Marine cyanolichens from different littoral zones are associated with distinct bacterial communities

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Abstract

Lichens symbioses, commonly described as fungal partnerships with a photosynthetic partner (green algae and/or cyanobacteria) are associated with a diverse bacterial community. Whereas the microbiome diversity and function of terrestrial lichens has been well studied, knowledge about the non-photosynthetic bacteria associated with marine lichens is still scarce. Since there is a clear vertical zonation of lichens across the littoral belt we hypothesized that this correlates with significant variations of bacterial communities associated with these lichens. To test this hypothesis, 16S rRNA gene illumina sequencing was used to assess the culture-independent bacterial diversity in the strictly marine cyanolichen species *Lichina pygmaea* and *Lichina confinis*, and the maritime chlorolichen species *Xanthoria* sp. collected from the French Brittany coast. These species occupied the lower eulittoral, upper eulittoral and supralittoral zones respectively. Inland terrestrial cyanolichens from Austria were also analysed as for the marine lichens to examine further the impact of habitat/lichen species on the associated bacterial communities. The *L. confinis* and *L. pygmaea* communities were significantly different from those of the terrestrial lichens with the Bacteroidetes phylum accounting for 50% of the sequences, whereas Alphaproteobacteria, notably *Sphingomonas*, dominated the terrestrial lichens. Bacterial communities associated with the two *Lichina* species were significantly different sharing only 33 core OTUs, half of which were affiliated to the Bacteroidetes genera *Rubricoccus*, *Tunicatimonas* and *Lewinella*, suggesting an important role of these species in the marine lichen symbiosis. The presence of multiple OTUs of these genera that were differentially distributed between the two *Lichina* species, could be suggestive of ecological speciation driven by adaptation to the different conditions of the intertidal and supratidal zones. Marine cyanolichens showed a higher abundance of OTUs likely affiliated to moderately thermophilic and/or radiation resistant bacteria belonging to the Phyla Chloroflexi, Thermi, and the families Rhodothermaceae and Rubrobacteraceae when compared to those of terrestrial lichens. This most likely reflects the exposed and highly variable conditions to which marine lichens are subjected to daily.

Introduction

The lichen symbiosis, commonly recognized as a partnership of a fungus (mycobiont), and a photosynthetic partner (photobiont) arose already with the conquest of land in the lower Devonian, according to the first clear fossil evidence (Honegger et al. 2013). The enclosure of the photobiont partner by protective layers of the fungal partner (which provides the scientific name of lichens) gave rise to a new morphological structure. This symbiotic structure is called the lichen thallus, which apparently mediates high degree tolerance to desiccation (Kranner et al. 2008) allows many lichens to thrive as poikilohdric organisms in environments characterized by periodic changes in environmental conditions. Therefore, lichens are typically found in habitats where other organisms have trouble to persist. This also includes the intertidal belt of coastal rocks, where lichens develop characteristically colored belts.

Bacteria were already found in the oldest microfossils that can reliably assigned to lichen thalli (Honegger et al. 2013), an observation that fits well to the observations of bacterial colonization of extant lichens (e.g., Cardinale et al 2008). Recent work suggests that the ubiquitous presence of bacteria in lichen thalli contributes to a more complex functional network beyond the interaction of fungi and algae [for a review see (Aschenbrenner et al. 2016)]. Bacteria were first cultured from lichens many years ago and were originally supposed to be involved in nitrogen-fixation (Henkel & Yuzhakova 1936). However, due to the low culturability of bacteria from many environments (e.g. Ferguson et al. 1984) and the tendency of culture methods to select for opportunistic species which rarely dominate in the natural environment (e.g. Suzuki et al 1998, Eilers et al. 2000), their diversity was not until recently fully recognized.

Culture-independent molecular studies of lichen microbial diversity and microscopic techniques revealed that bacteria belonging to the Alphaproteobacteria were the dominant microbial group associated with the lichens (Cardinale et al. 2008; Grube, et al. 2009; Hodkinson et al. 2012; Bjelland et al. 2011; Cardinale et al. 2012). In these studies, high abundances of Alphaproteobacteria were generally observed on the surface structures of the lichen thalli although some were observed in the fungal hyphae. More recently, the application of high throughput sequencing techniques to lichen bacterial community analysis confirmed that the composition of

the lichen bacterial communities could be more influenced by lichen species (mycobiont) rather than by their sample site (Bates et al. 2011), by the photobiont (Hodkinson et al. 2012), and also by the position in the lichen thallus (Mushegian et al. 2011).

Lichens exhibit clear specificity for substrate and microhabitat conditions, and a clear example of habitat specialization can be observed for marine lichens, which show vertical zonation in four characteristic belts along rocky coastlines (also known as sublittoral, littoral, supra-littoral and terrestrial zones; Fletcher 1973a, 1973b). The common littoral lichen *Lichina pygmaea* is immersed for several hours each day in the littoral zone, whereas *Lichina confinis* occurs higher up in the littoral zone, where it is perpetually subjected to splashing and sea-spray and submerged only during short periods of high tides. *Xanthoria* sp. can also occur in this zone and in the xeric supralittoral zone, which is exposed to sea-spray during storms but not submerged in seawater. Therefore, marine lichen species certainly experience different levels of stress, ranging from direct sunlight exposure, and temperature, salinity and wind variation according to their position in the littoral belts (Delmail et al. 2013).

The genus *Lichina* belongs to the class Lichinomycetes, which is characterized by cyanobacterial photobionts closely related to strains of the genus *Rivularia* (Ortiz-Alvarez et al. 2015). Even though the two lichen species above show a similar distribution range, their cyanobionts belong to separate groups that do not overlap at the OTU or even at the haplotype level (Ortiz-Alvarez et al. 2015). Apart from the cyanobacterial photobiont, the composition of the bacteria associated with marine lichens is poorly studied when compared to those of inland lichens. So far, only the common littoral black-belt forming *Hydropunctaria maura* was included in a culture-independent study (Bjelland et al. 2011). Bacterial communities of this lichen were different from inland lichens, with higher relative abundances of Actinobacteria, Bacteroidetes, Deinococcus, and Chloroflexi. A recent study of Icelandic marine lichens focused on culturable bacteria, but also revealed by fingerprinting (DGGE) analysis of 16S rRNA genes that the bacterial communities were different among different marine lichen species (Sigurbjornsdottir et al. 2014).

Such differences could be of interest for bioprospecting approaches since lichens are known to be a rich source of natural products (Boustie & Grube 2005; Parrot et al. 2016). However, lichen-associated bacteria have only recently been discovered as an additional contributor to the lichen chemical diversity, and even though only

few lichen-associated bacteria have been studied carefully in this respect, many of them produce secondary metabolites. Some of their compounds have pronounced bioactive properties [see Suzuki et al. (2016) for a review]. Nevertheless, the role of these compounds in the symbiosis is not well understood at this stage. So far, bioprospecting of the marine lichens *L. pygmaea* and *L. confinis* (and *Roccella fuciformis*) was carried out by a cultivation-based approach targeting Actinobacteria that lead to the isolation of 247 bacterial species including 51 different Actinobacteria (Parrot et al. 2015).

