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Structure and temporal dynamics of the bacterial communities associated to microhabitats of the coral *Oculina patagonica*

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Originality-significance statement: Corals are known to contain a diverse microbiota that plays a paramount role in the physiology and health of holobiont. However, few studies have addressed the variability of bacterial communities within the coral host. This is precisely the central point of our work, which makes it different from previous characterizations of coral microbiotas. We have characterized the bacterial community composition from mucus, tissue and skeleton of the coral *Oculina patagonica*, seasonally and at two locations in the Western Mediterranean Sea, to further understand how environmental conditions and the coral microbiome structure are related.

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Abstract

Corals are known to contain a diverse microbiota that plays a paramount role in the physiology and health of holobiont. However, few studies have addressed the variability of bacterial communities within the coral host. In this study, bacterial community composition from the mucus, tissue and skeleton of the scleractinian coral *Oculina patagonica* were investigated seasonally at two locations in the Western Mediterranean Sea, to further understand how environmental conditions and the coral microbiome structure are related. We used denaturing gradient gel electrophoresis in combination with next-generation sequencing and electron microscopy to characterize the bacterial community. The bacterial communities were significantly different among coral compartments, and coral tissue displayed the greatest changes related to environmental conditions and coral health status. Species belonging to the *Rhodobacteraceae* and *Vibrionaceae* families form part of *O. patagonica* tissues core microbiome and may play significant roles in the nitrogen cycle. Furthermore, sequences related to the coral pathogens, *Vibrio mediterranei* and *Vibrio coralliilyticus*, were detected not only in bleached corals but also in healthy ones, even during cold months. This fact opens a new view onto unveiling the role of pathogens in the development of coral diseases in the future.

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Introduction

Scleractinian corals form a collaborative consortium with a range of different microbial partners: endosymbiotic dinoflagellates, bacteria, archaea, and viruses, which together form the "coral holobiont" (Rohwer *et al*., 2002; Knowlton and Rohwer, 2003). Unicellular dinoflagellate algae from the genus *Symbiodinium* (Trench, 1979), also known as zooxanthellae, are by far the best-understood microbial associate of corals. This mutualistic symbiosis provides dinoflagellate photosynthetic products to the coral host, which comprises up to 95% of coral energy requirements (Muscatine, 1990). Bacterial symbioses are increasingly recognized as integral contributors to the coral holobiont, playing significant roles in coral physiology and health (Rosenberg *et al*., 2007; Wegley *et al*., 2007, Kimes *et al*., 2010; Bourne and Webster, 2013). Therefore, the coral holobiont is a very rich consortium whose components interact in complex ways that are still very poorly understood (Knowlton and Rohwer, 2003; Ainsworth *et al*., 2010; Krediet *et al*., 2013).

Corals have at least three different microhabitats (mucus, coral tissue and skeletal matrix) with unique physicochemical characteristics and different bacterial communities (Sweet *et al*., 2011). Hence the understanding of coral-associated bacterial assemblages requires a detailed knowledge of their distribution within the different coral compartments. The interphase zones between the coral and its surrounding environment are constituted by the surface mucus layer and the coral skeleton, which is a porous structure in contact not only with seawater but also with sediments. In contrast, bacterial communities within the coral tissues below the mucus are embedded in a more stable matrix and less exposed to environmental changes, and therefore they may be less affected by variations in environmental conditions.

coral-associated microbial communities. Some studies show that these communities are coral-host specific across geographically distant sites (Rohwer *et al*., 2002), suggesting that the coral host determines the composition of prokaryotes within the holobiont. However, more recent studies indicate that the influence of geographical and temporal factors in the coral microbial composition is noteworthy (Hong *et al*., 2009; Littman *et al*., 2009; Barott and Rohwer, 2012; Roder *et al*., 2015).

Temperature can drive changes in host physiology and corals may suffer bleaching (corals appear white because of the loss of their symbiotic zooxanthellae or their pigments, Brown, 1997) in response to elevated seawater temperatures (Hoegh-Guldberg, 1999; Hughes *et al*., 2003). Not surprisingly, coral health status has a significant influence on the composition of bacterial communities, which suffer changes during bleaching (Ritchie, 2006; Bourne *et al*., 2008; Koren and Rosenberg, 2008). However, the role of these microorganisms in the bleaching and whether their shifts are a cause or a consequence of this process are uncertain.

Bleaching of the coral *Oculina patagonica* was first observed along the Israeli shoreline in the summer of 1993 (Fine *et al*., 2001) and it has been monitored since then (Israely *et al*., 2001, Shenkar *et al*., 2005). The *V. shiloi*-*O. patagonica* model system of coral bleaching was studied intensively from 1996 to 2002 (reviewed by Rosenberg and Falkowitz, 2004). The pathogen *Vibrio mediterranei* (= *Vibrio shilonii*) was also reported in 1996 as the putative causative agent of the temperature-induced bleaching disease (Kushmaro *et al*., 1996, 1997). Since 2002, the corals apparently became resistant to infection by *V. mediterranei* (Reshef *et al*., 2006) and a subsequent study stated that bacteria were not directly involved in bleaching, but instead played a secondary role due to an increase of

(Ainsworth *et al*., 2008). Recent studies (Mills *et al.,* 2013), including a study carried out with the same coral samples analyzed in this work (Rubio-Portillo *et al*., 2014a), suggested that the coral pathogens *V. mediteranei*and *V. coralliilyticus* are indeed the causative agents of bleaching in the coral *O. patagonica.*

It is well established that interactions among host-associated bacterial communities are critical for the health of the coral holobiont (see revision by Krediet *et al*., 2013), but our limited knowledge about changes in resident microbiota associated with corals during non-diseased states hinders the construction of an ecological model to explain what shifts in coral microbiota are involved in coral diseases. Therefore, gaining knowledge about the normal coral microbiota and how it changes over time is very important in order to understand coral susceptibility to infection and its ability to survive bleaching.

The present study aims at describing the time-space variations of the microbial community associated with the three different microhabitats (mucus layer, coral tissue and skeletal matrix) present in the coral *O. patagonica*, which could have an influence on the coral health status, by using a combination of microbiome profiling by Illumina sequencing and DGGE of the 16S rRNA bacterial genes and transmission electron microscopy (TEM). Overall, our results indicate that the bacterial communities are different among coral microhabitats and that the coral tissue is the compartment whose microbiota best exhibit changes under different environmental conditions. This finding contrasts with the fact that, *a priori*, coral tissue would be expected to be steadier than the mucus or the skeletal matrix since it is more isolated from the environment.

Results and discussion

Microbial cells were clearly observed in the in the mucus and tissue of *O. patagonica* by

electron microscopy the presence of endolithic microorganisms in the skeletal matrix of *O. patagonica* (Rubio-Portillo *et al*., 2014b). Morphological diverse microbial aggregates were observed in the mucus layer (Fig. 1A). At the epidermis and the tissue layer, bacteria with coccoid and vibrio shapes are difficult to clearly differentiate from the abundant cytoplasmatic inclusions. The presence of filamentous cell structures inside the gastrodermal tissue was obvious in the samples collected in September 2011, during the bleaching event, at the Harbor area (Fig. 1B and C).

In this work a total of 40 samples of *O. patagonica* taken from Alicante Harbor and the Marine Protected Area (MPA) of Tabarca over a year, including a bleaching event on September 2011, were used to monitor the bacterial communities from mucus, tissue and skeleton compartments by DGGE analysis. A total of 12 gels were analyzed and 37 different bands were sequenced. Identification of band sequences was carried out using BLASTn with the GenBank database (Table 2).

DNA from six different tissue samples were selected for sequencing using the Illumina platform (see Experimental Procedures) and approximately 1.9 million reads were generated. After removing short- and low-quality reads, as well as chimeras, a total of 562,494 sequences were obtained. The results related to the richness indexes, including number of observed operational taxonomic units (OTUs) and Shannon´s diversity indexes, calculated as described in the Experimental Procedures, are summarized in Table 3. Most of the samples contained up to 1,000 OTUs and the Shannon's indexes ranged from 5.72 to 8.28.

