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RESEARCH ARTICLE

Diversity of deep-water coral-associated bacteria and comparison across depth gradients

Sigmund Jensen[1,*], Martin Hovland[2,3], Michael D. J. Lynch[4] and David G. Bourne[5,6]

[1]Department of Biology, University of Bergen, PO Box 7803, Bergen 5020, Norway, [2]Centre for Geobiology, University of Bergen, [3]Tech Team Solutions ASA, Stavanger, [4]Department of Biology, University of Waterloo, Canada, [5]College of Science of Engineering James Cook University, Townsville, Australia and [6]Australian Institute of Marine Science, Townsville, Australia

[*]**Corresponding author:** Department of Biology, University of Bergen, PO Box 7803, Bergen 5020, Norway. Tel: +47 55584400; E-mail: sigmund.jensen@bio.uib.no

One sentence summary: Corals located on the mid-Norwegian shelf host an abundance of poorly known bacterial species comparable to other deep-water corals though contrasts with tropical corals, reflecting environmental influence.

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ABSTRACT

Environmental conditions influence species composition, including the microbial communities that associate with benthic organisms such as corals. In this study we identified and compared bacteria that associate with three common deep-water corals, *Lophelia pertusa*, *Madrepora oculata* and *Paragorgia arborea*, from a reef habitat on the mid-Norwegian shelf. The 16S rRNA gene amplicon sequencing data obtained revealed that >50% of sequences were represented by only five operational taxonomic units. Three were host-specific and unclassified below class level, belonging to Alphaproteobacteria with affiliation to members of the Rhizobiales order (*L. pertusa*), Flavobacteria affiliated with members of the *Elisabethkingia* genus (*M. oculata*) and Mollicutes sequences affiliated with the *Mycoplasma* genus (*P. arborea*). In addition, gammaproteobacterial sequences within the genera *Sulfitobacter* and *Oleispira* were found across all three deep-water coral taxa. Although highly abundant in the coral microbiomes, these sequences accounted for <0.1% of the surrounding bacterioplankton, supporting specific relationships. We combined this information with previous studies, undertaking a meta-data analysis of 165 widespread samples across coral hosts and habitats. Patterns in bacterial diversity indicated enrichment of distinct uncultured species in coral microbiomes that differed among deep (>200 m), mesophotic (30–200 m) and shallow (<30 m) reefs.

Keywords: deep-water corals; coral-associated bacteria; depth gradient; bacterial diversity

INTRODUCTION

Corals are the structural building blocks of vast but vulnerable ecosystems on continental shelves (Freiwald 2002; Hovland 2008; Bourne and Webster 2013) and in fjords (Buhl Mortensen and Buhl Mortensen 2014). From shoreline to shelf break, environmental factors shape coral species distribution (Kitahara *et al.* 2010); however, we know very little about how environmental factors influence microbial species associated with coral and the implications of any microbial community changes for coral reef function. The study of coral microbiomes has focused on characterising the bacterial community associated with the coral colony. From the mucus, tissue and skeleton samples obtained, the bacterial communities appear structured,

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with many species and less-defined taxa postulated to interact and provide important functional roles to the host (Rohwer *et al.* 2002; Neulinger *et al.* 2008; Gray *et al.* 2011). In the least-understood coral reef environments of deep-sea regions (below 200 m depths), such as on the Norwegian shelf, chemical energy from captured plankton drives the building of coral reefs (Freiwald 2002). Here, corals are surrounded by a cold permanent darkness enriched with downwelling surface plankton (Thiem *et al.* 2006) including local plankton potentially sustained by the oxidation of compounds seeping from the seafloor (Hovland, Jensen and Indreiten 2012; Jensen *et al.* 2015). In shallower waters (30–200 m depths), a transition zone of mesophotic low-light conditions occurs (Olson and Kellogg 2010) that modifies the coral phenotype to maximise light-harvesting capabilities by becoming flatter, with changes in species dominance and shifts to photosymbiont-dominant corals. In the shallow, more oligotrophic waters of tropical coral reefs (0–30 m depth), sunlight drives Symbiodiniaceae (LaJeuneusse *et al.* 2018), dinoflagellate endosymbionts that provide carbohydrates and oxygen, allowing tropical scleractinians to lay down their hard calcium carbonate structures and form the framework for these reef ecosystems (Falkowski *et al.* 1993).

Information on the coral microbiome composition is mostly based on culture-independent approaches that typically analyse nucleic acid sequences extracted directly from the coral samples. In deep waters, the first studies focused on characterising bacteria derived from samples of black and bamboo corals in the Gulf of Alaska (Penn *et al.* 2006) and *Lophelia pertusa* corals from the central Mediterranean Sea (Yakimov *et al.* 2006). Subsequent efforts on samples from deep Norwegian reefs investigated *L. pertusa* in the Trondheimsfjord (Neulinger *et al.* 2008,; Neulinger *et al.* 2009) and *L. pertusa* and *Madrepora oculata* from inshore southern sites and off the mid-coast (Schöttner *et al.* 2012). These studies all demonstrated that the bacterial microbiomes of deep-water corals were diverse, but also displayed conserved host-specific associations similar to what has been observed for shallow tropical scleractinian corals (Rohwer *et al.* 2002; Mouchka, Hewson and Harvell 2010; Bourne and Webster 2013; Glasl *et al.* 2017). Recently a study (Kellogg 2019) which compared bacterial communites across seven deep-sea corals futher confirmed deep-sea corals shared conserved taxa. In shallow-water scleractinian species, shifts in the coral-associated bacterial communities have been explored between seasons and regions (Littman *et al.* 2009; Ceh, van Keulen and Bourne 2010; Roder *et al.* 2015; Sharp *et al.* 2017) and following disturbances (Rosenberg *et al.* 2007; Bourne *et al.* 2008; Bourne and Webster 2013; Jessen *et al.* 2013; Röthig *et al.* 2016; Paulino *et al.* 2017; Ziegler *et al.* 2017).

Corals and their microbiomes potentially interact and co-vary to fine-tune fitness (Torda *et al.* 2017; Webster and Reusch 2017). The microbiome may provide the flexibility needed to adapt to local environmental conditions and provide resilience against perturbation as demonstrated for shifts in Mediterranean Sea *Oculina patagonica* bacterial communities between summer and winter (Reshef *et al.* 2006). Increasing water depth is a strong driver of environmental changes such as light, temperature, nutrients or some combination that shifts the coral microbial communites (Pantos *et al.* 2015; Hernandez-Agreda *et al.* 2016; Glasl *et al.* 2017). The aims of this study were to investigate depth-driven variation of coral-associated bacterial communities. We use 16S rRNA gene amplicon sequencing of new samples in an original study with three common northeast Atlantic deep-water corals, the reef-building hard corals *L. pertusa* and *M. oculata* (subclass Hexacorallia) and the tree-like soft coral *Paragorgia arborea* (subclass Octocorallia) sampled from the Norwegian continental shelf, and combine these sequences in a meta-analysis with previously published datasets from a range of coral hosts across a depth gradient.

