**Research Article**

**Diversity of pufM genes, involved in aerobic anoxygenic photosynthesis, in the bacterial communities associated with colonial ascidians**

Manuel Martínez-García¹, Marta Díaz-Valdés² & Josefa Antón¹

¹División de Microbiología and Instituto Multidisciplinar para el Estudio del Medio Ramón Margalef, Universidad de Alicante, Alicante, Spain; and ²Centre d’Estudis Avançats de Blanes, CSIC, Girona, Spain

**Correspondence:** Manuel Martínez-García, Bigelow Laboratory for Ocean Sciences, McKown Point Road, PO Box 475, Boothbay Harbor, ME 04535-0475, USA. Tel.: +1 207 633 39650; fax: +1 207 633 39569; e-mail: m.martinez@ua.es

Received 9 September 2009; revised 29 October 2009; accepted 9 November 2009. Final version published online 9 December 2009.

DOI:10.1111/j.1574-6941.2009.00816.x

Editor: Michael Wagner

Keywords

pufM; ascidian; bacteria; association.

**Abstract**

Ascidians are invertebrate filter feeders widely distributed in benthic marine environments. A total of 14 different ascidian species were collected from the Western Mediterranean and their bacterial communities were analyzed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene. Results showed that ascidian tissues harbored Bacteria belonging to Gamma- and Alphaproteobacteria classes, some of them phylogenetically related to known aerobic anoxygenic phototrophs (AAPs), such as Roseobacter sp. In addition, hierarchical cluster analysis of DGGE patterns showed a large variability in the bacterial diversity among the different ascidians analyzed, which indicates that they would harbor different bacterial communities. Furthermore, pufM genes, involved in aerobic anoxygenic photosynthesis in marine and freshwater systems, were widely detected within the ascidians analyzed, because nine out of 14 species had pufM genes inside their tissues. The pufM gene was only detected in those specimens that inhabited shallow waters ( < 77 m of depth). Most pufM gene sequences were very closely related to that of uncultured marine bacteria. Thus, our results suggest that the association of ascidians with bacteria related to AAPs could be a general phenomenon and that ascidian-associated microbiota could use the light that penetrates through the tunic tissue as an energy source.

**Introduction**

Many marine invertebrates are symbiotically associated with microorganisms from the three domains (Munn, 2004). Studies based on 16S rRNA gene and environmental genomic and metagenomic analyses have revealed some insights into the metabolic and physiological properties of microorganisms associated with oligochaetes, corals, sponges, benthic animals from hydrothermal vents and squids (Duperron et al., 2005; Woyke et al., 2006; Grozdanov & Hentschel, 2007; Taylor et al., 2007; Wegley et al., 2007). However, the vast array of marine symbioses remains largely unexplored (McFall-Ngai & Rubi, 2000).

Ascidians are invertebrate filter feeders (phylum Chordata, class Ascidacea) that inhabit benthic marine environments, usually in shallow waters attached to substratum such as rocks and shells. Ascidians are characterized by the presence of a tunic, a differentiated tissue composed of structural proteins, polysaccharides and different eukaryotic cell lines (Endean, 1961; Smith, 1970; Daele, 1991; Dolce-mascolo & Gianguzza, 2004). There are three kinds of ascidians according to their biology and ecology: solitary, social and colonial. Colonial ascidians consist of many small individuals, called zooids, surrounded by a common tunic forming a colony from 1 cm up to > 30 cm in diameter (Fig. 1). Some ascidians store in their tunic metabolites with cytotoxic and antitumor properties (Torres et al., 2002; Prado et al., 2004; Martínez-García et al., 2007a) that, in some cases, are produced by the bacteria associated with the animal (Schmidt et al., 2005).