Do different coral compartments harbor different bacterial communities?

FPQuest analysis of the 16S rRNA PCR-DGGE amplicons from mucus, tissue and skeleton of *O. patagonica* showed different bacterial diversity in the three coral compartments

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(ANOVA, $p<0.05$). Mucus (Shannon diversity index, $H' = 3.379$) and tissue ($H' = 3.305$) harbored more diverse bacterial communities than skeletal matrix ($H'=2.986$). The two-dimensional NMDS plot (Fig. 2; stress value = 0.21) and cluster analysis (Fig. 2) also showed that the bacterial composition was different among coral microhabitats (ANOSIM, $R=0.514$; $p=0.001$), with the skeletal matrix harboring the most distinct bacterial assemblage. This fact, together with the previous study carried out by Koren and Rosenberg (2006) who observed different bacterial communities associated with mucus and tissues in *O. patagonica*, confirmed the compartmentalization of bacterial communities within the *O. patagonica* holobiont. This bacterial compartmentalization within the coral has also been observed in the corals *Acroporapalmata* by Sweet *et al*. (2011) and in *Portiteslutea* (Li *et al*., 2014), using DGGE and pyrosequencing, respectively.

Most of the OTUs retrieved from mucus layer (61.50%) and coral tissue (61.11%) of *O. patagonica* were classified as *Proteobacteria* (Fig. 3), with *Alphaproteobacteria* as the dominant class of the microbial community in both microhabitats (84.89% and 64.8% of *Proteobacteria*, respectively). *Gammaproteobacteria* were mainly detected in the tissue compartment (22.52 % of tissue *Proteobacteria*), and *Deltaproteobacteria* in skeletal matrix (33.91% of skeletal *Proteobacteria*). An analysis of contribution to similarities by SIMPER was performed to determine which OTUs were primarily responsible for the observed differences among the microhabitats studied (Supplementary Table 1). Data indicated that *Ruegeria* (OTU AP3) and *Pseudovibrio* (OTU AP1) of the *Rhodobacteraceae* family were identified (Supplementary Table 1) as important drivers of these differences in the mucus layer, which were also relevant in the coral tissue layer together with *Oceanicola* genus (OTU AP2). In the skeletal samples, OTU AP2 was the

skeletal samples.

Most of the OTUs retrieved from *O. patagonica* had high identities with members of *Bacteria* previously detected in other marine invertebrates (see Table 2), such as sponges and other coral species, even outside the Mediterranean Sea. This fact indicates that certain members of the *O. patagonica* microbiota are shared with other marine invertebrates and could be generalist symbionts displaying a broad host range and/or a widespread occurrence.

DGGE analysis gels showed changes only in the coral tissue bacterial assemblages among sampling time (Fig. 2) and coral health status (Fig. 4), while mucus and skeletal matrix communities remained stable. For this reason, coral tissue samples from healthy and bleached corals from the two localities during cold and warm months were also analyzed by Illumina sequencing in order to obtain more information about bacterial assemblages and the role of environmental conditions affecting coral holobiont health status.

Comparison of bacterial communities associated with healthy and bleached tissues

Many previous studies showed that microbial coral communities change during bleaching events (Bourne *et al.*, 2008; Reis *et al*., 2009; Lins-de-Barros *et al*., 2013). In *O. patagonica* tissues (Koren and Rosenberg, 2008) and other coral species the associated bacterial diversity increases in diseased specimens (Bourne, 2005; Bourne *et al*., 2008; Reis *et al*., 2009; Sunagawa *et al*., 2009). Our results showed significant changes in bacterial communities associated with bleached tissues (Fig. 4 and 5 for DGGE and Illumina results, respectively), but diversity trends were different between localities. In corals from Alicante Harbor, an increase in the bacterial diversity in bleached corals was observed, whereas in the MPA, the highest bacterial diversity was detected in healthy corals (Table 3).

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warm months were more similar in bleached corals than in healthy ones (Fig. 4 and 5). As both techniques used in this study indicated, a decrease of some OTUs belonging to *Rhodobacteraceae* family was observed in healthy corals from both localities, specifically of *Pseudovibrio* genus (Fig. 6 and Supplementary Table 1). Previous studies have emphasized the possible role of *Pseudovibrio* species, detected in healthy tissues of corals *Platygyracarnous* in Hong Kong (Chiu *et al*., 2012) and *Montrastreaannularis* in the Florida Keys, as denitrifying heterotrophs in the nitrogen cycle of the coral holobiont (Bondarev *et al*., 2013), as well as their capacity to inhibit the growth of coral pathogens (Nissimov *et al*., 2009; Rypien *et al*., 2010).

Even though the class *Gammaproteobacteria* was detected in both healthy and bleached corals, their proportion was always higher in bleached corals, in which *Vibrio* genus reached up to 60% of the Illumina reads. Among these sequences, those related to *V. mediterranei* and *V. coralliilyticus*, two coral pathogens that promote *O. patagonica* coral bleaching under aquaria conditions (Rubio-Portillo *et al*., 2014a), were retrieved from both healthy (including samples collected in cold months) and diseased corals (Fig. 7). Given the low resolution of 16S rRNA gene partial sequences to identify *Vibrio* species (Thompson *et al*., 2009), caution must be exerted to avoid over-interpretation of these results, although they are in agreement with our previous finding of *Vibrio* pathogens in otherwise healthy corals that were exposed to high temperatures under aquaria conditions (Rubio-Portillo *et al*., 2014a) and that subsequently underwent bleaching. In fact, the sequences detected here are more than 99% identical to the pathogens detected in these aquaria.

The detection of both *Vibrio* pathogens in healthy corals, even during cold months (0.01-0.04 % of the total sequences), suggests that these pathogens could be in a viable but non culturable (VBNC) state in coral tissues. The approach used in this study has not been

analyzed by a culture dependent approach (Rubio-Portillo *et al*., 2014a) that resulted in the isolation of known vibrio pathogens only from diseased corals, which suggests that the sequences detected here correspond to VBNC pathogens. This is in good agreement with the results of Sharon and Rosenberg (2010), who found VBNC *Vibrio* spp. in the mucus layer of *O. patagonica*. According to this hypothesis, the increase of seawater temperature during warm months could trigger *O. patagonica* diseases through the activation of the VBNC *Vibrio* communities associated to the coral tissue.

The *O. patagonica* core microbiome and its predicted roles in healthy corals

Analysis of the core microbiome of the coral *O. patagonica* from Illumina sequencing demonstrated that only 14 of all bacterial OTUs identified were present in 100% of the healthy corals (Table 4). Among these OTUs, most of them belonged to the *Alphaproteobacteria* class, although *Vibrio* genus was also present in the core microbiome, as we observed previously by culture analysis of the same coral samples (Rubio-Portillo *et al*., 2014a). In spite of the fact that PCRs with archaeal DGGE primers were always negative in all samples (data not shown), archaeal sequences were recovered from tissue samples sequenced by Illumina and ranged from 0.3 to 0.7% with the exception of one sample that was about 15%. Furthermore, *Thaumarchaeota* sequences related to *Nitrosopumilus* genus were present in 100% of healthy corals (Fig. 4) and were similar to those found in previous studies (Lins-de-Barros *et al*., 2010; 2013). Species from *Nitrosopumilus* are capable of ammonia oxidation and they may play a relevant role in the holobiont nitrogen cycle as observed previously in the ascidian *Cystodytesdellechiajei* (Martínez-García *et al*., 2008).

Predictive metagenomic analysis (PICRUST; Langille *et al*., 2013) was used to estimate the functional abundance of OTUs obtained in QIIME analysis of Illumina sequences. The

approach, based on Ainsworth *et al*. (2015), though rather speculative, could shed some light on the putative metabolic functions encompassed by the coral tissue associated microbiota detected here. This analysis suggested that ABC transporters, sugar transporters and ion-couple transporters would be the three most abundant prokaryotic genes (Table 5) in the *O. patagonica* core microbiome. Previous studies indicated that prokaryotic genes related to transporters of sugars and ions were very abundant in coral tissues and could be related to metabolic exchange between the coral host and bacterial microbiota (Ainsworth *et al*., 2015). Other key pathways linked to energy metabolism, such as the nitrogen cycle, were also highly abundant in the *O. patagonica* core microbiome. As mentioned above, the core microbiome included different bacteria such as *Pseudovibrio* species that could be involved in the nitrogen cycle in corals. Furthermore, *Vibrio* species associated to *O. patagonica* might have a positive effect on coral health by fixing nitrogen, as shown previously for other corals species (Chimetto *et al*., 2008).