METHODS

Sampling

Dives were performed to an unexplored coral reef (C30) at 345 m depth on the mid-Norway continental shelf (Fig. 1) in February 2010 using a working class remotely operated vehicle (ROV) from the multipurpose vessel 'Edda Fauna'. Within a 20 m × 20 m location on the seafloor the ROV manipulator arm collected triplicate 10–25 cm sized branches, 1–2 m apart, from colonies of living *L. pertusa*, *M. oculata* and *P. arborea*. The ROV returned to the location carrying a Niskin bottle and collected a seawater sample approximately 1.5–2 m above the seafloor. Samples of water (2 and 7 L) and corals (triplicates of three species) were frozen at –20°C and transported to the Department of Biology at the University of Bergen, where they were stored at –80°C until further processing.

DNA extraction

Coral branches were thawed on ice and fragments consisting of mucus, tissue and skeleton were sampled using a sterile scalpel. The soft octocoral *P. arborea* was cut into smaller pieces while the stony scleractinians *L. pertusa* and *M. oculata* were ground in separate sections of a sterile (autoclaved) and –80°C pre-cooled mortar. The two seawater samples were thawed overnight and filtered through a 0.2 μm and 47 mm wide cellulose nitrate filter on glass fiber C underlay filters (Millipore, Billerica, MA, USA). DNA was extracted from ∼50 mg samples, from pooled triplicates of each coral species and individually from each of the two filters, using the PowerPlant DNA isolation kit (MOBIO Laboratories, Carlsbad, CA, USA). The method utilises bead beating and enzymatic lysis to break open aggregates and cells and sodium dodecyl sulfate to remove proteins. In an attempt to improve yield, bead tube vortexing was extended by 2× 20 min, with pipetting in between, and DNA was pelleted prior to elution by extended centrifugation for 20 min at maximum speed (∼11 000*g*). DNA was further processed through the clean-up protocol (MOBIO Laboratories). Obtained DNA was assessed by quantification using a NanoDrop 1000 (Thermo Fisher, Waltham, MA, USA) and by size using 1% agarose gel electrophoresis (Sambrook and Russell 2001), before the DNA was stored at –20°C.

PCR and pyrosequencing of 16S rRNA genes

Amplicons for GS-FLX 454 Titanium pyrosequencing were generated from the variable and taxonomically informative V1–V3 region of the 16S rRNA gene, using a two-step protocol (Berry *et al.* 2011) as described previously (Jensen *et al.* 2015). Briefly, the PCR mixture contained 10–100 ng DNA, 0.5 μM of bacterial primer 9bfm (5′-GAGTTTGATYHTGGCTCAG-3′; Mühling *et al.* 2008) and universal primer 518rm (5′-ATTACCGCGGSTGCTGG-3′; Muyzer, De Waal and Uitterlinden 1993), 200 μM of each dNTP, 20 μg mL^{-1} bovine serum albumin (BSA) and 0.4 U Phusion DNA polymerase in 1× HF buffer (Finnzymes, Espoo, Finland). Reactions were performed in duplicate for each sample. The first step (50 μL reactions) was performed with the thermocycler PTC-200 (Bio-Rad, Hercules, CA, USA) set to denature at 98°C for 1 min

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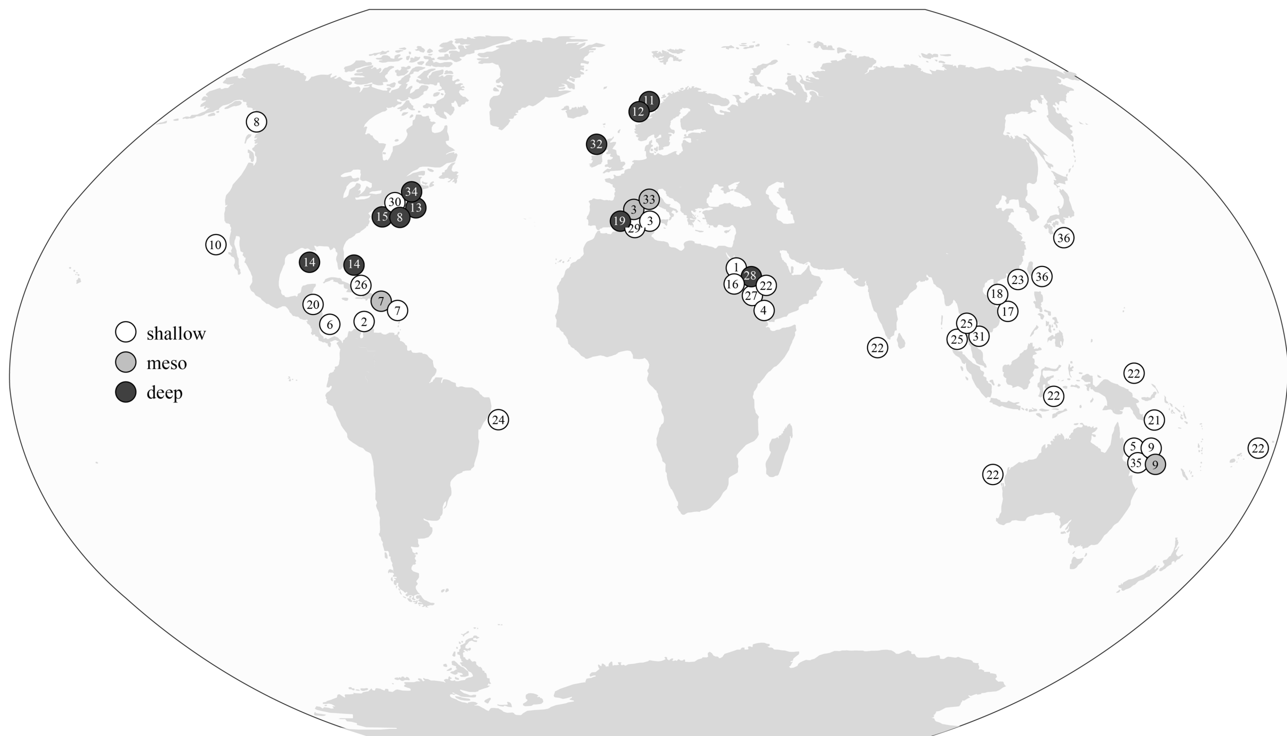