To address the paucity of knowledge on the total diversity and variation of bacteria associated with marine lichens, and to provide a base for future bioprospecting efforts, the uncultured microbial community of the cyanolichen species *L. confinis* and *L. pygmaea* and the chlorolichen *Xanthoria sp*. was characterised by Illumina sequencing of 16S rRNA gene fragments. In addition, other terrestrial cyanolichen species were characterized in parallel to allow us to address the following questions: i) what are the major bacterial groups associated to the *Lichina* genus lichens and are they different between the two species studied; ii) how does the culturable fraction of bacteria from *Lichina* spp. compare to the total bacterial community associated with these species; and iii) do terrestrial cyanolichens share a significant proportion of their symbionts with marine cyanolichens?

Materials and Methods

Sampling design and sample collection

The marine cyanolichens *Lichina pygmaea* and *Lichina confinis* were collected on 16 October 2013 from the Houssaye Point, Erquy on the coast of Brittany, France (Table 1, Fig. 1). Lichens were sampled in triplicate in a 3 m radius zone, that we defined as a sample cluster. Two other sample clusters of triplicates were sampled further along the littoral zone as indicated in Table S1. Duplicate seawater samples were also taken using sterile 50 ml centrifuge tubes. Similarly, one cluster of three *Xanthoria* sp. samples were also taken from Houssaye Point and another cluster of *Xanthoria* sp. was sampled from the rocky coastline of Banyuls-sur-mer on the Mediterranean Sea (Table 1, Table S1). The terrestrial cyanolichens were sampled at Kesselfalklamm, Austria. *Collema auriforme* and *Leptogium lichenoides* were sampled in the shaded humid zone near to a river whereas *Collema crispum* and *Collema flaccidum* were collected from rocks in an exposed dry zone (Table1, Table S1, Fig. 1). Lichens were sampled using a sterile scalpel and gloves, placed in sterile bags and stored at -80 °C.

DNA extraction

Genomic DNA was extracted using the PowerPlant Pro DNA Isolation Kit (MO-BIO Laboratories Inc). Lysis was achieved by first grinding the lichens to a powder with liquid nitrogen using a sterile pestle and mortar. The powder was added to the lysis tubes of the kit following the protocol according to the manufacturer's instructions, including a heating step of 65°C for 10 min before the addition of the RNase A solution. Homogenization was achieved using a standard vortex equipped with a flat-bed vortex pad. DNA quality was verified by agarose gel electrophoresis and DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo-Fisher) according to manufacturer's instructions.

PCR amplification of 16S rRNA genes and illumina sequencing

Bacterial 16S rRNA gene fragments were amplified using the primer pair 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) which show the best combination of domain and phylum coverage (Klindworth et al. 2012). The 341F primers were tagged at the 5' end with different 7 bp tags for each sample, and that were chosen from a set of tags designed to be robust to substitution, deletion and insertion errors incurred in massively parallel sequencing (Faircloth &

Glenn 2012; Table S2). We also included in parallel a control consisting of DNA from a synthetic mock community (Mock) of 20 bacterial species containing equimolar (even) rRNA operon counts (HM-782D, BEI Resources). Between 1-10 ng of each DNA sample (or 1 µl of Mock DNA) were amplified in duplicate 10 µl reactions containing 1X KAPA 2G fast mix (Clinisciences) and 0.5 µM of each primer. The PCR cycling conditions were 95 °C for 3 min followed by 25 cycles of 95°C for 15s, 55 °C for 15s and 72 °C for 2s, and a final extension of 72 °C for 30s. Duplicate reactions were pooled and PCR amplification verified by gel electrophoresis. To normalise the samples before pooling and sequencing, the Sequalprep Normalization Plate (96) kit (Invitrogen) was used according to manufacturer's instructions. After binding, washing and elution, the 37 environmental samples and the mock DNA sample were pooled into one tube. The clean-up of the PCR amplicon pool was achieved with the Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions with elution in 30 µl of molecular biology grade water (Sigma). DNA was quantified with the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen) according to manufacturer's instructions. Approximately 350 ng DNA was pooled with 700 ng of barcoded PCR products from a different project (sequencing the 16S rRNA genes of marine bacterioplankton) and sent out to Fasteris (Geneva, Switzerland) for library preparation and sequencing (described below). Library preparation involved ligation on PCR using the TruSeq DNA Sample Preparation Kit (Illumina) according to manufacturer's instructions except that 5 PCR cycles were used instead of 10 cycles. The library was sequenced on one Illumina MiSeq run using the 2 x 300 bp protocol with MiSeq version 3.0 chemistry and the basecalling pipeline MiSeq Control Software 2.4.1.3, RTA 1.18.54.0 and CASAVA-1.8.2. The error rate was measured by spiking the library with about 0.5 % of a PhiX library and mapping the reads onto the PhiX reference genome.

Sequence pre-processing

Paired-end illumina sequence data was processed with a custom pipeline (Supplementary Information) using mainly the USEARCH-64 version 8 package (Drive5; Tiburon, CA) with some commands from QIIME version 1.9.1 (Caporaso et al. 2010) and *mothur* version 1.33.3 (Schloss et al. 2009). Analysis of the Mock data set first allowed us to calibrate the different steps of the sequence processing pipeline ensuring that the assigned OTUs reflected as closely as possible the defined composition of the sample and allowed us to reveal potential sequencing artifacts

(errors, contamination). To minimise errors and reduce over-inflation of diversity, the following criteria were chosen in the pre-processing steps; a) zero mismatches allowed in the overlapping region when merging the paired end reads, b) quality filtering was carried out after merging using a stringent expected error of 1.0 (Edgar & Flyvbjerg 2015), c) zero mismatches allowed in the barcode when demultiplexing, d) exact matches to both primers required, e) removal of singleton sequences that are expected to have errors and that increase the number of spurious OTUs (Edgar 2013). Sequence data was submitted to the EMBL under the accession number: XXXXXXXXXXX.

Sequence data analysis

OTUs were defined with the UPARSE algorithm, part of the USEARCH-64 package, which clusters sequences with >97% identity to OTU centroid sequences (representative sequences that were selected based on their rank of read abundance after dereplication) and that simultaneously removes chimeras (Edgar 2013). Subsequent analysis steps were performed in QIIME or MACQIIME version 1.9.1. OTU taxonomy was assigned with the RDP classifier using the GreenGenes OTU database (gg_13_8_otus) defined at 97% identity. For the lichen dataset, the OTU table was filtered to remove eukaryotic, archaeal and organelle sequences (chloroplast, mitochondria). Due to the presence of the lichen photobiont, a large number of cyanobacterial sequences were recovered. The OTU table was filtered to separate non-cyanobacteria sequences from the cyanobacteria sequences. The representative sequences were aligned using the PYNAST aligner in QIIME and the 13_8 GreenGenes alignment (Supplementary Information), and the OTU table was further pruned by removing non-aligning OTUs. Data exploration and analysis was carried out with QIIME (see Supplementary Information) and with the package Phyloseq (McMurdie & Holmes 2013) in R (R. Core Team 2015). To examine the patterns of phylogenetic relatedness of the lichen-associated bacterial communities, the mean pairwise distance (MPD) and the mean nearest taxon distance (MNTD) were calculated using the Picante package in R (Kembel et al. 2010). Whereas MPD calculates the mean pairwise distance between all OTUs in each community, MNTD calculates the mean distance between each OTU in the community and its closest relative. To test the significance of the phylogenetic community structure observed, the standardized effect size (SES) was then calculated for the MPD and MNTD to compare the observed pattern to that of a null model of phylogeny. We used the null

model of randomly shuffling the tip labels. $SES_{(MPD)}$ and $SES_{(MNTD)}$ are equivalent to -1 times the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) respectively, reported previously (Webb et al. 2008).

