td>
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<tr>
<td>18</td>
<td>Flavobacteria</td>
<td><em>Cytophaga</em> sp. AB073566 (91)</td>
<td>2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Total in 18 clusters: 88 colonies, 60 in mucus, and 59 in tissue.

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From the summer coral samples was present in the 10 most-abundant clusters obtained by the DNA cloning technique. In addition to the 18 clusters (88 colonies) shown in Table 4, 59 colonies appeared only once. Thus, the total number of different culturable species was 77 out of the 147 colonies analyzed. Of these, 35 were novel species (<99% identity to bacterial 16S rRNA gene sequences in the GenBank database).

**TABLE 5. Distribution of most-abundant species between different coral fragments**

<table>
<thead>
<tr>
<th>Season</th>
<th>Closest match in Blast</th>
<th>Abundance (%)</th>
<th>Coral 1</th>
<th>Coral 2</th>
<th>Coral 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td><em>V. splendidus</em> AJ874360</td>
<td>35.4</td>
<td>39.1</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>V. splendidus</em> biovar II</td>
<td>3.8</td>
<td>2.3</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Prosthecochloris</em> sp.</td>
<td>13.3</td>
<td>8.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td><em>V. splendidus</em> AJ874360</td>
<td>35.7</td>
<td>34.1</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Prosthecochloris</em> sp.</td>
<td>14.0</td>
<td>0.7</td>
<td>2.7</td>
<td></td>
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<tr>
<td></td>
<td><em>Cytophaga</em> sp.</td>
<td>0</td>
<td>0</td>
<td>12.9</td>
<td></td>
</tr>
</tbody>
</table>

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*a* Dissection of clusters 1 and 2 (Table 1).

*b* Dissection of clusters 1, 4, 5, and 8 (Table 3).
teria in seawater determined by DNA analysis belonged to the genus *Synechococcus* (20.0%). The nine most-abundant bacterial clusters from clone libraries of seawater represented less than 1% of the bacteria in any of the coral samples.

**Distribution of the most-abundant clusters between coral fragments.** The data summarized in Tables 1 and 3 combine values from six different *O. patagonica* fragments, three from the winter and three from the summer. Table 5 presents the results for the most-abundant clusters obtained from the separate DNA libraries of each coral fragment. The most-abundant cluster, which has 16S rRNA gene sequences very close to the *V. splendidus* sequence with accession no. AJ874360, was present in higher abundance in all summer and winter fragments. The other abundant clusters showed large differences from summer to winter and from one fragment to another. For example, the cluster corresponding to the *V. splendidus* sequence with accession no. AB038030 represented 2.3 to 6.8% of the clones from the winter coral samples but was not detected in the summer coral samples, and the cluster corresponding to the δ-proteobacterium sequence with accession no. AY750148 represented 12.9% of the clones of summer coral 3 but was not detected in any of the other coral fragments.

**DISCUSSION**

As has been shown with several other corals (18, 20), the mucus layer of *O. patagonica* contains a high concentration of bacteria, 3 × 10^8/ml, 99.8% of which failed to produce colonies on MB agar under aerobic conditions. It should be pointed out that during the day, coral mucus and tissue are supersaturated with oxygen because of endosymbiotic algal photosynthesis (12), so that the presence of strict anaerobes is unlikely. In addition to the mucus bacteria, an even larger amount of bacteria was associated with the coral tissue, even after removal of the mucus by two centrifugation steps. It is not known if these latter bacteria are bound firmly to the outside of the coral or are present in coral tissues. What is clear is that the tissue bacteria are qualitatively very different from the mucus bacteria. No previous study of coral bacteria (e.g., reference 21) has examined the culturable and nonculturable bacteria, the mucus- and tissue-associated bacteria, and the bacteria present in different seasons. Since we now know the 16S rRNA gene sequences of the most-abundant tissue and mucus bacteria, it should be possible to use fluorescence in situ hybridization technology to locate the position of these bacteria on the coral surface or tissues.

By molecular methods, the most-abundant bacterium in *O. patagonica* mucus was *V. splendidus*, representing 68% and 50% of the clones from the winter and summer, respectively. At present, the species *V. splendidus* contains a rather broad group of bacteria (25). In this study, two clusters of *V. splendidus* were found. Each cluster consisted of sequences that showed more than 99.5% identity to the consensus sequence. However, the identity of the two consensus sequences to each other was only 98.7%. Since a few representatives of each of the *V. splendidus* clusters were present in the culturable bacteria, we are now able to study the physiological and biochemical properties of these bacteria in order to classify them more precisely and to attempt to understand the reason for their high concentration in mucus. Little is known about the role of coral mucus bacteria in coral health and disease (18).

Most studies of the bacterial populations of environmental samples, including the present one, have found very different data by culture and molecular methods. Since the number of colonies formed from the *O. patagonica* mucus and tissue samples represented only 0.2% of the total number of bacteria estimated from SYBR gold staining of the same samples, it is clear that the culturable bacteria are not representative of the total community. However, there is also no proof that the molecular methods, which employed DNA isolation, PCR with one set of primers, and cloning, did not introduce bias into the results. Furthermore, it has been reported (9) that *O. patagonica* contains a large number of filamentous, autofluorescent prokaryotes (probably cyanobacteria) tightly bound to the CaCO3 skeleton. The techniques used in this study would not reveal these bacteria because the skeleton was removed before analysis of the tissue.

The difference between the seawater temperatures at the times of sampling in the winter and summer was 10°C. By the molecular method, the only cluster that was present among the 10 most-abundant clusters in both seasons was the major *V. splendidus* cluster. By the culture method, the three most-abundant clusters were present in both seasons. We suggest that by changing the resident bacterial population, a coral holobiont may adapt more easily to different environmental conditions. The data presented in this report provide a comprehensive baseline for examining changes that occur when coral is stressed, e.g., during bleaching.

In considering the diversity of bacteria associated with *O. patagonica*, out of a total of 1,088 16S rRNA genes sequenced, ca. 400 were different OTUs. This is based on the arbitrary assumption that a bacterium or a group of bacteria that show less than 99.5% identity in their 16S rRNA gene sequence to any other bacterium represent a separate OTU. It has been reported that bacteria have multiple 16S rRNA operons and that the sequence divergence of these operons can exceed 5% (1). If this were the case with bacteria associated with *O. patagonica*, then our arbitrary assumption that a 16S rRNA gene sequence that differs from all other sequences by more than 0.5% is a separate OTU would lead to an overestimation of diversity. On the other hand, since most of the 400 OTUs appeared only once, it is clear that many more clones would have to be sequenced to estimate the full diversity of the coral bacteria. By using less than 99% identity to any 16S rRNA gene sequence in the GenBank database to define a novel species, 295 out of the 1,088 were novel OTUs. High bacterial diversity and novel species have been previously been reported for Caribbean corals (20). The bacterial communities associated with bleached *O. patagonica* and those found in dark caves (azooxanthellates) are under investigation.

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**REFERENCES**