Figure 1. Locations of coral reef habitats with sequences for this study. New samples were collected from a reef C30 on the continental shelf off mid-Norway (11; 65° 09′ 21′′ N, 06° 28′ 30′′ E). Habitat numbering refers to study (see supplementary Table S1 available online): *Acropora* (5, 21), *Agaricia* (7), *Alcyonium* (15), *Anthothela* (15), *Antillogorgia* (26), *Antipathes* (18), *Astrangia* (30), *Coelastrea* (31), *Corallium* (33), *Ctenactis* (1), *Dendrophyllia* (28), *Eguchipsammia* (28), *Eunicella* (3), *Fungia* (27), *Herpolitha* (1), *Lobophytum* (35), *Lophelia* (11, 14, 19, 32), *Madracis* (7), *Madrepora* (11, 19), *Montastraea* (2), *Muricea* (10), *Oculina* (29), *Pachyseris* (9), *Palythoa* (24), *Paragorgia* (11, 34), *Paramuricea* (13), *Platygyra* (23), *Pocillopora* (5, 16, 22), *Porites* (17, 20, 21, 25), *Primnoa* (8), *Pseudopterogorgia* (6), *Rhizotrochus* (28), *Sarcophyton* (16), *Scleronephthya* (36), *Seriatopora* (5), *Sinularia* (5), *Stephanocoenia* (7), *Stylophora* (4, 22) and seawater (1, 3, 5, 10, 11, 12, 22, 26, 27, 28, 30, 32, 33, 34).

and to cycle 20 times using denaturation at 98°C for 10 s, annealing at 53°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The second step (20 μL reactions) was performed with 2 μL first-step reactions for 10 cycles with the primers joined to adapters and sample-specific multiplex identifiers at the 5′ end (Lib-L chemistry). All amplicons were checked for size and purity by agarose gel electrophoresis followed by staining with ethidium bromide (Sambrook and Russell 2001). Duplicates were pooled and purified using Agencourt AMPure beads (Beckman Coulter Genomics, Beverly, MA, USA) and quantified using a Qubit fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA). The amplicons were frozen at –20°C and sent to the Research and Testing Laboratory LLC (Lubbock, Texas, USA) for emulsion PCR and Roche 454 FLX sequencing with the reverse primer.

Sequence analysis of C30 deep-water corals

The sequences were analysed in mothur (Schloss *et al.* 2009). Following the 454 standard operating procedure, sequences were extracted from fastq files and filtered for errors by mothur-implemented PyroNoise (Quince *et al.* 2011) using a minimum average quality score window of 30. Sequences with ambiguous nucleotides, barcodes, homopolymers (>7 nt), short length (<200 nt) and potential chimeras found relative to the more abundant sequences using UCHIME (Edgar *et al.* 2011) were discarded, but the primers were kept to ease recognition in the meta-data analysis. The C30 sequences were aligned in SILVA 128 (Quast *et al.* 2013) using kmer searching with 8mers and the Needleman-Wunsch method and screened to overlap in position 303 to 534 (*Escherichia coli* numbering). Sequences were normalized across samples by subsampling to the smallest sample, although in addition a compositional replacement of normalisation has been developed to avoid loss of low abundant sequences, as detailed by Gloor *et al.* (2017). Operational taxonomic units (OTUs) were clustered by the opticlust method at 97% nucleotide identity to define species. Sequence classification was performed to the lowest possible level using the method implemented by RDP (Wang *et al.* 2007) with the SILVA 128 alignment database and taxonomy reference (Quast *et al.* 2013) at a bootstrap confidence value of 75%. A total of 3.1% of the reef C30 sequences did not classify as Bacteria at the domain level (Archaea, Eukarya, chloroplast, mitochondria, unknown) and were discarded. OTUs unclassified beyond phyla represented 8.7% of the sequences. To explore poorly classified OTUs, and find nearest relatives and the origin of affiliates, BLASTN (Altschul *et al.* 1997) searches were performed. The sequences chosen to make the phylogenetic tree were representative of the dominant sequences recovered from the study. Reference sequences of each coral's most abundant OTU were downloaded from GenBank and imported into ARB (Ludwig *et al.* 2004). A filter was generated that excluded alignment positions with sequence ambiguity and positions where <50% of the sequences had a nucleotide. The alignment was exported for construction of a maximum-likelihood tree (DNAML) and bootstrapping (SEQBOOT, CONSENSE) in Phylip (Felsenstein 2013). The tree was imported into ARB for the addition of short sequences using the parismony tool. Pyrosequence flowgrams from the reef C30 samples are available at NCBI under BioProject PRJNA428587.

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Table 1. Overview of the data sets, normalized to 4665 sequences per sample for the C30 study (upper) and 2000 sequences per sample for the meta-study (lower).

Sample	Sequences	N (genera)	OTUs	Phyla	Shannon	Coverage (%)	Singletons
L. pertusa	6579	3 (1)	329	22	3.42	98.6	282
M. oculata	4665	3 (1)	116	14	1.67	99.0	56
P. arborea	7697	3 (1)	61	12	1.66	99.5	31
Seawater	7190	2 (–)	388	22	3.95	98.2	496
Deep	2935–213 141	54 (10)	11–238	4–33	0.75–4.32	96.3–100.0	0–7
Meso	3351–144 313	18 (6)	26–169	6–21	0.36–4.05	97.1–99.6	0–11
Shallow	2212–223 670	66 (27)	20–281	2–31	0.30–4.82	95.2–99.7	0–30
Seawater	2362–346 969	27 (–)	66–304	4–27	2.27–4.43	93.8–98.8	0–20

Sequence analysis of meta-data across depth gradients

Downloaded data sets were found by searching for citations to early literature (Penn *et al.* 2006; Yakimov *et al.* 2006; Kellogg, Lisle and Galkiewicz 2009) and by using keywords combined with wildcards (deep, cold, coral, bacteria) to search the sequence archives (NCBI, SRS). Sequences from all deep-water and mesophotic studies found were selected and contrasted with an arbitrary selection of sequences from complementary shallow-water studies. The sequences (454, MiSeq) retrieved from these samples, similar to sequences recovered from the C30 reef samples, had been PCR amplified from 16S rRNA gene fragments of total DNA extracted from samples of visibly healthy corals and surrounding seawater. The downloaded sequences were in the same way as the C30 sequences, quality checked, normalized (large data sets were also subsampled before the uchime step) and analysed by the mothur-implemented programs (Schloss *et al.* 2009). Because different PCR primers had been used to amplify these sequences (supplementary Table S1 available online) they do not all overlap in the same alignment space and were therefore clustered into OTUs by phylotype, by taxonomic identity using closed reference picking against the Silva 128 reference database (Quast *et al.* 2013), to the lowest possible level (label 1). Such phylotyping has, for example, already been used to compare human microbiomes across studies (Lozupone *et al.* 2013). Analyses of all sequences (C30 and downloaded) were performed with singletons removed from the OTU abundance table (shared file). The analyses estimated sample richness and evenness (alpha diversity), generated sample-based Venn diagrams, identified differentially abundant OTUs representative of strongest indicator species and generated a heatmap and a distance matrix from the meta-data (beta diversity). Matrices were calculated using Bray-Curtis distance metric based on alignment (C30) and on phylotypes (meta-study). The relationship between samples was obtained from the distance matrix and visualised using ordination by principal coordinate analysis (PCoA). Selected OTUs responsible for shifting samples along the two axes were included in the PCoA visualisation. To judge statistical significance a nonparametric group test (analysis of molecular variance, AMOVA) was performed ($P < 0.05$).