Since analysis of the Mock data revealed the presence of contaminant and/or sequences from the other project, potentially due to cross-talk during the Illumina sequencing, OTUs exhibiting a low uniform abundance amongst all the samples were filtered out by calculating between-sample variance on a relative abundance OTU table and removing those OTUs with a variance of $< 1 \times 10^{-6}$.

Comparison of sequences with cultured species, and the NBCI database

The 16S rRNA gene sequences of 421 bacterial cultures isolated previously from the lichens *L. pygmaea*, *L. confinis, Roccella fuciformis* and *Xanthoria* sp. (Parrot et al. 2015) were compared to the sequences from this study by *blastn* (Altschul et al. 1997) queries against a database of the representative OTU sequences in the current study using the standalone version of NCBI blast. In addition, the sequences top 30 OTUS from marine or terrestrial lichens were subject of *blastn* queries against the NCBI Genbank *nt* database.

Results

Control of sequence quality

Analysis of the 20-species mock community allowed us to check for potential contamination and also to refine our bioinformatic analysis pipeline to obtain the expected number and identity, of OTUs. Using the criteria described in the methods and the algorithm UPARSE of the USEARCH package for clustering OTUs at 97% identity, we obtained 19 OTUs (identical to the reference sequence (Edgar 2013)) which perfectly corresponded to the mock community (the two *Staphylococcus* sp. strains cluster together) plus 17 other OTUs. The 19 mock OTUs accounted for 99.5% of the sequences. Of the remaining 17 OTUs, 4 were affiliated to the Cyanobacteria class and 3 to chloroplasts that were the dominant OTUs found respectively in the lichen samples (this study) and in samples from the offshore bacterioplankton study loaded in the same MiSeq run. This is evidence of the sequencing artifact termed “cross-talk” which can be due to index misassignment where reads from one sample are wrongly assigned the indexes from a different sample. The remaining 10 mock OTUs accounted for 0.24% of the reads.

After the different sequence processing steps, a total of 509656 good quality sequences obtained were clustered into 93 cyanobacterial OTUs and 2519 bacterial OTUs. Examination of the OTU tables of the study samples also revealed cross-talk OTUs as observed in the mock sample that were mainly attributed to chloroplasts and cyanobacteria. This problem was partly resolved by removal of these lineages for subsequent analysis of the heterotrophic bacterial diversity but there were nevertheless some bacterioplankton OTUs present at consistently low abundances in all the lichen samples, which are dominant in the samples from different study (e.g., Pelagibacteraceae). Therefore, for certain analyses, the OTU table was filtered for low variance OTUs which allowed the removal of spurious OTUs.

Marine and terrestrial lichen-associated bacterial communities

For clarity, we use the term “marine lichens” to refer to the two *Lichina* species that are strictly marine, and “terrestrial lichens” to refer to *Xanthoria* sp. and the other lichens isolated from Austria. The term “coastal lichens” includes *Xanthoria* with the *Lichina* group since it is subject to maritime influences. “inland lichens” refers to the Austrian lichens. As expected, for cyanolichens, a high proportion of the sequences were affiliated to the Cyanobacteria lineage (including chloroplasts) and ranged from

30-60% for the marine lichens and from 40-85% for the terrestrial lichens (Fig. S1). The cyanobacterial sequences were then removed for further analyses. The number of non-cyanobacteria bacterial sequences per sample ranged from 2759 for *C. auriforme* to >14000 sequences for *Xanthoria* sp.

The alpha diversity of the non-cyanobacteria bacterial communities associated with the marine and terrestrial lichens was compared using different species diversity and richness indices (Table 2) calculated with non-normalised data. The same calculations performed on normalised (subsampling to an even sequence number for all samples) data ranked the diversity of the lichen communities in the same order. The lichens *C. auriforme* and *L. lichenoides* showed the highest diversity and species richness, followed by *C. crispum* and *C. flaccidum*, and the diversity of these inland lichens was significantly higher than the coastal lichen group composed of *L. confinis* and *L. pygmaea* and *Xanthoria* sp (t_{33} = 3.35, p= 0.001 for Inverse Simpson). *L. pygmaea* exhibited higher species richness than *L. confinis* (observed OTUs and ACE) but showed lower diversity as indicated by the Shannon and Inverse Simpson diversity metrics.

The taxonomic diversity at the phylum level of the non-cyanobacteria bacteria associated with the different lichens is shown in Fig. 2. Bacteroidetes and Proteobacteria were the dominant phyla in all the lichens. Ten further phyla were present at varying abundances in the different lichen species. The marine *Lichina* lichens showed a higher relative abundance of Bacteroidetes (>50% of the sequences) and Chloroflexi compared to the terrestrial cyanolichens that were dominated by Proteobacteria. The chlorolichen *Xanthoria* sp. collected from either the Mediterranean Sea or Atlantic Ocean coastlines were also associated with the Armatimonadetes and the candidate phylum FBP that were either absent, or associated at very low abundance with the other lichens. Interestingly, the phylum Thermi comprising many thermophilic species were predominantly associated with the coastal *Lichina* and *Xanthoria* lichens.

A comparison of the lichen beta diversity as assessed by Bray-Curtis dissimilarity of a rarefied OTU table and hierarchical clustering is shown in Fig 3A. The dendrogram shows a clear separation of the marine cyanolichens from the terrestrial cyanolichens with the seawater samples also well separated from the marine lichen cluster. The diversity of the bacterial communities associated with the terrestrial chlorolichen *Xanthoria* sp. clustered according to sampling location (Atlantic Ocean

or Mediterranean Sea coast) with the *Xanthoria* sp. cluster being more closely related to the other terrestrial lichens than to the strictly marine lichens. The bacterial communities associated with *C. auriforme* were more closely related to those of *L. lichenoides* than the other two species of the same genus, *C. crispum* and *C. flaccidum* that formed a separate cluster. The latter two species were isolated from an exposed rocky area whereas *C. auriforme* and *L. lichenoides* were collected from a humid forest gorge. We reanalyzed the data focusing on the most abundant OTUs associated with the lichens where the seawater samples were removed from the dataset and low variance OTUs were filtered from the OTU table reducing the OTU number from 2519 to 367. Despite this filtering step, the clustering of the lichen communities of the reduced dataset (Fig S2) was similar to that of the full dataset (Fig 3A).