Recently, our group showed that the colonial ascidian Cystodytes dellechiajei harbored inside its tunic a conserved and stable community that included ammonia-oxidizing Crenarchaeota and Alphaproteobacteria phylogenetically
related to aerobic anoxygenic phototrophs (AAPs) (Martínez-García et al., 2007b, 2008). Microscopy and molecular analyses indicated that part of the Alphaproteobacteria community was vertically transmitted to the next generation. In addition, the pufM gene, encoding the M subunit of the reaction center of aerobic anoxygenic photosynthesis (Béja et al., 2002), was being expressed inside the colony and larval tunics. AAPs are phototrophic bacteria that require oxygen for their growth and for bacteriochlorophyll a (Bchl a) synthesis. Given their capability of harvesting light energy, they represent an important fraction of the bacterioplankton in freshwater and marine environments (Suzuki et al., 2001, 2004; Béja et al., 2002; Yutin & Béja, 2005; Yutin et al., 2005, 2007; Cottrell et al., 2006; Jiao et al., 2007; Masin et al., 2008). Recent results have shown that AAP populations in marine systems are complex, diverse and dynamic, playing a particular role in the global oceanic carbon and energy cycles. Metagenomic analysis of water samples from Atlantic and Pacific Oceans showed that the composition of AAP assemblages changes between different oceanic regions, with specific bacterial assemblages adapted to open ocean or coastal areas (Yutin et al., 2007). Furthermore, a global ocean study carried out by Jiao et al. (2007) showed that the maximum AAP diversity was found in oligotrophic areas, whereas AAP abundance followed the opposite trend, positively correlated to the concentration of chlorophyll a. On the other hand, Bchl a measurements suggested that AAPs contribute up to 5% to the surface ocean photosynthetic electron transport (Kolber et al., 2001; Goericke, 2002; Jiao et al., 2007).

Because the ascidian C. dellechiajei harbored in its tunic bacteria related to AAPs that were expressing the pufM gene (Martínez-García et al., 2007b), we decided to extend the study to other Mediterranean ascidians in order to ascertain whether the association with bacteria related to AAPs is a common trend in colonial ascidians. Our results indicate that Gamma- and Alphaproteobacteria were the dominant bacterial fraction of ascidian-associated microbiota in the Western Mediterranean. In addition, data show that some Alphaproteobacteria detected in that tissue were phylogenetically related to known AAP bacteria. Furthermore, the pufM gene could be detected inside colonial ascidians that inhabited in the Western Mediterranean, indicating that the association of ascidians with bacteria related to AAPs could be a general phenomenon.

Materials and methods

Sample collection

Samples were obtained from one bottom trawl survey in the Western Mediterranean Sea (May and June 2007), on board the R/V Cornide de Saavedra. Trawl fishing grounds along the Spanish continental shelf and upper slope were surveyed. The gear used was a GOC73 trawl net. Its average horizontal and vertical openings were 16.4 and 2.8 m, respectively. A total of 164 hauls were made between 40 and 755 m depth. Samples were identified, counted, weighed and frozen at −20 °C.

Nucleic acid extraction

Colony samples were rinsed twice in filtered sterile seawater. Before DNA extractions, zooids were removed from the colonies under sterile conditions. Because zooids are the active filter feeder part of the animal, they were removed carefully to avoid contamination with marine planktonic microorganisms. Tunic sample pieces (1–1.3 g) were cut with a sterile razor blade and extracted only from the inner parts of tunic tissue, avoiding those tissue areas close to external surfaces of the colony, where epibionts occur (see Fig. 1). Then, selected samples were rinsed two times in filtered sterile seawater. Nucleic acid extractions from the colony tunic tissue were performed as described previously (Martínez-García et al., 2007b). The DNA pellet was air-dried at room temperature and resuspended in 100 µl ultrapure sterile water. Fifty microliters of DNA of each sample was purified using the Gene-clean® Spin Kit (MP Biomedicals, LLC, France) according to the manufacturer’s protocol.

PCR amplification of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE)

PCR amplifications of partial 16S rRNA genes from colony tunic samples were performed using primers 341-GC and
907R (Schäfer et al., 2001). Cycling conditions were as follows: 5 min at 94 °C, 1 min at 65 °C, 3 min at 72 °C, 10 cycles of 1 min at 94 °C, 1 min at 64 °C, 3 min at 72 °C, 23 cycles of 1 min at 94 °C, 1 min at 55 °C and 3 min at 72 °C, with a final extension step of 72 °C for 10 min in a PTC-100 thermal cycler (Peltier-Effect Cycling). Each 50-μl reaction contained 2.25 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 200 μM of each dNTP, 1.75 U Taq I DNA polymerase (Invitrogen), 0.4 μM of each primer and 100 ng of DNA. PCR product concentration and quality were determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 300–400 ng of PCR amplified products from each sample were separated by DGGE using a denaturing gradient of 40–65% (100% was defined as 7 M urea and 40% deionized formamide) as described previously (Martínez-García et al., 2007b). DGGE gels were stained with 10 μL SYBR Green (Molecular Probes) in 0.5 × Tris-acetic acid-EDTA (TAE) buffer, for 25 min, rinsed with 1 × TAE buffer for 20 min, visualized and photographed with Typhoon 9410 (Amersham Biosciences, UK). DGGE bands were excised and incubated overnight at 4 °C in 20 μL ultrapure sterile water for 16 h. The eluent was used as template DNA for reamplification under the same conditions. PCR products from each band were again analyzed by DGGE in order to check for the presence of a single band, and sequenced using an ABI PRISM TM310 DNA sequencer (Applied Biosystems).