RESULTS

Bacteria associated with corals from the C30 reef

Samples collected for this study were obtained from a reef C30 at 345 m depth on the mid-Norwegian coast (Fig. 1). In this region of the Halternbanken fishing ground, many coral reefs are scattered alongside small (<5 m diameter, <1 m deep) depressions in the seafloor known as unit pockmark craters, and among these reef C30 is located over a flat muddy seafloor at the hydrocarbon production field Morvin (Hovland 2008; Hovland, Jensen and Indreiten 2012). The C30 reef is about 100 m long, 20 m wide, 3–4 m tall and dominated by numerous bulbous visibly healthy colonies of *L. pertusa* up to 2 m in diameter. Environmental factors previously measured at Haltenbanken reefs revealed a temperature of 6°C, a top sediment content of 18 μM methane, 3 μM ethane, 1.8 μM propane and 0.8 μM n-butane (Hovland, Jensen and Indreiten 2012), and overlying seawater levels of 8 nM CH_4, 75 nM NO_2^-, 300 nM H_2S, 725 nM PO_4^{3-}, 8500 nM SiO_4^- and 12 350 nM NO_3^- (Jensen *et al.* 2012). The C30 *L. pertusa*, *M. oculata*, *P. arborea* and seawater samples collected provided 4665 normalized 16S rRNA gene sequences each (Table 1). Alignment and 97% identity clustering profiled the associated microbiomes into a combined richness of 648 OTUs from 25 bacterial phyla (Fig. 2A). The OTUs distributed unevenly between samples, some being unique to one sample, others being shared among samples, with increasing similarity by host subclass (supplementary Fig. S1 available online). The Proteobacteria dominated and represented 40–80% of sequences from any sample. By alpha diversity, Shannon's H' for *L. pertusa* was 3.42 and nearly twice that of *M. oculata* and *P. arborea* (Table 1). Beta diversity was skewed towards five OTUs, 1, 2, 3, 4 and 6, that represented >50% of retrieved sequences from corals but <0.1% of sequences from seawater. Three of these OTUs were unique, host-specific and dominated the deep-water coral microbiomes (Fig. 2A). Inserted into a phylogenetic tree (Fig. 2B), the most abundant OTU1 (2491 sequences) was recovered from *M. oculata* and affiliated (90% identity) with uncultured Bacteroidetes sequences within Flavobacteria from the tropical scleratinian coral *Muricea elongata* (Ranzer *et al.* 2007 unpublished DQ917866). OTU3 (2159 sequences) dominated *P. arborea* and affiliated (96% identity) with uncultured Tenerecutes within the Mollicutes genus *Mycoplasma*, previously recovered from seawater collected next to mesophotic sea pen octocorals in the Mediterranean Sea (Porporato *et al.* 2013) and samples of the deep-water *Cryogorgia koolsae* octocoral in the Aleutian Islands (Gray *et al.* 2011). The *P. arborea* also hosted a relatively abundant Rickettsiales-affiliated sequence type (OTU7, 939 sequences), similarly recovered from *C. koolsae*. OTU6 (1019 sequences) dominated the *L. pertusa* samples and affiliated (99% identity) with uncultured Alphaproteobacteria of the Rhizobiales from *L. pertusa* samples in the Gulf of Mexico (Kellogg, Lisle and Galkiewicz 2009). The *L. pertusa* microbiome included 354 of the 379 Verrucomicrobia-affiliated sequences from the

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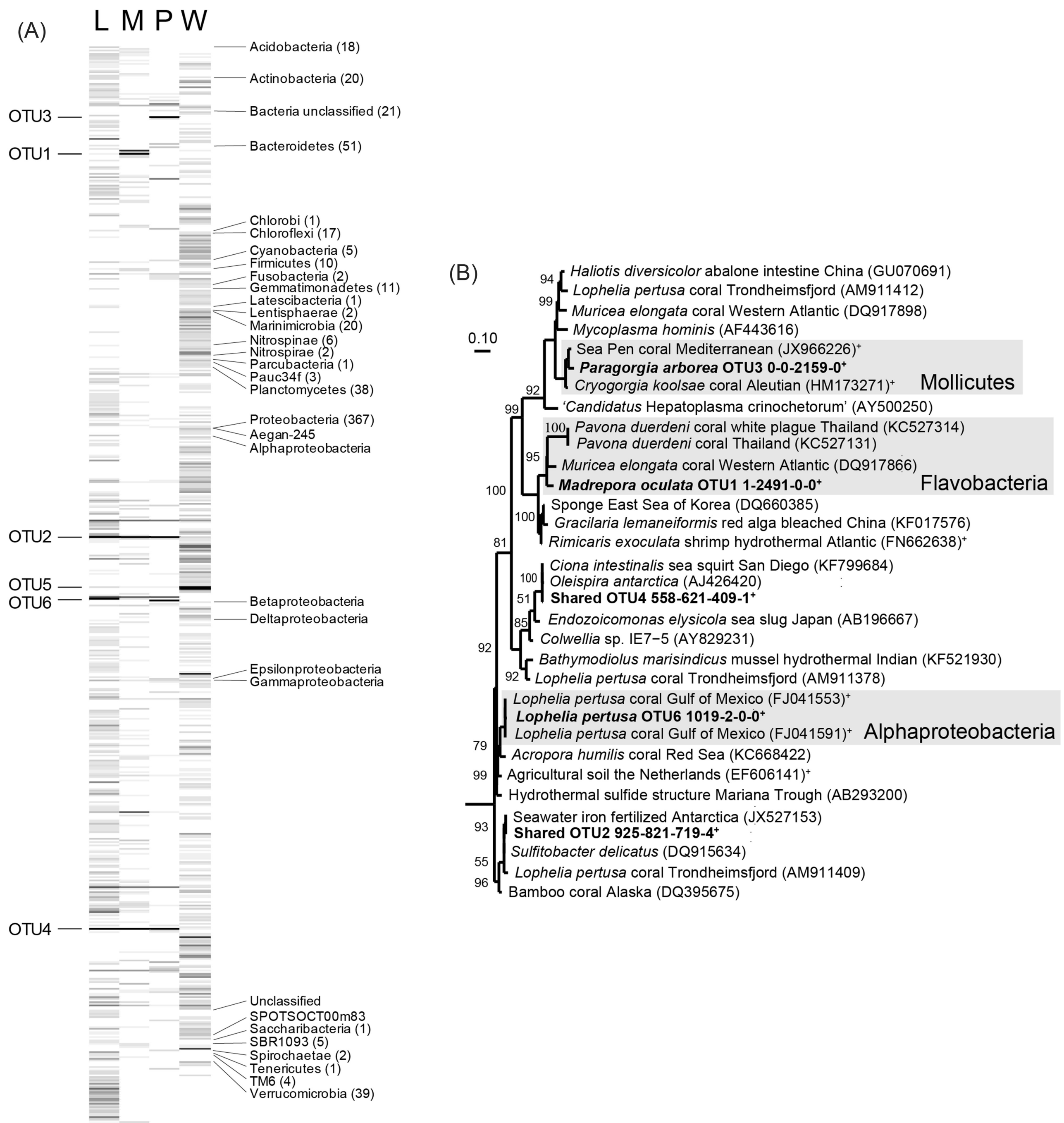