At the OTU level, the marine or terrestrial lichens were associated with very different bacterial communities as illustrated in Fig. 3B, which show the 30 most abundant OTUs for either the terrestrial or the marine lichens. The closest relatives of the OTU representative sequences assessed by *blastn* are presented in Table S3. Over 80% of the representative OTU sequences showed >97% identity hits to previously deposited sequences with OTUs with lower % identity belonging mainly to the Bacteroidetes phylum. Interestingly, one Chloroflexi OTU (OTU_12) that was recovered from *L. confinis* showed only a very low 91% identity to its closest previously described relative (Table S3) and half of the closest relatives for the marine lichen OTUs originated from marine intertidal outcrops (Couradeau et al. 2017).

The genus *Sphingomonas* (Alphaproteobacteria) was highly represented in the terrestrial cyano- and chlorolichens whereas a gammaproteobacterial OTU dominated in *L. pygmaea*. Although this OTU was also present in *L. confinis*, it was not the most abundant in the latter. where a more even distribution of the OTUs was reflected by the diversity metrics. Despite a relative spatial proximity of *Xanthoria* and *Lichina* lichens in the Atlantic coast, there were very few shared OTUs among those genera. One OTU affiliated to the candidate phylum FBP was present at low abundance in *L. confinis* and *Xanthoria* sp. but not in *L. pygmaea.* The microbial communities recovered from the *L. pygmaea* samples also showed very few OTUs in common with the seawater that was sampled in proximity to this lichen. This

suggests that the *L. pygmaea* communities were specifically associated to the lichen and very different from the free-living seawater communities.

Interestingly the *Lichina* lichens were associated with several OTUs belonging to the Rhodothermaceae family (Bacteroidetes) and the Deinococcus-Thermus and Chloroflexi phyla of which some members are meso- or thermophilic, highly resistant to UV radiation (Deinococcus) and phototrophic (Chloroflexi). Although the Lichina communities showed a similar genus-level composition with closely related OTUs (eg *Lewinella*, *Tunicatimonas*, *Rubricoccus*) present in both lichens, these OTUs often showed an inverse pattern of abundance between the two species, where OTUs in genera present in both species tended to have higher counts in only one of the species.

Finally, to determine the factors most likely affecting the non-Cyanobacteria bacterial community association with the lichen species, the level of phylogenetic clustering (environmental filtering) or over-dispersion (competitive effects) was estimated from a calculation of the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI; Webb et al. 2008). With the exception of the NRI for communities associated with *L. confinis*, there was evidence of phylogenetic clustering of the bacterial OTUs for the coastal lichens as observed by a positive NRI and NTI. For the inland lichens, significant NRI values were only observed for *L. lichenoides* and *C. auriforme* that were collected from the humid forest gorge, but none of the inland lichens showed significant phylogenetic clustering at the tree tips for the NTI (Table 3).

Core microbiome of marine lichens

To explore the degree of overlap between the *Lichina* bacterial communities in more detail, the OTUs consistently shared within the genus, and potentially indicative of a core microbiome were identified, grouped at the family (or order when identification not possible) level and represented considering their sequence counts (Fig 4A). Setting a very conservative threshold of 100% presence in all replicates of both lichen species, only 33 OTUs were shared by both species (Fig 4A). Of these, 15 were attributed to the Bacteroidetes families Saprospiraceae, Flammeovirgaceae and Rhodothermaceae (genera *Lewinella*, *Tunicatimonas* and *Rubricoccus* respectively) that accounted for around 60% of the sequences amongst the core OTUs and that showed similar relative abundances (Fig 4A). Other families included the Chloroflexi Caldilineaceae, and the Alphaproteobacteria families

Rhodobacteraceae and Erythrobacteraceae that were more abundant in *L. confinis* and a Gammaproteobacteria OTU in *L. pygmaea*.

When considering OTUs associated to each lichen species, 38 and 42 OTUs were specific (i.e. present at 100% of the replicates) for *L. confinis* and *L. pygmaea* respectively, and their relative abundances are shown in Fig 4B and 4C, grouped at the family (or order when identification was not possible at the family level) level. The distribution and relative abundance of the family level was very different between the two species. For *L. confinis*, the most noticeable differences included a higher relative abundance of Acidobacteria Ellin6075, Bacteroidetes Cytophagaceae and Flammeovirgaceae and the Chloroflexi order Rosiflexales. For *L. pygmaea*, the Actinobacteria family Euzebyaceae, and the Bacteroidetes families Saprospiraceae and Flavobacteriaceae dominated the specific core OTUs.

To determine the OTUs that showed statistically significant differential abundances between *L. confinis* and *L. pygmaea*, we used the DESeq2 negative binomial Wald test (Love et al. 2014). One third of the OTUs tested (120 out of 367 OTUs) showed a significantly different distribution between the two lichens ($P < 0.001$) and those OTUs with a mean abundance > 20 are shown in Fig 5. These OTUs were distributed in several shared families (left side of the plot) but we also observed families differentially distributed and that were distinct for each lichen. Interestingly, 29 out of the 33 core *Lichina* OTUs showed a statistically significant different distribution.

Xanthoria sp. collected above *L. confinis* on the littoral belt only shared one core OTU with the *Lichina* lichens that was assigned to the Gammaproteobacteria OTU, dominant in *L. pygmaea*. The *Xanthoria* sp. sampled from the Atlantic or Mediterranean shared 24 core OTUs (Fig S3) dominated by the Proteobacteria (mostly Alpha- and Betaproteobacteria) accounting for over 50% of the sequences for each species. The *Xanthoria* sp. sample collected from the dryer Mediterranean coast showed higher relative abundances of Acidobacteria and the family Acetobacteraceae compared to the sample from the Atlantic coast.

Photobiont diversity

The marine and terrestrial cyanolichens were associated with a different major photobiont. For *L. confinis* and *L. pygmaea*, the vast majority of photobiont sequences (97% of the reads) clustered into a single *Rivularia* sp. OTU whereas *Collema* species and *Leptogium lichenoides* were each associated with a different

Nostoc OTU. The marine lichens were associated with a higher diversity of cyanobacteria than the terrestrial lichens where cyanobacteria other than primary photobiont accounted for up to 10% of the cyanobacterial sequences (Fig S3).

Comparison to previously cultured strains from coastal lichens and C. auriforme

The 16S rRNA gene sequences of previously isolated strains originating from *L. confinis*, *L. pygmaea, Roccella fuciformis*, and *C. auriforme* were compared to the sequences in this study to investigate the representation of the culturable fraction relative to the bacterial diversity assessed by cultivation-independent molecular method. Forty-two strains out of the 421 previously isolated strains had 16S rRNA sequence identities > 97% to the OTUs identified in this study (Table S4). The majority of the OTUs that had 16S rRNA sequences with high identity (>99%) to the strains were of low abundance (< 0.5% of total sequences) in our study. The two exceptions included the abundant OTU_37 (*Sphingomonas*; ~4-14% of total sequences) that shared 99% identity to a strain isolated from *C. auriforme*, and OTU_144 (Microbacteriaceae) which comprised up to 1.65% of sequences in *Xanthoria* sp. (Atlantic) and with 99% sequence identity to a strain isolated from *L. pygmaea*. Finally, one of the top 30 most abundant OTUs from the marine lichens (OTU_21, Erythrobacteraceae) shared 98% sequence identity with a strain isolated from *L. pygmaea*. The majority of the OTUs with ≤98% sequence identity to the strains were also in low relative abundance in the lichen bacterial communities (<1%).