**DGGE data analysis**

A matrix was constructed taking into account the presence or absence of the individual DGGE bands. This matrix was used to calculate a distance matrix using the Jaccard index (shared characters/total characters) using the software sproc® 12.0 software (SPSS Inc., Chicago, IL). Finally, a similarity dendrogram of the DGGE patterns of the colony tunics was computed.

**PCR amplification of pufM genes and clone libraries**

PCR amplification of the pufM gene was carried out using pufM forward and pufM reverse primers (Béja et al., 2002) with the cycling conditions described previously (Martínez-García et al., 2007b). Each 25-μl reaction contained 2.25 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 200 μM of each dNTP, 1.5 U Taq I DNA polymerase (Invitrogen), 0.4 μM of each primer and 200 ng of DNA. Twenty microliters of PCR products were purified from a 2.5% MetaPhor agarose gel (EMC BioProducts, Rockland, ME) in 1 × TAE buffer using the GFX PCR DNA and gel band purification kit (Amersham Biosciences). Purified PCR products were cloned using TOPO TA Cloning (Invitrogen) according to the manufacturer’s instructions. Twenty-five clones from each clone library were sequenced from plasmid preparation (Wizard Plus SV Minipreps DNA purification system, Promega) using the primer M13 forward (5’-ACGTTGTAAAACGACGGCC-3’).

**Phylogenetic analysis of 16S rRNA and pufM genes**

16S rRNA gene sequences were analyzed using MALLARD 1.02 and PINTAIL 1.1 softwares (Ashelford et al., 2005, 2006) in order to detect chimeras and other artifacts. Phylogenetic analysis was carried out using the software program ARB as described in detail (Ludwig et al., 2004; Martínez-García et al., 2007b). Nucleotide gene sequences of pufM genes obtained in this study and the closest pufM gene sequences from GenBank were aligned using CLUSTALX 2.0.12. Then, phylogenetic analyses were conducted in MEGA 4.1 software. The evolutionary history was inferred using the maximum parsimony method (1000 bootstrap replications), and a consensus tree was calculated from the most parsimonious trees. Branches corresponding to partitions reproduced in < 50% trees were collapsed.

**Rarefaction analysis and estimation of pufM gene diversity**

The rarefaction curve analysis of the pufM sequence was computed using ANALYTIC RAREFACTION 1.4 (http://www.uga.edu/strata/software/) based on the algorithm described previously (Raup, 1975). The coverage percentage was defined as \([1 - (n/N)] \times 100\), where \(n\) is the number of singleton clones and \(N\) is the total number of sequences. Estimation of pufM sequence richness and diversity in colonial ascidians were calculated using the PAST software version 1.82b (Hammer et al., 2001) and BIODIVERSITY PRO version 2.0 (http://www.sams.ac.uk/).

**Nucleotide sequence accession numbers**

The 16S rRNA and pufM gene sequences were submitted to the NCBI database and assigned accession numbers FJ659118–FJ659158 and FJ669169–FJ669214, respectively.

**Results**

**DGGE of 16S rRNA genes from ascidian tunic tissues**

Twenty-eight specimens of colonial ascidians (detailed in Table 1) were collected from 14 different sampling points in the Balearic and Alboran Seas of the Western Mediterranean, ranging in depth from 40 to 508 m (Fig. 2). DGGE analysis was used to study and compare the bacterial 16S rRNA genes present in the different tunic tissues of the collected ascidians (Fig. 3). As shown, the number of DGGE bands for the
A single DGGE band in different ascidians was very diverse, obtaining from only one colonial ascidians were obtained. Depth and geographic coordinates are shown in Fig. 2.