Figure 2. Heatmap representing bacterial community profiles (A) and maximum-likelihood phylogeny (B) of OTUs associated with the C30 *Lophelia pertusa* (L), *Madrepora oculata* (M), *Paragorgia arborea* (P) and surrounding seawater (W). The profiles are marked with domininant OTUs 1-6 (left) as sorted alphabetically by phyla including OTU abundance (right; Proteobacteria sorted by class). The tree shows the dominant OTUs phylogenetic positions including sequence abundance in community profile order. Shaded boxes indicates OTUs dominating each coral host. The tree was constructed in PHYLIP (Felsenstein 2013) with a filter of 1088 aligned 16S rRNA gene postitions and in ARB (Ludwig *et al.* 2004) added sequences representative of dominant OTUs including six other short sequences (marked $^{+}$). Bootstrap values above 50% are indicated at the branch points. *Archaeoglobus fulgidus* was used as outgroup. The scale bar indicates 10% sequence divergence.

C30 samples and these distributed across 33 of 39 different OTUs. OTUs 2 and 4 were shared among all three coral host species and their sequences affiliated (99–100% identity) with known and cultured bacteria. These were (Fig. 2B) Alpha- and Gammaproteobacteria from various marine sources and recognised as the Rhodobacterales *Sulfitobacter* (OTU2) previously retrieved from iron-fertilised Southern Ocean seawater and the Oceanospirillales *Oleispira* previously retrieved from a sea squirt (OTU4). The dominant OTUs associated with these deep-water coral bacterial communities displayed aspects of the less abundant OTUs, including affiliation with unique coral restricted species, shared affiliation with previously characterised species

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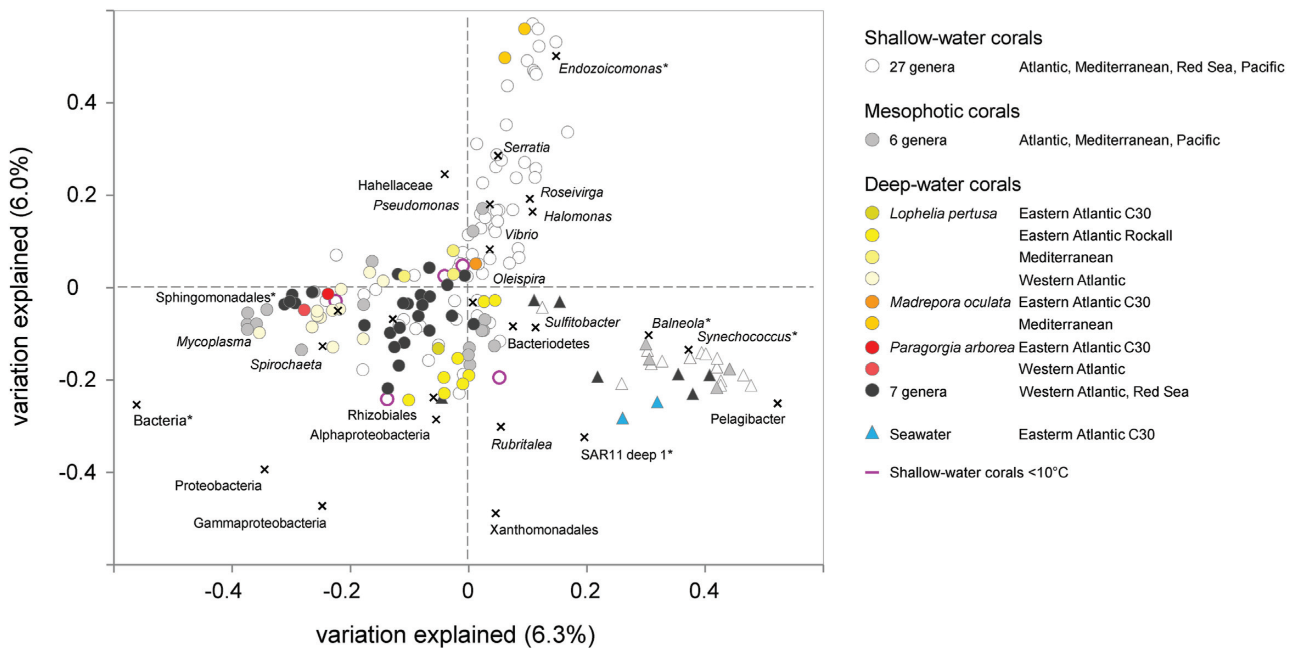

Figure 3. PCoA of pairwise Bray-Curtis distances summarizing bacterial community composition from 165 microbiome samples of coral (circles) and surrounding seawater (triangles). Symbol fill indicates sampling depths and fill colour indicates C30 samples and samples from similar host species. Crosses represent some of the most discriminating OTUs from the axes correlation analysis. Significant indicator species of highest relative abundance and frequency of occurrence are indicated with an asterisk (∗).

and many being different from sequences in the surrounding seawater. The reef C30 seawater was dominated by OTU5 (Fig. 2A) that included 1289 sequences affiliated (99% identity) to SAR11 surface 1 bacterioplankton of the '*Candidatus* Pelagibacter ubique'.