Discussion

The largest body of lichen research has been undeniably devoted to terrestrial lichens. We address this knowledge gap in applying for the first time, next generation Illumina sequencing to characterize the bacterial communities associated to marine cyanolichens growing on a rocky intertidal belt and compare them with those of terrestrial cyanolichens. We showed that there was no overlap between the marine and terrestrial bacterial communities and whereas two marine *Lichina* species, occurring in two neighbouring belts of the littoral zone, share some common OTUs, many are specific to each species.

Lichina bacterial communities

A comparison of the *Lichina* bacterial community diversity allowed us to identify potential core OTUs and specific OTUs for each species, reflecting the potential adaptation of *L. confinis* or *L. pygmaea* to their different littoral zone. The *Lichina* bacterial communities shared 15 of 33 core OTUs that were attributed to only 3 Bacteroidetes genera, *Rubricoccus*, *Lewinella*, and *Tunicatimonas*, as well as several Alphaproteobacteria OTUs from the Erythrobacteraceae and Rhodobacteraceae families, and a Chloroflexi OTU. *Tunicatimonas pelagia* which belongs to the Flammeovirgaceae family was initially isolated from a sea anemone (Yoon et al. 2012) and other members of this family are known to associate with corals (Apprill et al. 2016). *Lewinella* belongs to the Saprospiraceae family that was found as an epiphyte of red algae (Miranda et al. 2013) and on filamentous bacteria identified as Chloroflexi (Xia et al. 2008), and isolates from this genus were also cultured from marine lichens (Sigurbjornsdottir et al. 2014; Parrot et al. 2015). Notably, the bacterial groups associated with the *Lichina* lichens were also similar to those found in the surface layer of hypersaline microbial mats (Schneider et al. 2013) and in epiphytic communities on the red macroalga *Porphyra umbilicalis* (Miranda et al. 2013). The co-occurrence of these different bacteria in microbial mats and on various marine hosts suggests that they have adapted to similar environmental conditions together. In functioning as a consortium, this communities could potentially increase their robustness and that of their hosts by expanding their range of metabolic capacities and resistance mechanisms to stress (Hays et al. 2015).

Interestingly there were multiple *Lewinella*, *Tunicatimonas* and *Rubricoccus* OTUs in each lichen species and OTUs that were affiliated to the same genus were differentially distributed between the two Lichina species (e.g., see the Lewinella

OTUs 6, 7, 24 and 61 in Fig 3). This could suggest that the bacterial genera could have evolved separately in each lichen species as described for the *Rivularia* cyanobionts of *L. confinis* and *L. pygmaea* (Ortiz-Álvarez et al. 2015). The split between the *L. confinis* and *L. pygmaea Rivularia* cyanobionts is estimated to have occurred in the mid-Jurassic and the cyanobionts are thought to have diverged in each lichen species by allopatric speciation (Ortiz-Álvarez et al. 2015). This mechanism of niche adaptation is thought to occur in extreme environments and could also apply to the bacteria associated to these marine lichens, which became adapted to the intertidal or supratidal zones. The presence of these closely related clusters was also supported by the positive and significant NTI values (Webb et al. 2002) that indicate phylogenetic clustering of the *Lichina* bacterial communities.

Rocky tidal zones are thought to be one of the most stressful habitats on earth (Miller et al. 2009) and both marine lichen species bacterial communities were associated with several groups of bacteria that are known to be heat- or radiation-resistant. These include several OTUs identified as core OTUs that were affiliated to the genus *Rubricoccus* of the Rhodothermaceae (Park et al. 2011), to the phylum Chloroflexi (that contains moderately thermophilic/thermophilic groups), and to the family Truperaceae in which the isolated species are thermophilic and nearly as radiation resistant as *Deinococcus* (Albuquerque et al. 2005).

Despite sharing 33 core OTUs, the two marine Lichina species *L. confinis* and *L. pygmaea* were associated with significantly different bacterial communities at the OTU level and remarkably the Bray Curtis dissimilarity distance between these communities was greater than that of the distance between the communities associated with two different terrestrial cyanolichen genera, *Collema auriforme* and *Leptogium lichenoides* (Fig 3). This difference was also reflected in the high number of OTUs (>120) that displayed a significantly different abundance between the two species (Fig 5). These significant differences were despite the relatively short distance that separated the species on the vertical gradient (height on the littoral belt). Other studies show evidence that height on the littoral zone is the most important factor explaining the rocky coastline species variance with slope, exposure, substrate and orientation explaining less (Chappuis et al. 2014). *L. confinis* occurs higher up on the littoral belt, being mostly exposed to the air and would be subject to higher temperatures and UV radiation. This could explain why some thermophilic groups such as Chloroflexi and the Actinobacterial genus *Rubrobacter* that include

thermophilic species resistant to radiation (Ferreira et al. 1999) were four-fold and 13-fold respectively more abundant in *L. confinis* than *L. pygmaea*. The bacterial groups that were more abundant in *L. pygmaea* when compared to *L. confinis* included the Gammaproteobacteria OTU related to a seagrass epiphyte *Granulosicoccus* (Kurilenko et al. 2010) an OTU affiliated to the Actinobacterial genus *Euzebya*, recovered from a sea cucumber (Kurahashi et al. 2010) and several Flavobacteriaceae and Rhodobacteraceae OTUs. Marine epiphytic Actinobacteria, and *Phaeobacter* (Rhodobacteraceae), associated with a variety of marine plants and animals, are known to produce bioactive molecules (Valliappan et al. 2014; Rao et al. 2007). Such molecules have been shown to inhibit settlement of algal spores, fungi and larvae and may contribute to the host's defence against biofouling (Rao et al. 2007). As *L. pygmaea* is immersed for half the day, it may be more subject to biofouling than *L. confinis* and hence the biofilm communities covering the *L. pygmaea* may play an important role in the inhibition of attachment of undesirable organisms.

Despite the significantly different bacterial communities observed in the two vertically segregating *Lichina* species in the littoral belt, we did not find clear evidence for significant differences between the samples of each species across horizontal distances. This was indicated by beta-diversity analyses both on the whole or filtered dataset which included the most abundant OTUs and which revealed a lack of clustering of the replicates according to site. A lack of geographical influence on the lichen associated bacterial communities was also observed in other studies where sampling was carried out on a small spatial scale (Grube, Cardinale, Joao Vieira de Castro, et al. 2009; Bates et al. 2011). In a study where lichen sampling was carried out at a much greater spatial scale, across the North Amercian continent, a significant effect of geographical location on bacterial communities associated with a specific species was observed (Hodkinson et al. 2012).