Seasonal changes of the *O. patagonica* microbiome

The phylum *Proteobacteria* was always the dominant group in coral tissues and constituted 54 to 93% of the bacterial Illumina reads in coral tissues (Supplementary Figure 1). The dominance of *Proteobacteria* in coral tissues has been observed previously in other studies using both DGGE (Rohwer *et al*., 2002; Littman *et al*., 2009) and pyrosequencing techniques (Wegley *et al*., 2007; Pantos *et al*., 2015). However, the relative proportion of sequences belonging to different classes within the phylum changed depending on the technique (Fig. 3 for DGGE and Supplementary 1 for Illumina), most likely due to the bias exerted by different ribosomal operon copy numbers. For example, the number of ribosomal operons in members of the *Vibrionaceae* and *Rhodobacteraceae* families varies

analysis, the abundance of sequences belonging to one given species could depend on its ribosomal operon copy number and thus we may be detecting several sequences that belong to the same organism, such as the case of *Vibrionaceae* family members.

In general terms, both techniques showed a decrease of *Alphaproteobacteria* class, mainly the order *Rhodobacterales*, and an increase of *Gammaproteobacteria*, in particular *Vibrionales* in *O. patagonica* tissues during warm months (Fig. 3 for DGGE and Supplementary Figure 1b for Illumina), which was consistent with the results obtained by Koren and Rosenberg (2006). Specifically, the OTUs belonging to the genus *Pseudovibrio* (AP1 in DGGE and 19 and 20 in Illumina), as well as the order *Vibrionales* (GP2 in DGGE and OTUs 37-51 in Illumina) were identified as the OTUs that mainly contributed to the differences between samples taken in cold or warm months. Therefore, the microbiota in the tissues of *O. patagonica* experience shifts during warm months that may have an effect on coral health, either due to the decrease of *Pseudovibrio* representatives known to inhibit the growth of coral pathogens, or to the direct increase of pathogenic *Vibrio* species. These shifts are likely due to the increase of seawater temperature although other factors not considered here could also be involved.

Comparison among localities

There was no clustering of the DGGE microhabitats samples by sampling location, and the ANOSIM test confirmed that bacterial communities were not significantly different among environments (Harbor and Tabarca, R=0.019; p=0.710). Differences between localities were detected, however, in Illumina libraries as observed by UniFrac-based principal coordinate analysis (Fig. 5), mainly due to the deeper resolution of the technique. Consistent with our results, Lee *et al*. (2012) suggested that the sensitivity and resolution of DGGE is low in comparison to pyrosequencing.

NGS based16S rRNA gene analysis, which allows for detecting differences among bacterial communities associated with corals from sites with different environmental conditions, including water nutrient content.

In Illumina tissue libraries, the Shannon's indexes were higher in the Harbor corals (7.59±0.76) than in the MPA (5.28±0.36), at 0.03 cutoff. In both sampling locations the bacterial community was dominated by the phylum *Proteobacteria*, but the class *Gammaproteobacteria* showed a larger proportion in the Harbor than in Tabarca, where the class *Alphaproteobacteria* was dominant. Hence, not only the diversity but also the composition of the bacterial communities associated to *O. patagonica* differed substantially from pristine to disturbed areas (previously characterized in terms of chlorophyll and organic matter concentrations in Rubio-Portillo *et al*., 2014c), which could also contribute to differences in coral susceptibility to diseases. This is in agreement with previous findings (Vezzulli *et al*., 2013) that also reported higher bacterial diversity associated with the coral *Paramuriceaclavata* in Mediterranean locations affected by humans.

Comparison of Illumina and DGGE to profile bacterial assemblages in *O. patagonica*

Although, overall, Illumina sequencing of 16S rRNA genes and PCR-DGGE have considerable overlap in the main OTUs found in the *O. patagonica* core microbiome, there were some differences regarding the bacterial composition they each revealed. Although the primers used in both techniques match with similar percentages of classes belonging to *Proteobacteria*phylum gene sequences in the non-redundant Silva database (data not shown), the presence of some minority classes (less than 5% of the sequences in Illumina libraries), like *Betaproteobacteria* or *Epsilonproteobacteria*, was detected in *O. patagonica* tissues solely by NGS analysis. The proportions of *Alphaproteobacteria* and

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the number of ribosomal operons in both classes (see above). In any case, we should take into account that neither of these techniques is suitable for quantification purposes since both involve a PCR amplification step (Polz *et al*., 1999). In fact, when four prokaryotic species, *H. walsbyi*, *S. ruber*, *V. mediterranei* and *V. splendidus* were analyzed by DGGE and Illumina 16S rRNA gene sequencing, both techniques failed at describing the actual proportion of each species (Table 6). This fact confirms that DGGE band intensity should not be used as a quantitative measure of relative abundance of organisms in environmental samples as previously described (Murray *et al*., 1996). This is to be expected since it includes a 30-cycle PCR step. In addition, the ribosomal operon copy number may also introduce a bias in an Illumina analysis, as discussed above. Thus, both techniques might be calibrated by other methods like FISH or by metagenomic analyses, in which DNA is sequenced without a prior amplification (Sharpton, 2014).

Therefore we can conclude that: i) OTUs belonging to the core microbiome with relative abundance up to 1% (see Table 4) can be detected by DGGE and NGS, in agreement with the detection threshold for DGGE (Muyzer at al., 1993); ii) DGGE is a good technique to describe differences among bacterial assemblages associated with the three different compartments present in the coral holobiont, and is a suitable tool to check the similarity among sample replicates; iii) Illumina 16S rRNA gene sequencing is better than DGGE to detect rare taxa including coral pathogens or shifts in bacterial composition due to environmental changes; and iv) neither of these two techniques is suitable to measure the relative abundance of organisms in environmental samples. The combination of both techniques provides a better picture of the coral holobiont than either technique on its own.

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Conclusions

Here we have shown that 16S rRNA gene PCR-DGGE and Illumina sequencing approaches are suitable for providing useful qualitative information about microbial communities associated to corals and their time-space variations, as well as changes in microbiota related to coral health status. The use of these techniques unveils high bacterial diversity within the three microhabitats (i.e. mucus, tissue and skeleton) of the coral *O. patagonica*. Bacterial communities appear strongly compartmentalized within the coral, with the tissue as the compartment that best reflected changes in environmental factors such as seawater nutrient concentration or temperature. Furthermore, our results suggest the presence of coral pathogens within the tissues of healthy specimens as well as the detection within the coral microbiota of bacteria typically present in other marine invertebrates. These findings, together with the ongoing expansion of *O. patagonica* throughout the Mediterranean Sea (Sartoretto *et al*., 2008) could have significant implications for disease propagation in the present global change scenario.

Experimental procedures

Sample collection

Five *O. patagonica* samples were seasonally collected (December 2010, February 2011, June 2011 and September 2011, when a bleaching event was detected by Rubio-Portillo *et al*. 2014c) at two locations: Alicante Harbor (38º20’11.1"N, 00º29’11.8“9W) and the Marine Protected Area (MPA) of Tabarca (38º09′59″N, 0º28′56″W) on the Alicante coast, South East of Spain (Western Mediterranean Sea), covering different environmental conditions based on organic matter and chlorophyll concentration in seawater, as reported previously by Rubio-Portillo *et al*. (2014c). A total of 40 full-colony samples were

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this study (Table 1). The samples were immediately placed in plastic bags underwater and transported to the laboratory in a cooler (within <2 h). The three different microhabitats (mucus, coral tissue and skeletal matrix) were separated from coral fragments. Firstly coral fragments were placed in 50-ml centrifuge tubes and centrifuged for 3 min at 2,675xg twice, using new tubes each time, to remove the mucus. After centrifugation, the coral pieces were crushed in sterile seawater with a mortar and pestle and, after allowing the $CaCO_3$ skeleton to settle during 15 min, the tissue was removed from the supernatant (Koren and Rosenberg 2006) and the skeleton was washed with sterile seawater in order to eliminate any residual tissues. The coral health status was estimated by chlorophyll a measurements obtained from coral tissues as previously described by Rubio-Portillo *et al*., (2014a).