Meta-analysis of bacterial communities across depth gradients

Previous culture independent studies were used to contrast with the bacterial taxa characterised from the C30 reef sampled corals (supplementary Table S1 available online). Classification of 2000 normalized sequences from each of 54 deep-water, 18 mesophotic and 66 shallow-water coral host samples plus 27 surrounding seawater samples (Fig. 1) profiled the bacterial communites into a combined richness of 1317 taxa from 49 phyla (Table 1). Comparable to the C30 samples, the taxa were distributed unevenly (supplementary Fig. S2 available online) and were also dominated by the Proteobacteria (supplementary Fig. S3 available online). Alpha diversity indices were very different across all samples in each depth zone and Shannon's H' ranged from 0.3 in the photosynthetic *Coelastrea aspera* to 4.8 in the non-photosynthetic *Astrangia poculata* (Table 1, supplementary Fig. S3 available online), but average values did not differ significantly for corals in any depth zone (t-test, $P > 0.20$). Beta diversity was skewed and sequence abundance for taxa representing dominant reef C30 associated OTUs 1, 3 and 6 varied (supplementary Fig. S3 available online). The 24 other *L. pertusa* samples were dominated by Spongiibacteraceae (Cellvibrionales, Gammaproteobacteria) from Rockall Bank samples (Van Bleijswijk *et al.* 2015), Anaplasmataceae (Rickettsiales, Alphaproteobacteria) from Mediterranean Sea samples (Meistertzheim *et al.* 2016) and Sphingomonadales (Alphaproteobacteria) from Western Atlantic samples (Kellogg, Goldsmith and Gray 2017). The two other *M. oculata* samples were dominated by *Endozoicomonas* (Oceanospirillales, Gammaproteobacteria) from the Mediterranean Sea samples (Meistertzheim *et al.* 2016). The other *P. arborea* sample was dominated by unclassified Bacteria from the Gulf of Maine (Weiler, Verhoeven and Dufour 2018). Sequences most abundantly associated with the *L. pertusa*, *M. oculata* and *P. arborea* samples were represented by <88 sequences from any seawater sample. More than 50% of the coral bacterial sequences fell into 22 taxa. The largest taxon was unclassified Bacteria, representing 7.7% of the coral sequences and present in 94% of the coral samples, with a maximum sequence abundance of 95% in *Pachyseris speciosa* samples from mesophotic depths (supplementary Fig. S3 available online). Corals abundantly associated with taxa only classified to the kingdom Bacteria had all been sampled at water depths >30 m or they lacked photosymbionts. Ordination of Bray-Curtis distances among all taxa using PCoA (Fig. 3) clustered the C30 *L. pertusa* microbiome nearest to *L. pertusa* corals from the Rockall Bank (Van Bleijswijk *et al.* 2015). The C30 *M. oculata* microbiome clustered away from other *M. oculata* samples (Meistertzheim *et al.* 2016), while the C30 *P. arborea* microbiome clustered with the other *P. arborea* (Weiler, Verhoeven and Dufour 2018) sample plus Western Atlantic *L. pertusa* (Kellogg, Goldsmith and Gray 2017) and *Anthothela grandiflora* (Lawler *et al.* 2016) samples. The PCoA (Fig. 3) further clustered deep-water and mesophotic coral microbiomes together and away from shallow-water corals (AMOVA Fs = 5.59, $P < 0.001$). Collectively, the coral microbiomes differed significantly from the surrounding SAR11-dominated bacterioplankton (AMOVA Fs = 12.78, $P < 0.001$).

Bacteria associated with coral depth zonation

Analysing for taxa responsible for shifting the coral microbiomes along the two PCoA axes revealed the least classified Bacteria, Proteobacteria and Gammaproteobacteria groupings to

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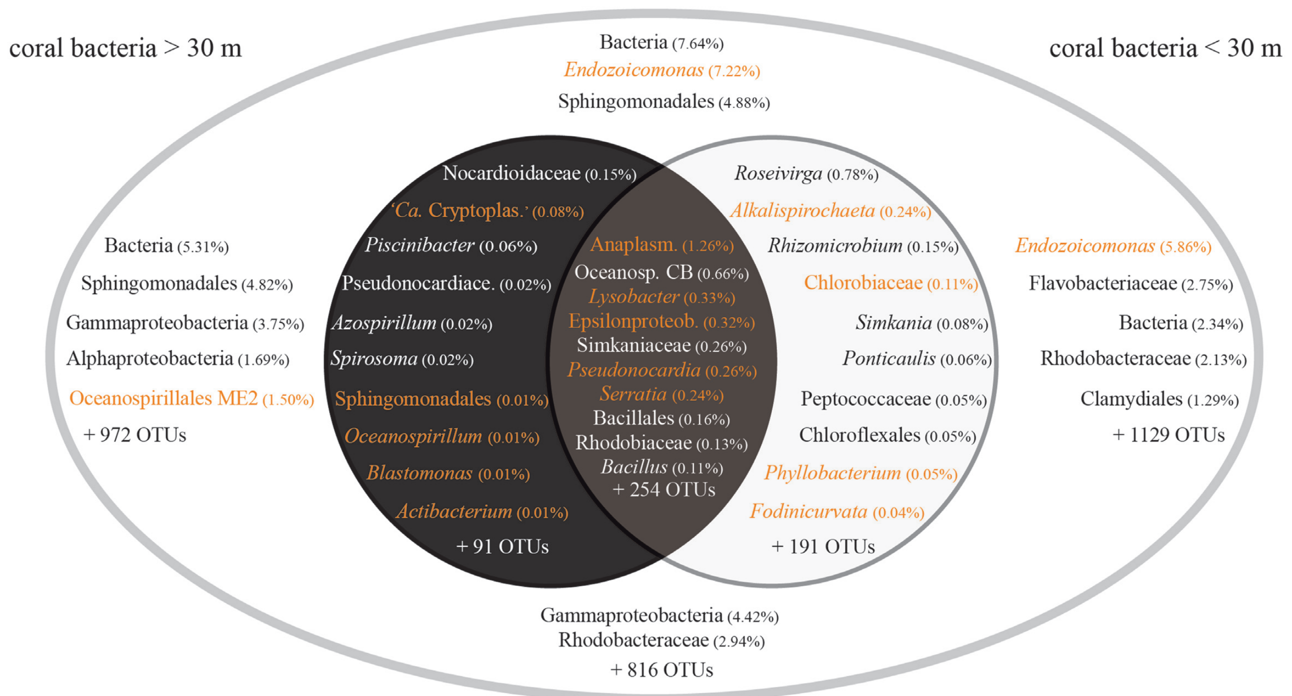

Figure 4. Bacterial taxa enriched in coral microbiomes across depth ranges. The inner left circle indicates sequences exclusively from corals at greater than 30 m depth and the inner right circle indicates sequences exclusively from corals at shallower than 30 m depth. Overlap between circles indicates sequences from both depth ranges. Taxa surrounding the circles include sequences from seawater but are similarly grouped according to depth. Abundance is given relative to the 275 757 sequences from all the 138 coral samples (parentheses). Colour indicates coral sample source as the closest BLASTN hit.