Low culturability of marine lichen associated bacteria

Culturing of bacterial isolates from novel environments is essential for natural product discovery and the harsh environments, in which the *Lichina* lichen species are found, could provide a wealth of bioactive products, particularly from the potentially thermophilic/radiation resistant bacteria identified in our study. However, only 42 OTUs showed significant similarity to the 247 cultured isolates previously isolated (Parrot et al. 2015), even when lowering the similarity threshold to 97% and

the 3 OTUs that were 100% identical to isolates were present at low abundances in the dataset. Whereas the Bacteroidetes phylum accounted for around 53% of the 16S rRNA sequences from the marine lichens, only 4 strains from this phylum were cultured. These results can be partly explained by the culturing strategy which was intentionally skewed towards the isolation of *Actinobacteria* by using Actinobacteria Isolation Agar (Parrot et al. 2015). The low culturability of bacteria from certain environments is well known (Amann et al. 1995) and our results further highlight the importance of parallel culture independent molecular methods to gain a more complete picture of the microbial diversity. Such results, combined with metagenomic approaches to target gene clusters of interest (Vester et al. 2015) and novel isolation strategies incorporating lichen extracts into culture media (Biosca et al. 2016) should improve the bioprospecting of marine lichens. Nevertheless the fact that some of the previously cultured strains were relatively close to the community described by our study, suggests that some of the bacteria associated with lichens might not be as recalcitrant to culture when compared to bacteria from other environments, highlighting the interest of these associations as sources of cultured microbial diversity.

Marine versus terrestrial lichens: challenging the paradigm of Alphaproteobacterial dominance

Here we show that marine lichen bacteria communities are completely different from terrestrial counterparts and that this difference is immediately visible at the phylum level with 50% of the sequences assigned to Bacteroidetes in *Lichina*, with a lower proportion of Proteobacteria (20-30%), the converse of the inland lichens from the humid wooded zone (15-19% Bacteroidetes and 50% Proteobacteria). At the OTU level, the differences were even more marked, with no overlap between the marine and terrestrial cyanolichens. The *Xanthoria* sp. communities (sampled above *L. confinis* on the rocky shore) only shared a single OTU with the *Lichina* lichens (Gammaproteobacteria OTU_15) which was only present at low abundance (0.05%). High abundances of Bacteroidetes were also observed in a different marine lichen *Hydropunctaria maura* (Bjelland et al. 2011) and supports the idea that this group of bacteria are major components of the bacterial communities associated with marine lichens, whereas Alphaproteobacteria are well confirmed by several studies as the dominant group associated with terrestrial lichens (Grube et al. 2009; Cardinale et al. 2008; Hodkinson et al. 2012; Bates et al. 2011; Mushegian et al. 2011).

In marine environments Bacteroidetes are specialized in degrading polymeric organic matter and are adapted for an attached lifestyle by the production of adhesion proteins (Fernández-Gómez et al. 2013). Although Bacteroidetes are often reported attached to particles they are also known to be epiphytic on a variety of marine algae from temperate regions and often co-occur with Proteobacteria (Miranda et al. 2013; Burke et al. 2011; Wahl et al. 2012; Lachnit et al. 2011). Although high abundances of Alphaproteobacteria sequences have been detected associated with the green alga *Ulva australis* from Botany Bay NSW Australia (Burke et al. 2011), Bacteroidetes was the most abundant group associated with the red alga *Porphyra umbilicalis* (Miranda et al. 2013) in Maine, US and with red and brown algae sampled during summer months in Kiel Bight, Germany (Lachnit et al. 2011), and it appears this bacteria in this phylum might be an important member of epiphytic and perhaps epilichenic communities, although this will need to be shown by microscopy based (FISH) approaches

Other differences between the marine and terrestrial lichens included the relative abundance of the non-cyanobacterial sequences compared to the photobiont sequences that were as low as 20% in *C. auriforme* but accounted for 50-60% in the marine lichens. The marine lichen *H. maura* also had a higher estimated concentration of bacterial cells in the lichen thallus compared to inland lichens (Bjelland et al. 2011). These observations suggest that bacteria associated with marine lichens might play a greater role in the functioning of the lichen symbiosis but this would need to be confirmed by microscopy analyses and for example, metabolite profiling.

Several roles of lichen-associated bacteria have been suggested and include nutrient supply (e.g. by nitrogen fixation and phosphate scavenging), nutrient recycling, protection against UV radiation, resistance to abiotic and biotic environmental stressors, and thallus degradation (Eymann et al. 2017). Unlike terrestrial lichens, marine lichens would need to be adapted to much more variable gradients of UV light, salinity (osmotic) stress, heat, desiccation and mechanical stresses from wave action and therefore we would expect the associated bacteria to play a role in resisting these particularly variable conditions. Although marine lichens can provide their own UV protection by the production of a parietin derivative in the case of *Xanthoria* and mycosporine-like amino acids in the case of the *Lichina* sp. (Roullier et al 2011), it is possible that the bacteria that colonize the surface of the

lichen like a biofilm (Cardinale, Steinova, et al. 2012) could provide a protective layer conferred by other photoprotective pigments. The majority of the identified OTUs were affiliated to pigmented bacterial families, and several potentially phototrophic bacterial groups such as the aerobic anoxygenic phototropic (AAP; e.g. Chloroflexi, Erythrobacteraceae, Rhodobacteraceae) or rhodopsin-containing bacteria (e.g. Flavobacteriaceae, Yoshizawa et al. 2014), Rhodothermaceae (Vavourakis et al. 2016), Deinococcus-Thermus) that contain carotenoids may have a photoprotective role.

To alleviate salt stress and resist desiccation, marine lichens and their photobionts accumulate compatible solutes that are mainly sugars, sugar alcohols and complex sugars, lowering the water potential and hence reducing water movement out of the cells (for a review see Delmail et al. 2013). Indeed *L. pygmaea* which is immersed in seawater for half the day was shown to resist salt stress better than *Xanthoria aureola*, which is located higher up on the littoral belt and only receives sea spray (Delmail et al. 2013). Marine biofilms are considered to be analogous to a multicellular organism in that it functions like a “second skin”, replacing the hosts top layers as the new interface between the host and the environment (Wahl et al. 2012). Such a function could be imagined for the bacteria associated with *L. pygmaea* that could contribute to the osmotic adaptation of the lichen by maintaining a layer of cells in osmotic balance with the surrounding water. This could be achieved by different mechanisms including the synthesis and uptake of organic osmolytes and by using a sodium pumping rhodopsin as inferred from Bacteroidetes metagenomes recovered from hypersaline lakes (Vavourakis et al. 2016). While this is speculative, bacterial communities inhabiting the rhizosphere of coastal plants were shown to confer salt stress acclimatization to the plants, potentially though the use of ion and compatible solute transporters (Yuan et al. 2016).

In cyanolichens or tripartite lichens, fixed carbon and nitrogen are supplied to the mycobiont by the photobiont whereas phosphate was shown to be a limiting macronutrient since cyanolichen growth was stimulated after immersing the thalli in a phosphate solution (McCune & Caldwell 2009). This was also supported by metagenomic and proteomic analysis of two terrestrial lichens that attributed an important role of phosphate solubilization genes to the associated microbial community, more specifically to the Alphaproteobacteria (Grube et al. 2015;

Sigurbjornsdottir et al. 2015). The *Lichina* lichens may be less subject to phosphate limitation because *L. pygmaea* is immersed in seawater for half a day and *L. confinis* receives sea spray in a coastal area that is not particularly phosphorus limited. Nevertheless, some of the associated bacteria may play a role in phosphate uptake from the seawater or in the recycling and transfer of phosphate from senescing parts of the thallus to the growing apices as was shown for *Cladonia* (Hyvärinen & Crittenden 2000). Such nutrient recycling may be carried out in part by the Bacteroidetes that are known to produce a wide range of enzymes capable of degrading polymeric organic matter including glycoside hydrolases and peptidases (Fernández-Gómez et al. 2013).