Microscopy analyses

Three samples from each locality and each seasonal sampling time were processed for Scanning Electron Microscopy (SEM) studies (see Rubio–Portillo *et al*., 2014c). Coral fragments were fixed (3% glutaraldehyde followed by 1% OsO_4), dehydrated in an ethanol series, and embedded in LR-White acrylic resin. After polymerization, the blocks were cut and finely polished. The fine-polished surfaces of the cross sections were carbon coated and finally examined using a DMS 960 Zeiss SEM equipped with a four-diode, semiconductor BSE detector. The microscope operating conditions were: 0º tilt angle, 35º X-ray take-off angle, 15 kV acceleration potential, 15 mm working distance and 1–5 nA specimen current range. After that, the SEM ultrastructural study areas of interest were removed from the polished sample and embedded for transmission electron microscopy (TEM) study in LR-White acrylic resin. In this study only TEM images are shown. Then ultrathin sections were

(Reynolds, 1963) and observed in a LEO 910 TEM (80KV) using a BioscanGatan 792 digital camera.

DNA extraction and polymerase chain reaction amplification of 16S rRNA genes

Coral mucus, tissue and skeletal samples were centrifuged at 9,300×g for 15 min, and pellets were used for DNA extraction, using the UltraClean Soil DNA Kit (MoBio; Carlsbad, CA) following the manufacturer's instructions for maximum yield. The extracted genomic DNA was used for PCR amplifications of bacterial 16S rRNA genes by using the specific bacterial primer 341f-GC (Muyzer *et al*., 1993) and the reverse universal primer 907R (Muyzer *et al*., 1993). Each PCR mixture contained 5 µl of 10x PCR reaction buffer (Invitrogen), 2.5 µl of 50 mM $MgCl_2$,1 µl 10 mMdNTP mixture, 1 µl of 10 µM of each primer, 1 units of Taq polymerase, sterile MilliQ water up to 50 µl and 30 ng of the extracted DNA. The PCR program was: 94ºC for 5 min, 65ºC for 1 min, 72ºC for 3 min and 9 touchdown cycles of: 94ºC for 1 min, 65ºC (with a decreasing of 1ºC in each cycle) for 1 min, 72ºC for 3 min, followed by 20 cycles of: 94ºC for 1 min, 55ºC for 1 min, 72ºC for 3 min (Muyzer *et al*., 1993); a step at 72ºC for 30 min was added to minimize double band formation (Janse *et al*., 2004). PCR products were diluted 10-fold and used as templates for a five cycle reamplification in order to eliminate heteroduplexes (Thompson *et al*., 2002). Since DGGE band migration patterns of replicate corral tissue samples taken at the same time did not show significant differences, six of them, three from Alicante Harbor and three from Tabarca, (two of the three samples were healthy corals, one collected in February and the other in June, and the third sample was a bleached coral collected in June) were selected for massive sequencing of 16S rRNA gene amplicons to analyze in depth the bacterial communities associated to *O. patagonica* coral tissues. (Table 1). The V3-V4 region of the 16S rRNA gene was amplified using universal bacterial primers Bakt_341f and Bakt_805r

(Herlemann *et al*., 2011) containing Ilumina-specific adapter sequences. Each PCR mixture contained 5 µl of 10x PCR reaction buffer (Invitrogen), 1.5 µl of 50 mM $MgCl_2$, 1 µl 10 mMdNTP mixture, 1 µl of 100 µM of each primer, 1 units of Taq polymerase, 3 µl of BSA (New England BioLabs), sterile MilliQ water up to 50 µl and 10 ng of DNA. The amplification products were purified with the the GeneJET PCR purification kit (Fermentas, EU), quantified using the Qubit Kit (Invitrogen), and the quality (integrity and presence of a unique band) was confirmed by 1% agarose gel electrophoresis.

Analysis of bacterial community composition by denaturing gradient gel electrophoresis (DGGE)

DGGE was performed by using the DCode System (Bio-Rad, Hercules, CA). PCR products (500 ng) were separated by electrophoresis at 100 V during 16 h in a linear gradient from 40% to 60% (where 100% of denaturant consists of 7 M urea and 40% formamide) in a 6% (w/v) polyacrylamide gel (acrylamide-bisacrylamide gel stock solution 37.5:1; Bio-Rad), in 1x TAE buffer (40 mMTris, pH 8.0; 20 mM acetic acid; 1 mM EDTA). DGGE gels were stained for 30 min with SYBR Green, visualized under UV light and photographed with a Typhoon 9410 (Amersham Biosciences) system.

DGGE gel images were analyzed using the FPQuest Software Version 5.10 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion due to electrophoresis, the DGGE patterns were aligned and normalized using an external ladder, made by PCR products from specific marine bacteria. Bands that were visible under UV light were excised from DGGE gels using sterile scalpel blades and soaked overnight in 20 µl of MilliQ water. Two µl of each band were then reamplified with the same primer set and checked again by DGGE to ascertain that they corresponded to single bands and to

GeneJET PCR purification kit (Fermentas, EU) and sequenced with primer 907R using an ABI 3730xl sequencer (Applied Biosystems). Partial 16S rRNA gene sequences were compared with reference sequences using the BLAST (Basic Local Alignment Search Tool) software and the reference National Centre of Biotechnology Information database (http://www.ncbi. nlm.nih.gov/). The band sequences are available from Genbank under accession numbers KU936838-KU936871. Sequences of different bands were clustered into OTUs at 98.7%, the threshold recommended as the best for the definition of members of a species by Stackebrandt and Ebers (2006) similarity using RDP.

Statistical analysis

The presence/absence of OTUs in each sample, obtained from DGGE analysis, was used to construct a binary matrix that represented the banding patterns, and multivariate analyses were performed with a Primer 5 software package (Clarke and Gorley, 2001). A distance matrix was constructed using Bray–Curtis similarity, and hierarchical clustering analysis (CLUSTER) (similarity dendrogram) and non-metric multidimensional scaling (NMDS) were used to explore groupings of the samples, since replicates of the same coral status collected from the same locality at the same sampling time were grouped into the same plot since there were no differences among them. Analyses of similarity (ANOSIM) was used to determine if coral microhabitat (mucus/tissue/skeleton), sampling time (cold months: December and February / warm months: June and September), coral status (healthy/bleached), or sampling location (Harbor/Tabarca) had an effect on the bacterial communities. Similarity percentage (SIMPER) was used to identify species that could be potentially responsible for these differences.

Illumina high-throughput 16S rRNA gene sequencing and bioinformatic analyses

PCR products of the V3-V4 region were sequenced using a paired-end, 2×250-bp cycle run

on an IlluminaMiSeq sequencing system (Genomics Service at the Universidad Autónoma de Barcelona, Spain) and raw reads were deposited in the NCBI Sequence Read Archive (SRA) database under BioProject ID PRJNA315808. Downstream bioinformatic analyses were performed using QIIME 1.8.0 (Caporaso *et al*., 2010). Paired-end reads were assigned to their respective samples according to their barcodes and then sequences were screened by quality and size, and de-replicated with the script split_libraries.py (Caporaso *et al*., 2010). Sequences were then subjected to the following procedures with QIIME scripts: (1) chimeras were removed, (2) sequences were clustered at 97% similarity using UCLUST (Edgar, 2010), (3) cluster representatives were selected, (4) SILVA_119 database (July 2014 edition) was used for taxonomic assignments of selected representatives by BLAST and (5) tables with the abundance of different operational taxonomic units (OTUs) and their taxonomic assignments in each sample were generated. Representative OTUs were also aligned using PyNAST (Caporaso *et al*., 2010) with the SILVA_119 database as a reference. The number of reads was normalized to 6071 (the lowest number of the post-assembly and filtered sequences in a sample) per sample. The number of OTUs and Shannon diversity index values corresponding to 6071 sequences per sample were calculated with QIIME. The similarity among different microbial communities was assessed using phylogenetic information using jackknifed UPGMA (unweighted pair group method with arithmetic mean) clustering based on the unweighted UniFrac (Lozupone and Knight, 2005) distances between samples implemented in the QIIME pipeline. For functional metagenome prediction, the PICRUSt software package (http://picrust.github.com/picrust/) (Langille *et al*., 2013) was applied, which predicts the gene content of a microbial community from the information inferred from 16S RNA genes

of microbial communities. Metabolic predictions were made based on copy-number normalized OTUs and using only healthy samples.