Table 1. Details of ascidian samples collected from the trawl survey carried out in the Western Mediterranean

<table>
<thead>
<tr>
<th><em>Ascidian species</em> (abbreviation)</th>
<th>Suborder/family</th>
<th>Collection location (sampling point/latitude, longitude)</th>
<th>Depth (m)</th>
<th><em>Sample</em></th>
<th><em>puM</em> gene detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclinidae species (Up)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°31′N, 3°31′E</td>
<td>76</td>
<td>Up1</td>
<td><em>NA</em></td>
</tr>
<tr>
<td>Aplidium proliferum (Ap)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>37°55′N, 0°34′E</td>
<td>75</td>
<td>Ap1</td>
<td>–</td>
</tr>
<tr>
<td>Cystodytes dellechiae (Cd)</td>
<td>Aplousobranchia/Polyctenidae</td>
<td>39°31′N, 2°39′E</td>
<td>508</td>
<td>Cd1</td>
<td>+</td>
</tr>
<tr>
<td>Aplidium densum (Ad)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°31′N, 2°39′E</td>
<td>62</td>
<td>Ad1</td>
<td>+</td>
</tr>
<tr>
<td>Polyclinella azemai (Pb)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°31′N, 3°31′E</td>
<td>76</td>
<td>Pb1</td>
<td>+</td>
</tr>
<tr>
<td>Synoicum blochmanni (Sb)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°19′N, 2°44′E</td>
<td>60</td>
<td>Sb1</td>
<td>+</td>
</tr>
<tr>
<td>Aplidium elegans (Ae)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°16′N, 2°26′E</td>
<td>358</td>
<td>Sb2</td>
<td>–</td>
</tr>
<tr>
<td>Aplidium haouarianum (Ah)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°13′N, 2°39′E</td>
<td>62</td>
<td>Ae1</td>
<td>+</td>
</tr>
<tr>
<td>Aplidium nordmanni (An)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°31′N, 3°31′E</td>
<td>76</td>
<td>Ah2</td>
<td>–</td>
</tr>
<tr>
<td>Aplidium conicum (Ac)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>37°43′N, 0°31′E</td>
<td>113</td>
<td>Ac1</td>
<td>–</td>
</tr>
<tr>
<td>Botryllus schlosseri (Bs)</td>
<td>Stolidobranchia/Styeliidae</td>
<td>39°19′N, 2°44′E</td>
<td>60</td>
<td>Bs1</td>
<td>+</td>
</tr>
<tr>
<td>Pseudodistoma cymusense (Pc)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°18′N, 3°16′E</td>
<td>93</td>
<td>Pc1</td>
<td>–</td>
</tr>
<tr>
<td>Pseudodistoma obscurum (Po)</td>
<td>Aplousobranchia/Polyclinidae</td>
<td>39°31′N, 3°31′E</td>
<td>76</td>
<td>Pc2</td>
<td>–</td>
</tr>
<tr>
<td>Díazona violacea (Dv)</td>
<td>Phlebobranchia/Cionidae</td>
<td>38°41′N, 0°17′E</td>
<td>69</td>
<td>Dv1</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>41°32′N, 2°41′E</td>
<td>71</td>
<td>Dv2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>41°28′N, 2°43′E</td>
<td>106</td>
<td>Dv3</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*Species were identified using morphological characteristics.
1*Abbreviation and number of samples used in Figs 3 and 4.
2See the Mediterranean Sea map (Fig. 2) to locate the sampling point.
3Undetermined specimen at the genus and the species level. Not analyzed for *puM* gene detection.

Fig. 2. Mediterranean Sea map showing the sampling points where colonial ascidians were obtained. Depth and geographic coordinates are indicated in the map.

DGGE patterns from different samples, a similarity dendrogram was calculated (Fig. 4). As shown, in general, specimens belonging to the same ascidian species, with the exception of *Polyclinella azemai* and *A. elegans*, were grouped together forming separate clusters, thus indicating that their bacterial DGGE patterns were more similar to each other than to the resulting patterns of other ascidian species. In addition, different ascidian species shared some DGGE bands (from 20% to 50%), which would indicate that the same bacterial phylotype could be present in different ascidians. On the other hand, in general, high intraspecific heterogeneity in the DGGE pattern was detected within each ascidian species.