Conclusions

Here we show the striking differences between bacterial communities associated with marine and terrestrial lichens and even between marine lichens inhabiting different littoral zones. This represents a first piece in the puzzle in understanding the role of the associated bacteria in the marine lichen symbiosis, which is lagging much behind that of the terrestrial lichens. To begin to elucidate the functions of these communities, future work should include a multidisciplinary approach combining microscopy techniques to determine the spatial organization of the core microbiome members and “omic” approaches to understand their functional role. An integrative study using metagenomics, metatranscriptomics and metabolomics could be performed on *L. pygmaea* during high and low tide to reveal a wealth of information on how the symbiotic partners interact and adapt to these extreme conditions. This could be a particularly interesting endeavor with lichens from the littoral zones, as these are possibly the pioneering habitats for the symbiosis-assisted conquest of land. Moreover, such information would not only further our understanding of the marine lichen symbiosis but could also guide future bioprospecting efforts.

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Figure Legends

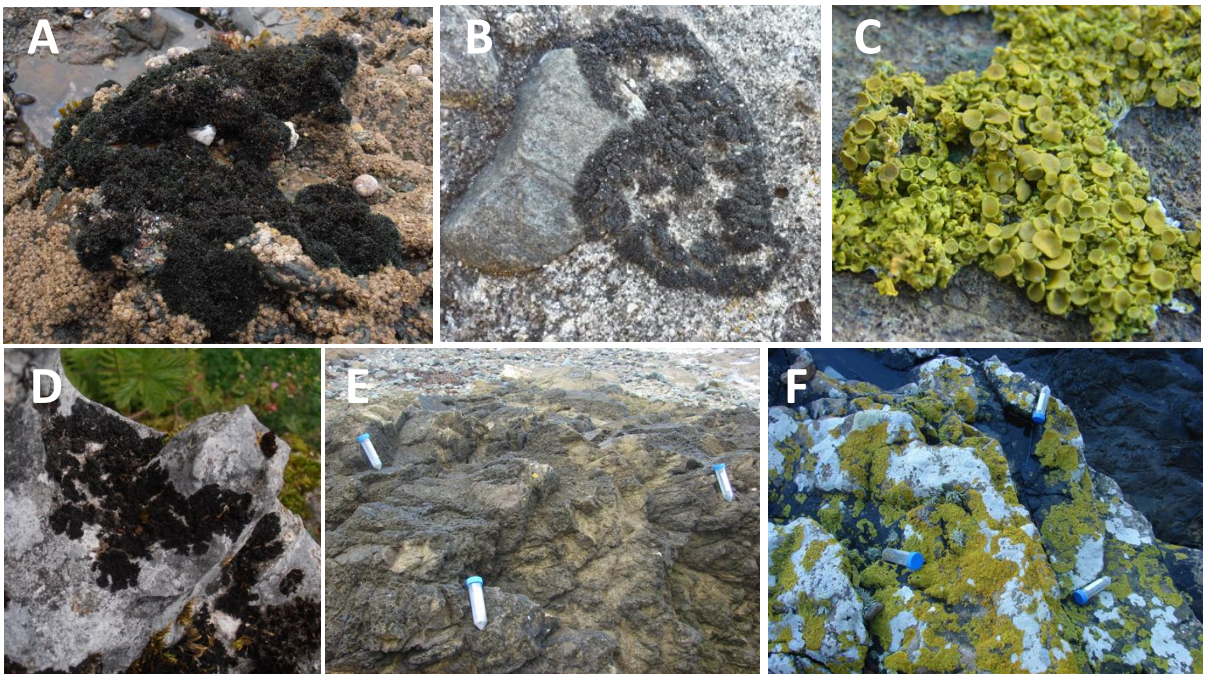
Figure 1. Examples of lichens collected in this study. A, *Lichina pygmaea*; B, *Lichina confinis*; C, *Xanthoria* sp.; D, *Collema auriforme*; E, *L. confinis* sample cluster; *Xanthoria* sp. sample cluster.

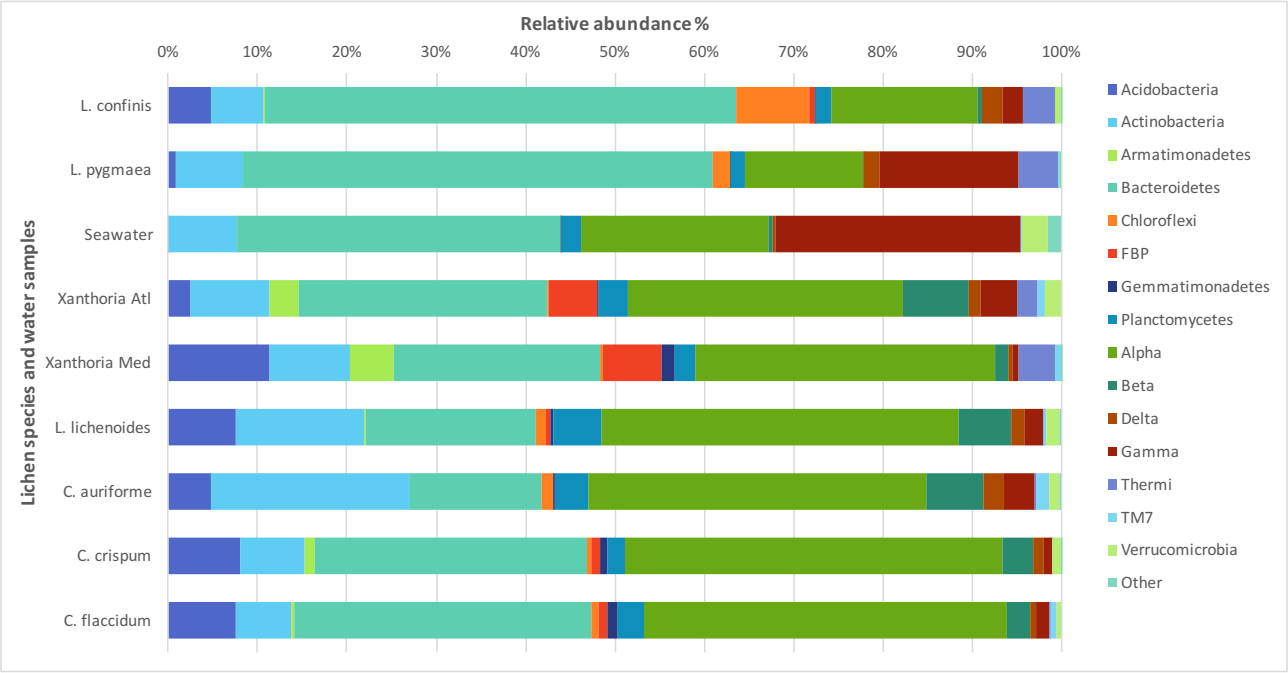
Figure 2. Phylum-level phylogenetic diversity of the bacteria associated to different lichen species or in seawater.