Sequences corresponding to the coral vibrio pathogens, *V. mediterranei* and *V. coralliilyticus*, previously isolated from the same coral samples used in the present study (Rubio-Portillo *et al*., 2014a), were searched for in Illumina 16S rRNA gene libraries using Basic Local Alignment Search Tool (BLAST 2.2.31+) with the following command 'blastn –perc_identity 98.7 –evalue 0.00001 –num_alignments 0 –maz_target_seqs 300000'.

Comparison among DGGE and Illumina sequencing techniques

In order to compare the results obtained by these two techniques, two controls of DNA mixtures with different cell proportions were obtained from pure cultures of *Haloquadratumwalsbyi*, *Salinibacterruber*, *Vibrio mediterranei* and *Vibrio splendidus* (Table 6) with a known number of cells previously determined by 4'-6-diamidino-2-phenylindole (DAPI) staining. These two controls were amplified with DGGE primers, and electrophoresis was performed and stained as explained above. In parallel, the mock communities were analyzed by Illumina sequencing as described above. DGGE band intensities were quantified using PyElph 1.4 software and the relative contribution of individual bands corresponding to each isolate to the total band intensity in the lane was compared with the proportion of sequences obtained from Illumina sequencing of the same DNA mixtures.

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Figure legends

Figure 1. Representative transmission electron micrographs of *Oculina patagonica* tissues from September Harbor samples. A. Diverse morphological microbial cells from mucus layer present in the external part of the coral. B. Filamentous bacteria (arrows) in the gastrodermis in the area around mesoglea (M). C. The filamentous bacteria seem to penetrate the coral tissue in an invasive way (arrows). Bars:1 µm.

Harbor

Figure 2. Non-metric Multidimensional Scaling plots of the first two dimensions based on Bray-Curtis dissimilarities for mucus (circles), tissue (squares), and skeletal matrix (triangles), in gray samples collected in cold months (December and February) and in black in warm months (June and September).

Figure 3. Cluster analysis and taxonomic composition (dominant bacterial sequence affiliations grouped into dominant ribotypes at the class level) from DGGE analysis of only healthy corals, with replicates grouped in the same plot, of mucus (M), tissue (T) and skeletal (S) collected in cold (C) or warm (W) months from Alicante Harbor (H) or the

Marine Protected Area of Tabarca (T).

Figure 4. Non-metric Multidimensional Scaling plot, from all tissue profiles of DGGE, of the first two dimensions based on Bray-Curtis dissimilarities. Alicante Harbor (circles) and Marine Protected Area of Tabarca (squares), with black for healthy samples and white for bleached ones.

Figure 5. Bacterial communities, detected by Illumina sequencing, associated to *Oculina patagonica* tissues clustered using coordinated analysis of the weighed UniFrac distance matrix. Each point corresponds to a coral sample from Alicante Harbor (circles) and Marine Protected Area of Tabarca (squares), with black for healthy samples and white for diseased ones.TCH: Tabarca Cold Healthy; TWH: Tabarca Warm Healthy; TWU: Tabarca Warm Bleached; HCH: Harbor Cold Healthy; HWH: Harbor Warm Healthy; HWU: Harbor Warm Bleached.

Figure 6. Heatmap summarizing the alpha diversity and abundance of the dominant prokariotic OTUs, from Illumina analysis, (those present at more than 1% of abundance) associated to *Oculina patagonica* tissues from Alicante Harbor and the Marine Protected Area of Tabarca. TCH: Tabarca Cold Healthy; TWH: Tabarca Warm Healthy; TWU: Tabarca Warm Bleached; HCH: Harbor Cold Healthy; HWH: Harbor Warm Healthy; HWU: Harbor Warm Bleached.

Figure 7. Percentage of Illumina reads belonging to Vibrionacea genus and the percentage of them that showed more than 98.7% of identity with *Vibrio mediterranei* and *Vibrio coralliilyticus* sequences. See Table 3 for sample identifiers.

Supplementary Figure 1. Representative denaturing gradient gel electrophoresis (DGGE) profiles of the coral tissue bacterial communities from samples collected in February in the Alicante Harbor and the Marine Protected Area of Tabarca. Specific bands are marked

and named as Table 2. M: reference ladder.

Supplementary Figure 2. Taxonomic classification of Illumina libraries into (a) phylum and (b) class levels. See Table 3 for sample identifiers.

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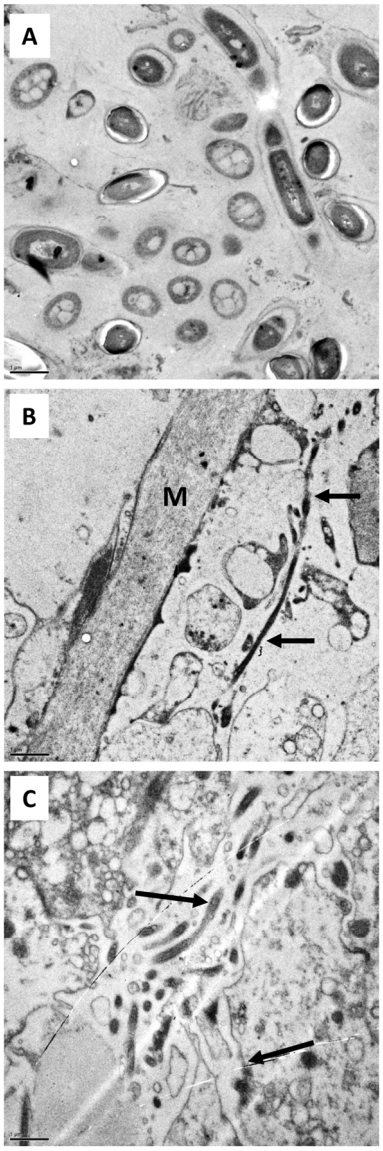

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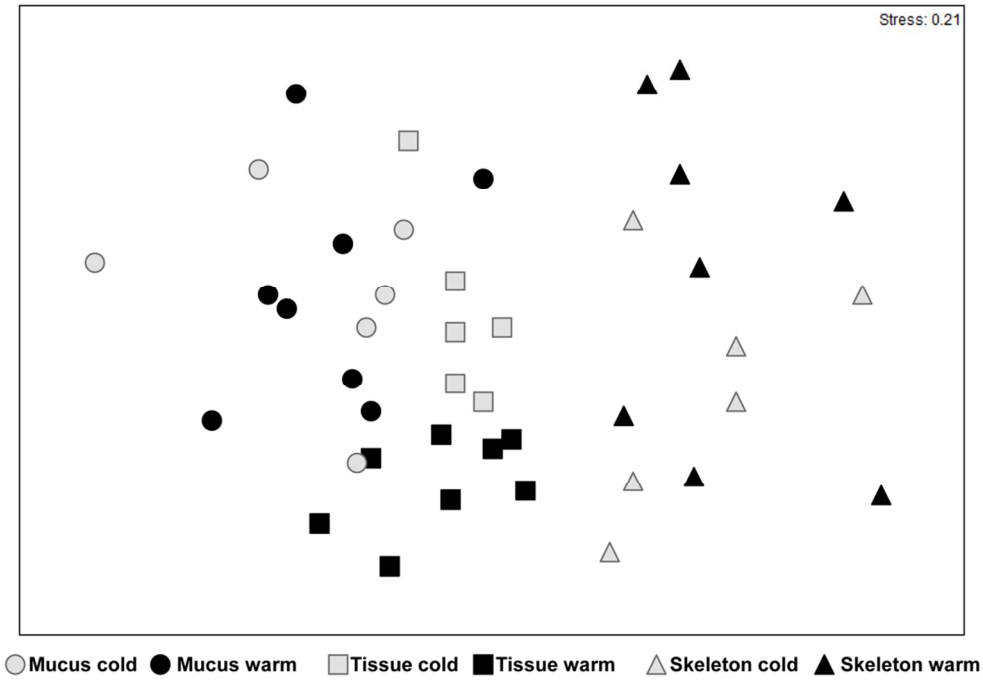