be more strongly correlated with deep-water and mesophotic corals (Fig. 3). Separation by depth was further contributed by distinct indicator species. The strongest indicator species for coral microbiomes below 30 m depth affiliated with taxa classified under the general gammaproteobacterial grouping [indicator value (iv) = 62]. Throughout, the strongest indicator species were Sphingomonadales at below 200 m depth (iv = 51), Bacteria at mesophotic 30–200 m depth (iv = 55) and *Endozoicomonas* at shallow above 30 m depth (iv = 54). For the surrounding bacterioplankton (Fig. 3), the corresponding depth horizons were characterised by the indicator species (iv = 63–95) SAR11 deep 1 (deep), *Balneola* (mesophotic) and *Synechococcus* (shallow). Bacterioplankton sequences were absent from nearly half the 1290 coral-associated taxa (Fig. 4). Among these 566 taxa, the most frequent and the most enriched species were found across all depth ranges and belonged to Alphaproteobacteria. The most frequent belonged to unclassified species of the family Bradyrhizobiaceae, shared across 25% of corals with a maximum 60 sequences retrieved from the Western Florida *L. pertusa* sample (Kellogg, Goldsmith and Gray 2017). The most enriched affiliated to unclassified species of the family Anaplasmataceae, representing 1.3% of all the coral sequences obtained, with a maximum of 1538 sequences in a Mediterranean *L. pertusa* (Meistertzheim *et al.* 2016). Below 30 m depth these most enriched coral represented taxa belonged to the Actinobacteria Nocardioidaceae (0.15%) and the Alphaproteobacteria 'Ca. Cryptoplasma' (0.08%), the latter enriched below 200 m water depth (Fig. 4).

DISCUSSION

To date, culture-independent molecular techniques have profiled the bacterial communities associated with 13 species of corals from waters greater than 200 m depth (Penn *et al.* 2006; Yakimov *et al.* 2006; Kellogg, Lisle and Galkiewicz 2009; Van Bleijswijk *et al.* 2015; Kellogg, Ross and Brooke 2016; Lawler *et al.* 2016; Meisterzheim *et al.* 2016; Kellogg, Goldsmith and Gray 2017; Röthig *et al.* 2017; Goldsmith *et al.* 2018; Weiler, Verhoeven and Dufour 2018), including two from Norwegian waters (Neulinger *et al.* 2008; Schöttner *et al.* 2012). Our reef C30-sampled bacterial communities reflect many of the previously identified community patterns, emphasising conservation of some potentially important bacterial taxa in microbiomes of deep-water corals.

Along the Northeast Atlantic margin *L. pertusa* represents an important builder of deep coral reefs (Freiwald 2002; Hovland 2008). In the C30 reef, *L. pertusa* hosted the most diverse coral bacterial communities and included 21 of the 25 bacterial phyla associated with the C30 samples. All these represented phyla sequences associated with other deep-water corals analysed through the meta-analysis, however at variable abundance. For example, the dominant alphaproteobacterial sequences (OTU6) of *L. pertusa* at reef C30 shared 99% identity with less abundant 4753K3 clone sequences derived from *L. pertusa* samples from 460 m depth at station VK826 in the Gulf of Mexico (Kellogg, Lisle and Galkiewicz 2009). These sequences affiliated to Fhm clone sequences from a hydrothermal mound (95% identity) and may be related to the widespread coral associated family Bradyrhizobiaceae (meta-study), which for shallow-water corals have been characterised based on the presence of *nifH* sequences, suggesting potential nitrogen-fixation roles (Lema, Willis and Bourne 2012). The OTU6 sequence type may represent conserved (specialists) taxa analogous to sponge-specific clades in deep water (Jensen *et al.* 2017). The formation of these clades may reflect

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different adaptive strategies (Reshef *et al*. 2006), resource partitioning or the heterotrophic diet, each which may help different coral host species to share the same habitat (Meistertzheim *et al*. 2016). The *L. pertusa* was also characterised by hosting most of the Verrucomicrobia sequences retrieved from reef C30 and all sequences of the 11 OTUs classified as *Rubritalea*. Neulinger *et al*. (2008) suggested *L. pertusa*-associated *Rubritalea* in the Norwegian Trondheimsfjord to be involved with the degradation of cellulose present in food particles. Similarly, cellulose maybe degraded by *L. pertusa*-associated Cellvibrionales at the Rockall Bank off the coast of Ireland (Van Bleijswijk *et al*. 2015).

Previous studies on the bacterial communites of *M. oculata* identified *Endozoicomonas*-affiliated sequences as dominant in samples from the Northwestern Mediterranean Sea (Meistertzheim *et al*. 2016) and Rockall Bank (Hansson *et al*. 2009). Interestingly, *Endozoicomonas* belongs to a clade of marine invertebrate-associated Oceanospirillales and is a putative symbiont typically associated with healthy shallow-water corals and a range of other widespread marine invertebrates (Bayer *et al*. 2013a; Neave *et al*. 2016). No sequences classified as *Endozoicomonas* were recovered from the C30 *M. oculata*, though BLASTN analysis of 98 unclassified gammaproteobacterial sequences (OTU16) did exhibit 97% identity with *Endozoicomonas elysicola*. The C30 *M. oculata* samples were instead dominated by a novel coral-associated Flavobacteriales (OTU1) that displayed 90% identity with an *Elizabethkingia* sequence (DQ917866). Interestingly, *Elizabethkingia* was the third largest OTU in the Mediterranean *M. oculata* coral samples (Meistertzheim *et al*. 2016), while *Endozoicomonas* was the fifth largest OTU in C30 *M. oculata* samples (BLASTN). Variation in relative bacterial abundance across coral samples may be attributed to sampling different microniche habitats within the corals, for example the skeleton was not included with the samples of Meistertzheim *et al*. (2016) and Hansson *et al*. (2009) although it was included in the current study. In addition, local environmental characteristics (Kellogg, Goldsmith and Gray 2017), such as food, with reef C30 being rich in copepods as indicated from plankton sampled from nearby areas (Jensen *et al*. 2015), and water temperatures also likely influence the associated bacterial communities (Rosenberg *et al*. 2007; Bourne *et al*. 2008). At the Mediterranean site the temperature was 13°C, while it was 6°C at the C30 site. Notably, corals with a broader niche range have been reported to possess higher variability in their bacterial communities (Glasl *et al*. 2017) and *M. oculata* belongs to the polyphyletic familiy Oculinidae, with members in both deep and shallow waters, with and without photosymbionts (Kitahara *et al*. 2010).

Culture-based studies with glycerol artificial seawater agar isolated *Vibrio* strains from the deep-water coral species *P. arborea* from the Aleutian Islands (Gray *et al*. 2011). By culture-independent assessment of the *P. arborea* bacterial community, *Vibro* sequences were not identified in the C30 samples, but some were retrieved from the Gulf of Maine samples (Weiler, Verhoeven and Dufour 2018). Instead a distinct *Mycoplasma* (OTU3) dominated the bacterial community of *P. arborea* from reef C30. It affiliated most closely (>95% identity) with *Mycoplasma* sequences derived from Octocorals including a Mediterranean sea pen (Porporato *et al*. 2013) and Aleutian *C. koolsae* (Gray *et al*. 2011). *Mycoplasma* sequences also dominated the Gulf of Maine *P. arborea* samples (Weiler, Verhoeven and Dufour 2018), but whether these two *P. arborea* mycoplasmas belong to the same phylogenetic clade is unclear, because of the non-overlapping, poorly classified sequences. Less affiliated (<95% identity) were mycoplasmas from Aleutian *Plumarella superba* (Gray *et al*. 2011) and the '*Ca*. Mycoplasma corallicola' from *L. pertusa* (Neulinger *et al*. 2008; Kellogg, Lisle and Galkiewicz 2009). The second largest OTU, *Rickettsiales* (OTU7), affiliated with *Anaplasma* and *Wolbachia* (96–98% identity) and may be derived from coral-associated microfauna as suggested by Gray *et al*. (2011).