Sequencing indicated that most DGGE bands belonged to *Gamma- and Alphaproteobacteria* (56% and 31%, respectively, of all DGGE bands), although members of *Bacteroidetes, Deltaproteobacteria* and *Actinobacteria* were also found (Fig. 5). All DGGE bands shared by most of the samples corresponded to *Alpha*- or *Gammaproteobacteria*. As shown...
in Fig. 5, most of the Gammaproteobacteria sequences (18 out of 23) were phylogenetically related (with identities from 94% to 99.9%) to bacteria associated with marine invertebrates such as the coral *Oculina patagonica*, the ascidian *C. dellechiajei* and the sponges *Halichona gellius*, *Tethya aurantium*, *Axinella verucosa* and *Halichondria okadai*. The rest of the DGGE sequences belonging to Gammaproteobacteria had their best matches with *Serratia marcescens* (95.3% similarity), *Pseudoalteromonas* sp. (97.1% similarity) and *Psychrobacter* sp. (95–97.1% similarity). Strains of these two last bacterial genera have also been isolated from the tunic tissues of other colonial ascidians (Romanenko et al., 2002; Mai-Pochnow et al., 2004; Martinez-Garcia et al., 2007a, b).

Most (58%) of the DGGE bands belonging to the Alphaproteobacteria class were related to uncultured Alphaproteobacteria symbiotically associated with invertebrate marine animals: the sponges *H. gellius* and *Aplysina aerophoba*, the marine star *Ophiopholis aculeata* and the oligochaete *Olavius loisae*. Three DGGE band sequences were related to the widely distributed AAP *Roseobacter* sp. (3, 25, 42, 43 and 44), and an additional DGGE sequence was related to *Ahrensia* sp., a dinoflagellate-associated alphaproteobacterium that harbors *pufLM* genes in a plasmid (Pradella et al., 2004).

DGGE sequences related to Bacteroidetes, more specifically to *Tenacibaculum* sp., were only recovered from *C. dellechiajei*, *Aplidium conicum* and *Pseudodistoma cyrnusense*. Finally, two sequences were found in the tunic tissue, one belonging to Deltaproteobacteria, and the other one to Actinobacteria, which were related to uncultured marine bacteria.

**pufM gene analysis and diversity inside the tunic tissue of colonial ascidians**

The *pufM* gene could not be amplified from any specimen of *Synoicum blochmanni*, *Aplidium nordmanni*, *P. cyrnusense*...
and *Aplidium proliferum*. However, for the rest of the species, the *pufM* gene could be amplified from the tunic tissue of the specimens that inhabited at depth < 77 m, while those collected from deeper points (samples Ac1, Dv3 and Ad2) did not yield amplification products (see Table 1). A clone library of the *pufM* gene was constructed with every PCR product and a minimum of 25 clones per clone library were sequenced, yielding a total of 250 *pufM* sequences. A total of 24 different gene sequences were detected from the colonial ascidians (Table 2). It is remarkable that all the sequences, except clones 30-28 and 30-38, displayed their best match in GenBank with uncultured microorganisms, most of them with the *pufM* genes from uncultured bacteria from marine and brackish environments. These environmental sequences were found at depths < 200 m, and some of them from the surface of the Atlantic and Pacific Oceans. The rest of the sequences obtained in this study (34%) had their best match in GenBank with uncultured microorganisms, most of them with the *pufM* genes from uncultured bacteria from marine and brackish environments. These environmental sequences were found at depths < 200 m, and some of them from the surface of the Atlantic and Pacific Oceans. The rest of the sequences obtained in this study (34%) had their best match with *pufM* gene of bacteria from freshwater environments, such as the Soap and Chaohu Lakes, located in Washington and China, respectively. In addition, two sequences were related to the AAPs, *Roseobacter denitrificans* strain OCH114 and the alphaproteobacterium *Rhodobacteraceae bacterium* strain BS110, which have been widely detected in the Mediterranean Sea (Oz *et al.*, 2005). Overall, the *pufM* gene similarities found ranged between 82% and 98%. Maximum parsimony analysis was performed in order to ascertain the phylogenetic position of *pufM* gene sequences from the ascidians. As shown in the phylogenetic tree (Fig. 6), all the sequences, except the clone 20-11, were clustered into a wide group formed mainly by *pufM* sequences from uncultured marine microorganisms, as well as some sequences from freshwater environments. In addition, *pufM* sequences from cultured AAPs belonged to Alphaproteobacteria and Gammaproteobacteria, such as *Roseobacter* spp. or *Methylobacter* sp., were found inside the cluster composed by the *pufM* sequences from the ascidians.