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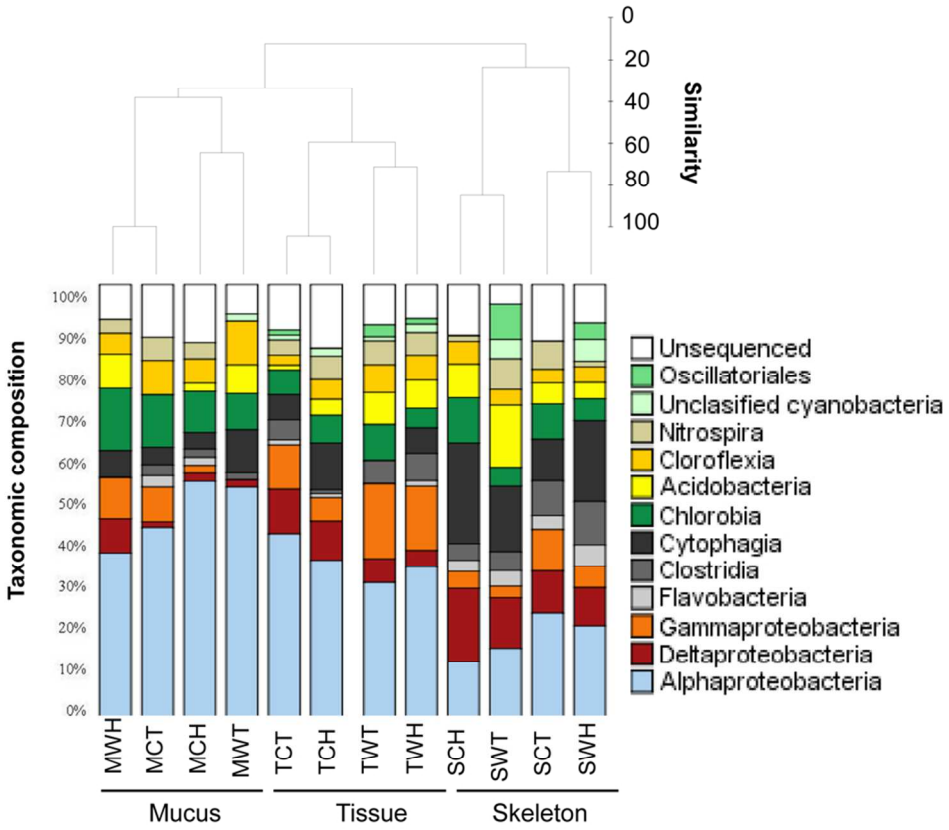

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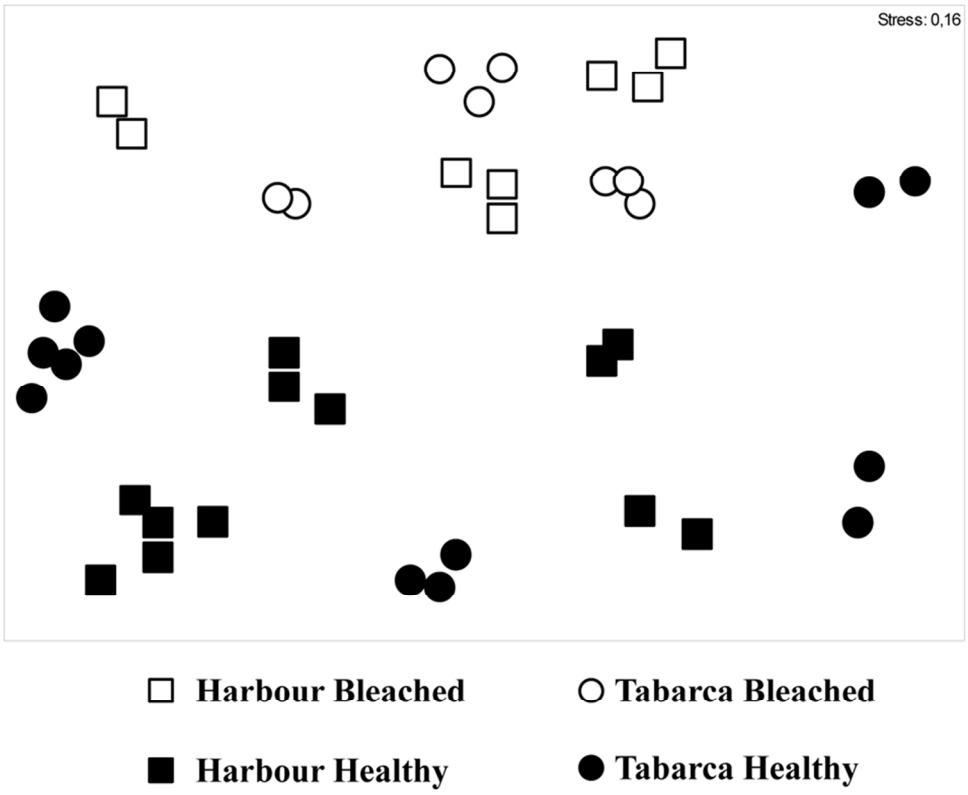

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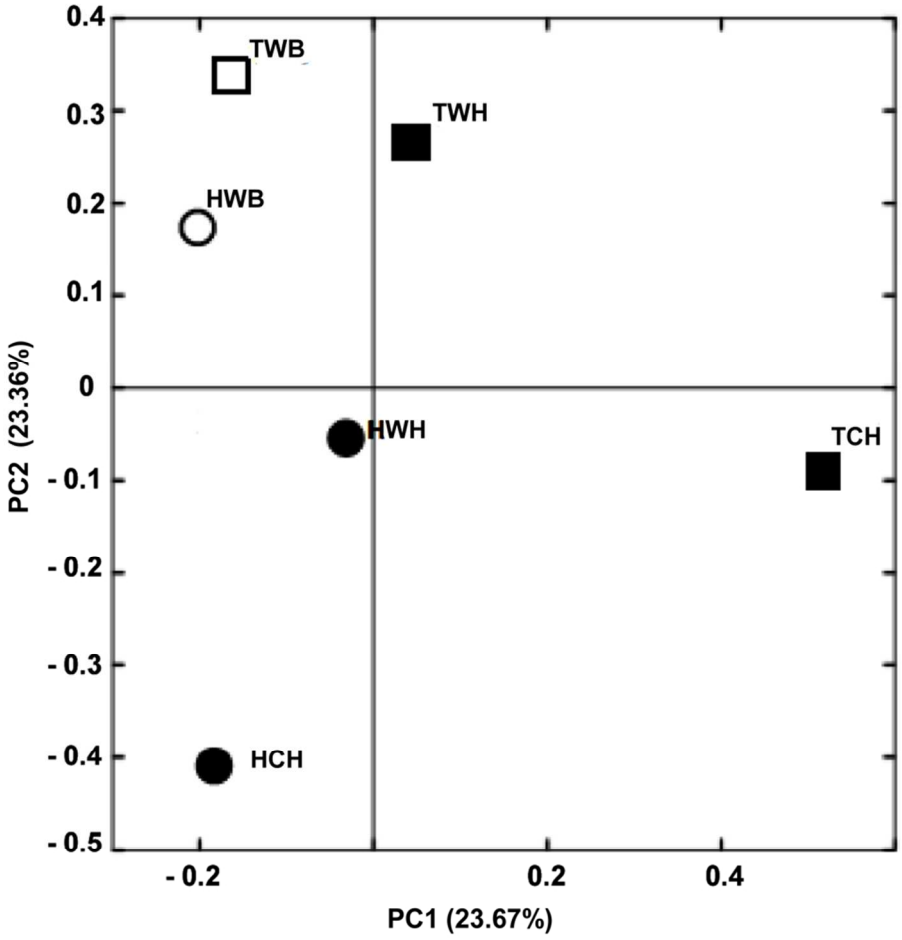