Reef C30 is located at a site with glacimarine clay-rich and organic-poor sediment having pockmark craters indicative of perennial gaseous hydrocarbon seepage (Hovland, Jensen and Indreiten 2012). Abundant taxa found shared among the three C30 corals belong to the genera *Sulfitobacter* (OTU2) and *Oleispira* (OTU4) and these associations could be driven by elevated concentrations of hydrocarbons. Corals from the VK826 site in the Gulf of Mexico are also adjacent to localised seepage of hydrocarbons and also displayed an abundance of potential hydrocarbon degraders, *Novosphingobium* and *Pseudonocardia* (Kellogg, Lisle and Galkiewicz 2009). Furthermore, Röthig *et al*. (2017) documented *Oleibacter*-affiliated sequences, associated with *Dendrophyllia* corals in the warmer Red Sea and similarly postulated hydrocarbons as a potential carbon source driving the association. The *Sulfitobacter* may be abundant due to utilisation of sulfur compounds derived from hydrocarbons seeping from the sediments. These sequences were found shared among different hosts in the same depth range, potentially indicating these are transient environmentally influenced host associates (generalists).

The most abundant taxon identified in the meta-analysis (which include OTU3 from the C30 reef samples) was the unclassified Bacteria, which was enriched in coral samples below 30 m depth. In total, about 6500 (one quarter) of these unclassified Bacteria sequences displayed 90–97% BLASTN identity with sequences from characterised bacteria, of which about 4000 affiliated with the phylum Firmicutes and possibly the order Clostridiales and genus *Desulfitispora*. These species are anaerobic halophiles with a restricted respiratory metabolism, growing with pyruvate and lactate as the electron donor and sulfite, thiosulfate or elemental sulfur as the electron acceptor (Sorokin and Muyzer 2010). The association of deep-water corals with bacteria potentially involved with the sulfur cycle was noted in early studies (Penn *et al*. 2006; Neulinger *et al*. 2008; Kellogg, Lisle and Galkiewicz 2009). In the algae-devoid coral tissue (Falkowski *et al*. 1993) oxygen minimum zones are likely and this may enrich for sulfur-reducing symbionts that under low oxygen conditions uses host-fermentation products, as observed in a gutless marine worm (Blazejak *et al*. 2006) and discussed for the *Geodia barretti* sponge (Jensen *et al*. 2017).

Recently, Sharp *et al*. (2017) suggested environmentally driven changes to largely influence the coral microbiome by modifying host physiology. These pattern may be reflected in the bacterial communities associated with *Astrangia poculata* sampled from Rhode Island (Sharp *et al*. 2017) and *Primnoa pacifica* sampled from the Gulf of Alaska (Goldsmith *et al*. 2018), which are species living in shallow yet cold waters (3–5°C), though clustered among deep-water corals, analogous to the clustering of bacterial communities from some *M. oculata* (13°C) among shallow-water corals. The meta-analysis separated bacterial communities of shallow-water corals from those of deep-water and mesophotic corals. This was driven by a strong correlation of abundant *Endozoicomonas* and often co-occurring *Roseivirga* with the shallow-water corals. Environmental imprinting, including temperature (Rosenberg *et al*. 2007; Bourne *et al*. 2008; Ziegler *et al*. 2017), salinity (Röthig *et al*. 2016), light levels (Olson and Kellogg 2010), biogeography (Schöttner *et al*. 2012) and carbon or other nutritional inputs (Jessen *et al*. 2013; Paulino *et al*. 2017), has previously been found to influence the coral host-associated microbiomes. Many of

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these conditions change by water depth, thereby contributing to the variability observed among the coral microbiomes. This bacterial species zonation observed by water depth is also not surprising given that deep-water corals are phylogenetically distinct species (Kitahara *et al.* 2010) and that previous studies have suggested some host conservation of microbiomes in shallow (Rohwer *et al.* 2002; Mouchka, Hewson and Harvell 2010; Bourne and Webster 2013), mesophotic (Olson and Kellogg 2010; Pantos *et al.* 2015; Hernandez-Agreda *et al.* 2016; Glasl *et al.* 2017) and deep-water (Neulinger *et al.* 2008; Kellogg, Lisle and Galkiewicz 2009; Kellogg 2019) corals. Indeed, though coral reef species vary across depth and geographic gradients, just like species in forest ecosystems, this does not mean that species swap climate zones to compensate for environmental change.

Although there are limitations in the approach of the current study, due primarily to bias associated with different DNA extraction approaches and 16S rRNA primers and target regions used for PCR amplification, results statistically separate out host bacterial communities by depth zones. This is supported by the large number of host samples and associated sequences incorporated into the meta-analysis. Interestingly the PCoA separated coral bacterial communities by depth zones; however, more OTUs were shared among deep- and shallow-water corals. This is likely a result of more coverage of bacterial communities for the shallow- and deep-water corals, with currently few mesophotic coral microbiomes characterised. The analyses were all performed for sequences clustered the same way, either for well-defined OTUs generated from alignments or for variable but similar across group OTUs generated from classification. The systematic compositional differences appeared large enough to outweigh technical variation (Lozupone *et al.* 2013).

In conclusion, the diversity of bacterial communities associated with samples from *L. pertusa*, *M. oculata* and *P. arborea* corals from the mid-Norwegian shelf reef C30 differed among host species and represented 402 OTUs from at least 21 phyla. Abundantly associated with the corals were five distinct OTUs from the Proteobacteria, Bacteroidetes and Tenericutes, all of which were rare in the surrounding seawater. Three of these species were conserved by coral hosts and represented poorly known Alphaproteobacteria, Flavobacteria and Mycoplasma, suggesting tissue-associated specialists. Two species *Oleispira* and *Sulfitobacter* were shared by hosts and also found abundant in non-coral samples, suggesting weaker association and generalists. Insights from these diversity patterns were expanded by the comparison with bacterial communities from widespread corals across depth zonation. A pattern emerged with clear separation for deep and mesophotic corals from shallow-water species, indicative of both host and environment influencing the microbiomes. Finally, evidence is accumulating that distinct bacterial communities inhabit deep-water corals.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* Journal online.

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