On the other hand, rarefaction curves were calculated in order to estimate the *pufM* diversity recovered in the clone libraries from colonial ascidians. Results showed that the coverage for the *pufM* clone libraries was close to saturation (77%), which indicates that most of the *pufM* diversity present in that system was recovered. The data obtained were very similar to other studies, like the one carried out in Delaware Bay, where the coverage was 76% (Waidner & Kirchman, 2008).

The Chao 1 and Shannon indexes were used, respectively, to estimate the *pufM* gene richness and diversity inside the tunic tissue. *Cystodytes dellechiajei* and *A. densum* were the species that harbored higher *pufM* gene richness and diversity in their tissues, whereas *P. azemai*, *Pseudodistoma obscurum* and *Botryllus schlosseri* showed the lowest indexes. The Chao 1 values obtained for the ascidian tissues...
Fig. 5. Phylogenetic analysis of bacterial 16S rRNA gene sequences obtained from colonial ascidian tissues. Maximum-likelihood tree based on the 16S rRNA gene sequences reamplified from DGGE bands (520 nucleotide positions). The tree was calculated with the nearly complete reference sequences using TREE-PUZZLE program of the ARB package and then partial sequences from DGGE analyses (indicated in bold and named as ‘DGGE band-number’) were added using the ARB parsimony tool. Quartet puzzling support values for each branch are shown at the branch nodes. The archaeon Halobacterium halobium was used as outgroup. The horizontal scale bar represents the number of substitutions per site.
isms in the ocean. The ecological and biogeochemical role of these microorganisms remains poorly understood, which is critical to ascertain the contribution of photoheterotrophs to the flow of energy and carbon. The ecophysiology of AAPs is controversial, and the contribution of photosynthesis to the respiratory metabolism of ascidians has been the subject of a significant amount of recent research. However, most of these studies have been focused on the symbiotic association between ascidians and unicellular cyanobacteria Prochloron or Prochlorothrix. Although it has been proved that the ascidian tunic harbors bacteria related to AAPs that synthesized pufM gene, it has been involved in aerobic anoxygenic photosynthesis in marine and freshwater environments. Although that gene is also involved in anaerobic anoxygenic photosynthesis, different studies have shown that it is limited to aerobic anoxygenic photosynthesis in marine and freshwater environments. Our results indicate that colonial ascidians from the Western Mediterranean harbor bacteria with the pufM gene, which is known to be involved in aerobic anoxygenic photosynthesis in marine and freshwater environments. Although that gene is also involved in anaerobic anoxygenic photosynthesis, different studies have shown in situ by microsensor measurements the oxic conditions inside the tunic tissue of colonial ascidians (Kühl & Larkum, 2002; Kühl et al., 2005; Martínez-García et al., 2008). In addition, the ascidian tunic is a differentiated tissue formed by different eukaryotic cell lines that displayed fundamental biological functions for the sustainability of the animal and that thus it needs oxygen for its metabolic processes, such as respiration (Hirose et al., 1994; Rottmayr et al., 2001).

There are few reports on the microbiota associated inside the tunic tissue of ascidians besides the characterization of C. dellechiajei (Martínez-García et al., 2007a,b, 2008), and most of them are focused on the symbiotic association among tropical didemnid species with the unicellular cyanobacterium Prochloron sp. (Münchhoff et al., 2007). Although it has been proved that the ascidian C. dellechiajei harbored bacteria related to AAPs that had the pufM gene, it