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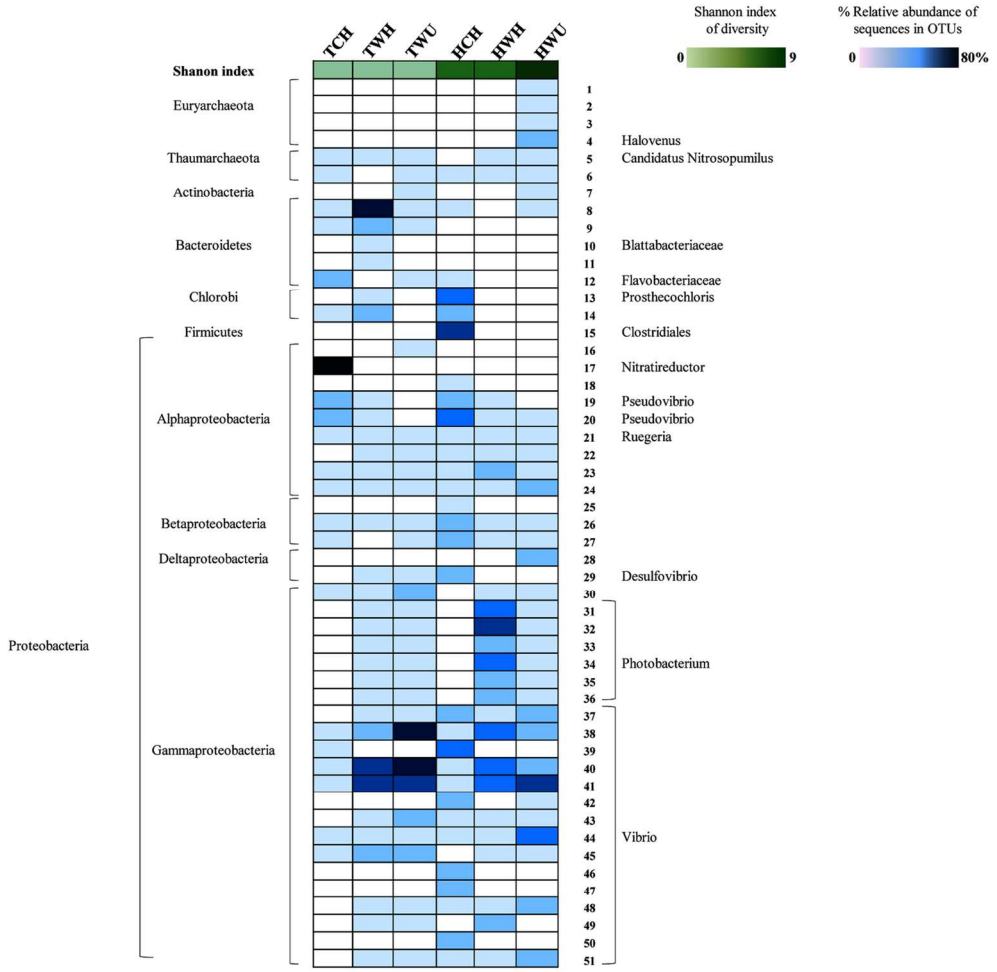

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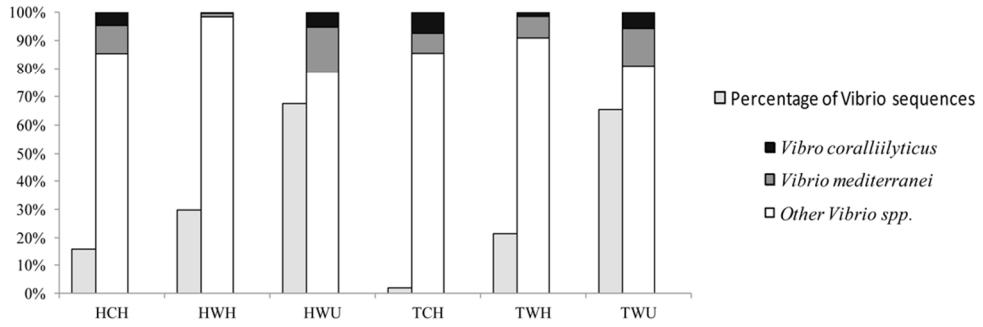

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Table 1. Summary of samples collected in this study.

Sampling Location	Sampling Time	Coral status	Replica	Techniques
Alicante Harbour	December 2010	Healthy	1-3	DGGE
		Bleached	4-5	DGGE
	February 2011	Healthy	1-4	DGGE
			5	DGGE and Illumina
	June 2011	Healthy	1-2	DGGE
		Bleached	3-5	DGGE
	September 2011	Healthy	1	DGGE and Illumina
			2	DGGE
		Bleached	3	DGGE and Illumina
			4 and 5	DGGE
MPA of Tabarca	December 2010	Healthy	1-3	DGGE
		Bleached	4-5	DGGE
	February 2011	Healthy	1-4	DGGE
			5	DGGE and Illumina
	June 2011	Healthy	1-2	DGGE
		Bleached	3-5	DGGE
	September 2011	Healthy	1	DGGE and Illumina
			2	DGGE
		Bleached	3	DGGE and Illumina
			4 and 5	DGGE

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Table 2. Bacterial 16S rRNA sequencesof selected DGGE bands.

OTU	Band	Sequence length (bp)	Phylogenetic group	Best hit in NCBI Source (% sequence identity, accesion no.)	Closest type strain (% sequence identity, accesion no.)
BC1	B1	513	Bacteroidetes (Cytophaga)	Uncultured Cytophaga Marine Sediments (88-89, AJ240979)	*Alkaliflexus imshenetskii* *Marinilabiliaceae* (86, AJ784993)
	B4	498			
	B6	531			
	B7	483			
BC2	B2	496	Bacteroidetes (Flavobacteria)	Uncultured Bacteroidetes bacterium Sponge-associated (99,AM259925)	*Vitellibacter aestuarii* (89, EU642844)
BC3	B3	318	Bacteroidetes (Cytophaga)	Uncultured Rhodothermaceae bacterium Sponge-associated (100, JQ612356)	*Rhodothermus profundi* (92, FJ624399)
BC4	B5	449	Bacteroidetes (Cytophaga)	Uncultured Cytophaga Hydrothermal vent chimney (97,FJ640814)	*Marivirga serice* (92, AB078081)
BC5	B8	455	Bacteroidetes (Flavobacteria)	*Coralibacter albidofladus* Hard coral (99, AB377124)	*Pseudozobellia thermophila* (93, AB084261)
CB1	B9	493	Chlorobia	Prosthecochloris vibrioformis Marine aquaculture pond water (99, AM690798)	*Prosthecochloris vibrioformis* (98, M62791)
	B11	535			
CX1	B10	486	Chloroflexi	Uncultured bacterium Sponge-associated (98,FJ900573)	*Bellilinea caldifistulae* (81,AB243672)
	B14	406			
AB	B16	485	Acidobacteria	Uncultured bacterium KM3-173-A5 Mediterranean Sea (93, EU686629)	
AP1	B15	484	Alphaproteobacteria (Rhodobacterales)	*Pseudovibrio japonicus* Abalone	*Pseudovibrio japonicus* (100, AB246748)

	B23	493		(99, HE584768)	
	B18	466	Alphaproteobacteria	Rhodobacteraceae bacterium	*Pseudovibrio denitrificans*
	B19	512	(Rhodobacterales)	Coral-associated (99-100, JF411476)	(99, AY486423)
AP2	B17	522	Alphaproteobacteria (Rhodobacterales)	Uncultured bacterium Seawater (89, HQ203925)	*Oceanicola batsensis* (99, AY424898)
AP3	B20	516	Alphaproteobacteria	Uncultured bacterium	*Ruegeria atlantica*
	B22	498	(Rhodobacterales)	Seawater (100, KC120680)	(99, D88526)
	B21	498	Alphaproteobacteria (Rhodobacterales)	*Ruegeria sp*. JZ11ML32 Marine sponge (100, KC429919)	*Ruegeria conchae* (98,HQ171439)
	B24	493	Alphaproteobacteria (Rhodobacterales)	*Roseobacter sp*. 7m33 Soil (99, JQ66197)	*Ruegeria halocynthiae* (98, HQ852038)
AP4	B28	488	Alphaproteobacteria (Rhodospiralles)	Uncultured Alphaproteobacteria Sponge-associated (98, JF824774)	*Nisaea nitritireducens* (94, DQ665839)
DP1	B25	354	Deltaproteobacteria	Uncultured bacterium Marine sediments (99, EU488075)	*Sandaracinus amylolyticus* (92, HQ540311)
DP2	B35	488	Deltaproteobacteria	Uncultured microorganism Sponge-associated (100, JN002375)	*Desulfonatronum thiosulfatophilum* (85, FJ469578)
	B36	489			
GP1	B29	501	Gammaproteobacteria (Vibrionales)	*Photobacterium sp*. 1983 Phytoplankton culture (99, HF549205)	*Photobacterium frigidiphilum* (99, AY538749)
GP2	B30	380	Gammaproteobacteria (Vibrionales)	Vibvrionales bacterium SWAT-3 (98%, AAZW01000075)	*Vibrio orientalis* (97, X74719)
	B31	455	Gammaproteobacteria (Vibrionales)	*Vibrio parahaemolyticus* (100, AAWQ1000319)	*Vibrio rotiferianus* (100, AJ316187)
GP3	B37	392	Gammaproteobacteria	*Psychrobacter glacincola*	*Psychrobacter piscatorii*

			(Pseudomonadales)	Sea ice (99, U85879)	(99, AB453700)
NI1	B26	498	Nitrospirae	Uncultured bacterium Sponge (99, EU035954)	*Nitrospira moscoviensis* (89, X822558)
FI1	B27	429	Firmicutes	*Clostridium sp*. AN-AS8 Sediments (97, FR872934)	*Defluviitalea saccharophila* (95, HQ020487)
CY1	B32	505	Cyanobacteria	*Oscillatroia corallinae* (99, X84812)	*Loriellopsis cavernicola* (93, HM748318)
CY2	B33	493	Cyanobacteria	Filamentous cyanobacterium Coral (96, EU196366)	*Prochlorothrix hollandica* (89, AM709625)

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Table 3. Counts of paired-end rRNA gene sequences obtained from Illumina (preassembly) and following assembly and screened (postassembly) for the libraries included in this study. OTUS and Shannon diversity index were calculated from filtered sequences. H: Harbour; T: Tabarca; C:Cold;W: Warm; H:Healthy and B: Bleached

Samples	Location	Time	Coral Status	Nº sequences		Nº OTUS	Shannon diversity
				Preassembly	Postassembly and filtered		
HCH	Harbour	Cold	Healthy	3661044	222966	1779	7.75
HWH	Harbour	Warm	Healthy	126618	18268	1031	6.22
HWB	Harbour	Warm	Bleached	120202	15627	1748	8.19
TCH	Tabarca	Cold	Healthy	146276	275958	1537	5.33
TWH	Tabarca	Warm	Healthy	109296	6071	1005	5.68
TWB	Tabarca	Warm	Bleached	1396170	23604	1263	5.54

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Table 4. Core microbiome community in *Oculina patagonica* (OTUs present in 100% of the samples analyzed by Illumina). Identity percentage indicates similarity to reference sequence used for taxonomic assignment. *: OTUs with more than 98% of identity with DGGE bands.

Silva Tag ID	Identity %	Taxonomic affiliation	Type strain (LTP database)	Identity %	Realtive abundance based on Illumina sequences (Mean±SD)	Detected by DGGE
KF758600	99	Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*	*Vibrio gigantis* (EF094888)	99	6.33 ± 4.19	*
ABXL01000053	99	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Pseudovibrio*	*Pseudovibrio denitrificans* (AY486423)	99	3.02 ± 2.54	*
EU854926	99	Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*	*Vibrio pelagius* (AJ293802)	99	2.32 ± 1.89	*
HE574865	99	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Uncultured bacterium	*Ruegeria conchae* (HQ171439)	99	1.17 ± 0.88	*
JF344078	99	Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Uncultured bacterium	*Pelagibius litoralis* (DQ401091)	99	1.10 ± 1.1	*
EF629830	99	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae ; *Ruegeria*	*Ruegeria atlantica* (D88526)	99	0.84 ± 0.65	*
FR693291	99	Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; *Pelagibius*	*Pelagibius litoralis* (DQ401091)	95	0.82 ± 0.53	*
GQ140332	99	Betaproteobacteria; Burkolderiales; Comamonadaceae;	*Diaphorobacter nitroreducens* (AB064317)	98	0.53 ± 0.49	

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		Variovorax				
JN606966	99	Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*	*Vibrio crassostreae* (EF094887)	99	0.44 ± 0.31	
AY654819	99	Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae	*Fodinicurvata halophila* (HG764424)	95	0.39 ± 0.37	
CU914838	99	Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; *Tepidibacter*	*Tepidibacter mesophilus* (GQ231514)	98	0.06 ± 0.02	
JX411936	99	Gammaproteobacteria; Alteromonadales; Colwelliaceae; *Colwellia*	*Thalassomonas haliotis* (AB369381)	99	0.05 ± 0.03	
GQ906610	98	Archaea; Thaumarchaeota; Marine Group; Candidatus *Nitrosopumilus*	*Nitrososphaera viennensis* (CP007536)	95	0.05 ± 0.02	
EU236284	99	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Roseovarius albus* (HF546052)	99	0.03 ± 0.02	

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Table 5. Most abundant predicted KEGG pathways from the *Oculina patagonica* core microbiome using PICRUSt and copy-number normalized OTUs from healthy corals samples.

KEGG pathway	Posible function in symbiosis (based on Langille *et al*., 2013)	Mean Abundance (%±SD)
Transporters	Transport of substrates (ions, sugars, lipids)	5.80 ± 0.49
ABC transporters	Transport of substrates (ions, sugars, lipids)	3.87 ± 0.23
Secretion systems	Secretion of proteins, including those involved in nutrient uptake	2.58 ± 0.65
Other ion-coupled transporters	Transport of substrates (ions, sugars, lipids)	2.46 ± 0.28
Purine metabolism	Nucleic acid metabolism	1.94 ± 0.21
Pyrimidine metabolism	Nucleic acid metabolism	1.33 ± 0.14
Ribosome	Genetic information processing	1.56 ± 0.32
Oxidative phosphorylation	Energy metabolism	1.28 ± 0.22
Nitrogen metabolism	Energy metabolism	1.18 ± 0.29
Pyruvate metabolism	Energy metabolism	1.11 ± 0.1

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Table 6. Relative levels of isolates in control samples C1 and C2 as estimated by different molecular methods, including DGGE band intensity and proportion of sequences detected by Illumina sequencing.

Samples	Proportion of cells	% in DGGE analysis	% in Illumina library
C1			
Salinibacter ruber	48.9	57.84	35.3
Haloquadratum walsbyi	48.9	0	2.5
Vibrio mediterranei	2.1	22.93	16.3
Vibrio splendidus	0.1	18.01	45.8
C2			
Salinibacter ruber	25	21.22	9.09
Haloquadratum walsbyi	25	0	0.01
Vibrio mediterranei	25	50.58	85.5
Vibrio splendidus	25	28.22	5.3

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