### Table 2. Summary of the pufM gene clone library results obtained from each ascidian species

<table>
<thead>
<tr>
<th>pufM gene</th>
<th>Clone (GenBank accession number)</th>
<th><em>Best match</em></th>
<th>Ascidian species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
</tr>
<tr>
<td>1</td>
<td>30-28 (FJ669172)</td>
<td>90% <em>Roseobacter denitrificans</em> CP000362</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td>20-18 (FJ669174)</td>
<td>91% Uncultured bacterium from the Chinese Marginal Sea AY731161</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>12-14 (FJ669191)</td>
<td>89% Uncultured bacterium from the Soap Lake EU921777</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>14-16 (FJ669171)</td>
<td>93% Uncultured bacterium from a brackish environment AM162701</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>31-41 (FJ669169)</td>
<td>87% Uncultured bacterium from the Soap Lake EU921777</td>
<td>X</td>
</tr>
<tr>
<td>6</td>
<td>30-34 (FJ669176)</td>
<td>85% Uncultured bacterium from a brackish environment AM162713</td>
<td>X</td>
</tr>
<tr>
<td>7</td>
<td>22-10 (FJ669178)</td>
<td>86% Uncultured bacterium from a brackish environment AM162716</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>12-7 (FJ669179)</td>
<td>87% Uncultured bacterium from the Atlantic Ocean EU862415</td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td>30-2 (FJ669186)</td>
<td>87% Uncultured bacterium from the Atlantic Ocean EU862436</td>
<td>X</td>
</tr>
<tr>
<td>10</td>
<td>32-37 (FJ669187)</td>
<td>90% Uncultured bacterium associated with an ascidian DQ858466</td>
<td>X</td>
</tr>
<tr>
<td>11</td>
<td>22-26 (FJ669188)</td>
<td>85% Uncultured bacterium from the surface of the Atlantic Ocean EU862418</td>
<td>X</td>
</tr>
<tr>
<td>12</td>
<td>31-49 (FJ669191)</td>
<td>82% Uncultured alphaproteobacterium from a marine environment AM944083</td>
<td>X</td>
</tr>
<tr>
<td>13</td>
<td>12-29 (FJ669194)</td>
<td>88% Uncultured bacterium from the Soap Lake EU921777</td>
<td>X</td>
</tr>
<tr>
<td>14</td>
<td>30-38 (FJ669196)</td>
<td>90% <em>Rhodobacteraceae bacterium</em> EU009369</td>
<td>X</td>
</tr>
<tr>
<td>15</td>
<td>30-48 (FJ669199)</td>
<td>87% Uncultured bacterium from the Lake Chaohu FJS9103</td>
<td>X</td>
</tr>
<tr>
<td>16</td>
<td>5-27 (FJ669198)</td>
<td>98% Uncultured bacterium from the Mediterranean Sea DQ080989</td>
<td>X</td>
</tr>
<tr>
<td>17</td>
<td>30-19 (FJ669200)</td>
<td>86% Uncultured bacterium from the Lake Chaohu FJS9105</td>
<td>X</td>
</tr>
<tr>
<td>18</td>
<td>32-4 (FJ669201)</td>
<td>87% Uncultured bacterium from a paddy soil AB510456</td>
<td>X</td>
</tr>
<tr>
<td>19</td>
<td>3-11 (FJ669214)</td>
<td>89% Uncultured bacterium from the Indian Ocean EU862453</td>
<td>X</td>
</tr>
<tr>
<td>20</td>
<td>12-33 (FJ669206)</td>
<td>84% Uncultured bacterium from the surface of the Pacific Ocean EU862459</td>
<td>X</td>
</tr>
<tr>
<td>21</td>
<td>20-33 (FJ669207)</td>
<td>86% Uncultured bacterium from a brackish environment AM162700</td>
<td>X</td>
</tr>
<tr>
<td>22</td>
<td>20-11 (FJ669209)</td>
<td>84% Uncultured bacterium from a brackish Antarctic Lake AY177979</td>
<td>X</td>
</tr>
<tr>
<td>23</td>
<td>30-41 (FJ669211)</td>
<td>88% Uncultured bacterium from the Lake Chaohu FJS9103</td>
<td>X</td>
</tr>
<tr>
<td>24</td>
<td>22-26 (FJ669121)</td>
<td>87% Uncultured bacterium from an eutrophic lake FJS9120</td>
<td>X</td>
</tr>
</tbody>
</table>

*Best match obtained by BLAST at the NCBI database.

†See Table 1 for abbreviations of ascidian species samples.

( < 41.25) were significantly lower than planktonic environments, where for instance, the index can reach up to 65, like in the Delaware estuary (Waidner & Kirchman, 2008). However, the Shannon indexes obtained for the different ascidian species, which ranged from 0.8 to 1.8, were comparable to those obtained for different planktonic environments from the Pacific Ocean and the China Sea, where the Shannon index ranged from 1.059 to 1.808 (Jiao et al., 2007).
was unknown whether that association was a general trend in colonial ascidians. In this sense, because specimens of 14 species belonging to three ascidian families from the Mediterranean Sea have been analyzed, the present study widens substantially the scarce knowledge about pufM gene presence inside tissues of marine invertebrates. Given the procedure carried out to extract the tissue sample (see Materials and methods), the 16S rRNA and pufM genes
found here could only come from the inner parts of the tunic tissue. Thus, our results indicate that the bacterial community associated with the tunic tissue could be more diverse than previously reported, because Gammaproteobacteria, Bacteroidetes, Actinobacteria and Deltaproteobacteria could also form a part of ascidian-associated microbiota.

Most ascidians collected for this study belonged to the Polyclinidae family (see Table 1), which lacks a vascular system linking the zooids and the tunic, and so nutrients filtered by zooids cannot be transported to the tunic tissue (Hirose et al., 1994; Rottmayr et al., 2001; Turon et al., 2005). It has been suggested that the mechanism to sustain the tunic tissue system, which comprises around 95% of the total biomass of these ascidians, could be the phagocytosis and lysis of their own microorganisms (Martínez-García et al., 2007b).

The results obtained on the pufM gene detection from ascidians at different depths are in agreement with Jiao et al. (2007), who showed in a global oceanic survey that the AAPs were confined within a zone that ranged from 50 to 100 m depth, and that below 150–200 m, AAPs did not thrive and were practically undetectable. Because ascidians usually inhabit shallow waters, its detection and collection from deeper areas is difficult and uncommon. Although the pufM gene could not be amplified from any of the samples collected from deeper areas, we cannot rule out its presence within specimens inhabiting below 100 m because the sampling size from these areas was small. However, given the pufM gene function, it seems reasonable to find it more easy in ascidians inhabiting areas where light penetrates.

Both 16S rRNA and pufM gene analyses point to Gammaproteobacteria and Alphaproteobacteria as the dominant bacterial fraction in the tunic tissue of colonial ascidians. These bacteria constitute a group very diverse and widely distributed in the planktonic AAP populations in the Mediterranean Sea and other ocean environments (Béja et al., 2002; Oz et al., 2005; Yutin & Béja, 2005; Yutin et al., 2005, 2007; Jiao et al., 2007). Overall, pufM diversity detected in colonial ascidians somewhat resembles that reported for the Mediterranean and Red Seas and Pacific and Atlantic Oceans (Béja et al., 2002; Oz et al., 2005; Yutin & Béja, 2005; Yutin et al., 2005, 2007), with both Alpha- and Gammaproteobacteria dominating the microbial community. On the other hand, the global oceanic study carried out by Jiao et al. (2007) showed that the maximum AAP diversity was found in oligotrophic areas, like in the Western North Pacific Gyre (Shannon index 2.33). Interestingly, in the present study, the highest pufM diversity Shannon index data were found in species such as C. dellechiajei, inhabiting oligotrophic waters far away from human activity (sampling points 12 and 14). On the other hand, the lowest indexes were detected for those ascidians, such as B. schlosseri and P. obscurum, collected from points very close to industrial and touristic ports (Alicante and Palma de Mallorca ports, sampling points 6, 7, 8 and 9).

The correlations of molecular data obtained from 16S rRNA gene and pufM have to be looked at cautiously. For instance, we have detected sequences related to pufM genes of Methylobacter sp., whereas the corresponding 16S rRNA gene sequences were not retrieved by DGGE. Different factors could be contributing to this finding: the high bacterial diversity found in the tunic, which is not being completely recovered in this study, and the lack of correlation between phylogenetic and physiological markers in many microbial groups. Accordingly, the possibility that some ascidian-associated bacteria not phylogenetically related to AAPs indeed belong to this metabolic group cannot be ruled out. Conversely, bacteria related to AAPs could have a different metabolism. This would explain the fact that some specimens, such as A. nordmanni, did not display pufM genes inside their tissues despite showing sequences apparently related to AAPs (Figs 3 and 5). On the other hand, although data provided by DGGE analysis of 16S rRNA gene could not reflect the entire actual diversity present inside the tunic tissue of ascidians, it has been proven that DGGE analysis for the ascidian C. dellechiajei-associated microbiota was consistent with clone libraries, FISH and catalyzed reporter deposition–FISH results (Martínez-García et al., 2007b, 2008).

In conclusion, this study shows that the association among bacteria and colonial ascidians is widespread in the Western Mediterranean Sea, and that the pufM gene, involved in aerobic anoxygenic photosynthesis, was present in the tunic of most ascidians analyzed that inhabited in shallow waters ( < 77 m depth). To adapt these findings into a more ecological framework, further studies should ascertain whether bacteria living inside the tunics of ascidians are indeed using light as an energy source.

**Acknowledgements**

This project was funded by the grant CGL2006-12714-C02-01 from the Spanish Ministry of Science. We thank Dr Enric Massuti and his group for the sampling, and also the European Project EVAMED from the Spanish Oceanographic Institute.

**References**


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