Figure 3. A. Beta diversity of bacterial communities associated with the lichens or seawater assessed by Bray Curtis dissimilarity and average linkage clustering, **B.** Heatmap showing the relative abundance of the 30 most abundant OTUs among either marine or terrestrial lichens. The mean sequence abundance was calculated for each lichen sample cluster or the seawater samples. Cf, *C. flaccidum*; Cc, *C. crispum*; Ca, *C. auriforme*; Ll, *L. lichenoides*; XaM, *Xanthoria* sp. from the Mediterranean coast; XaA, *Xanthoria* sp. from the Atlantic coast; SW, seawater; Lc, *L. confinis*; Lp, *L. pygmaea*.

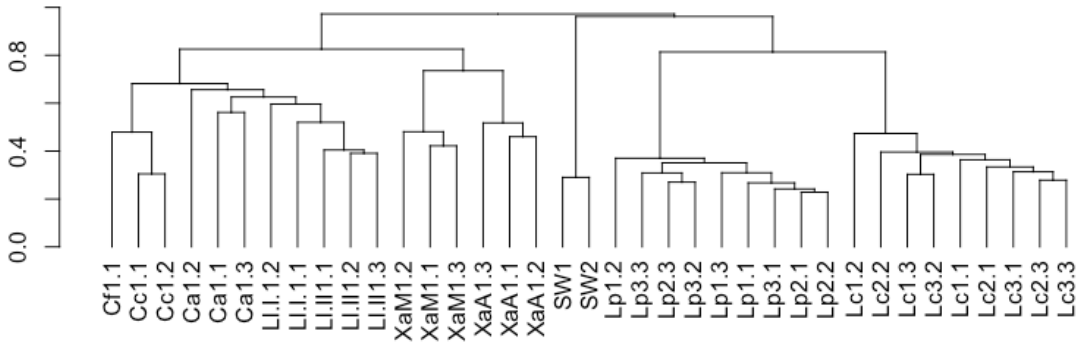
Figure 4. Comparison of relative abundance of core OTUs present in: **A.** all replicates of both *L. confinis* and *L. pygmaea* (Lichina core) or **B.** the core OTUs specific for either species grouped at the family level. When family level assignment was not available, the next higher taxonomic level assigned was used. **C.** Venn diagram showing the number of core OTUs shared or specific to *L. confinis* and *L. pygmaea***.**

Fig 5. OTUs with significantly different relative abundances in *L.confinis* and *L. pygmaea* assessed by the DESeq2 package using an adjusted p value < 0.001 and a base mean threshold > 20. NA : OTUs not assignable at the particular taxonomic level (order or family).


A
B
C
D
E
F


Relative abundance %
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Lichen species and water samples
L. confinis
L. pygmaea
Seawater
Xanthoria Atl
Xanthoria Med
L. lichenoides
C. auriforme
C. crispum
C. flaccidum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chloroflexi
FBP
Gemmatimonadetes
Planctomycetes
Alpha
Beta
Delta
Gamma
Thermi
TM7
Verrucomicrobia
Other

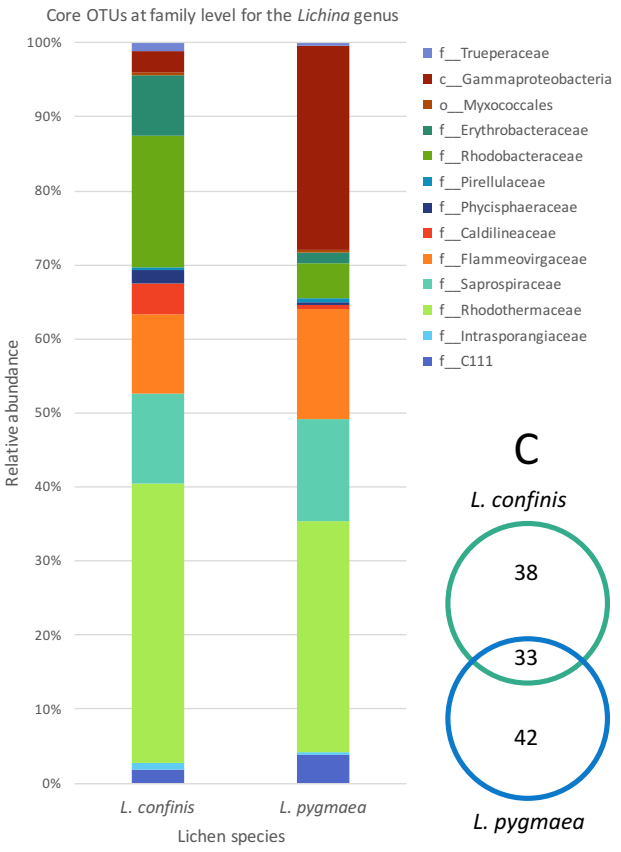
A



B

OTU	Class	Order	Family	Genus
15	Gammaproteobacteria			
19	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Jannaschia
21	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	
60	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
94	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	
5	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
40	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
7	[Saprospirae]	[Saprospirales]	Saprospiraceae	Lewinella
6	[Saprospirae]	[Saprospirales]	Saprospiraceae	Lewinella
24	[Saprospirae]	[Saprospirales]	Saprospiraceae	Lewinella
61	[Saprospirae]	[Saprospirales]	Saprospiraceae	Lewinella
17	Cytophagia	Cytophagales	Flammeovirgaceae	Tunicatimonas
10	Cytophagia	Cytophagales	Flammeovirgaceae	Tunicatimonas
75	Cytophagia	Cytophagales	Flammeovirgaceae	Tunicatimonas
26	Cytophagia	Cytophagales	Flammeovirgaceae	
20	Cytophagia	Cytophagales	Flammeovirgaceae	
186	Cytophagia	Cytophagales	Cytophagaceae	Persicitalea
250	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
50	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
25	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
147	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
79	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
42	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Rubricoccus
12	Chloroflexi	[Roseiflexales]		
46	Anaerolineae	Caldilineales	Caldilineaceae	
18	Nitriliruptoria	Euzebyales	Euzebyaceae	Euzebya
104	Acidimicrobiia	Acidimicrobiales	C111	
9	Deinococci	Deinococcales	Trueperaceae	
76	Deinococci	Deinococcales	Trueperaceae	
47	[Chloracidobacteria]	RB41	Ellin6075	
37	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas
59	Alphaproteobacteria	Sphingomonadales		
51	Alphaproteobacteria	Sphingomonadales		
16	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
34	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
137	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
468	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
54	Alphaproteobacteria	Rhizobiales		
165	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	
44	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
66	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	
95	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium
182	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana
187	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
100	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
31	[Chloracidobacteria]	RB41	Ellin6075	
63	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Pseudonocardia
122	Actinobacteria	Actinomycetales	Micromonosporaceae	
138	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Pseudonocardia
22	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma
112	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma
174	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma
23	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter
29	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter
235	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
39	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
77	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
130	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Flavisolibacter
32				
28	[Fimbriimonadia]	[Fimbriimonadales]	[Fimbriimonadaceae]	Fimbriimonas

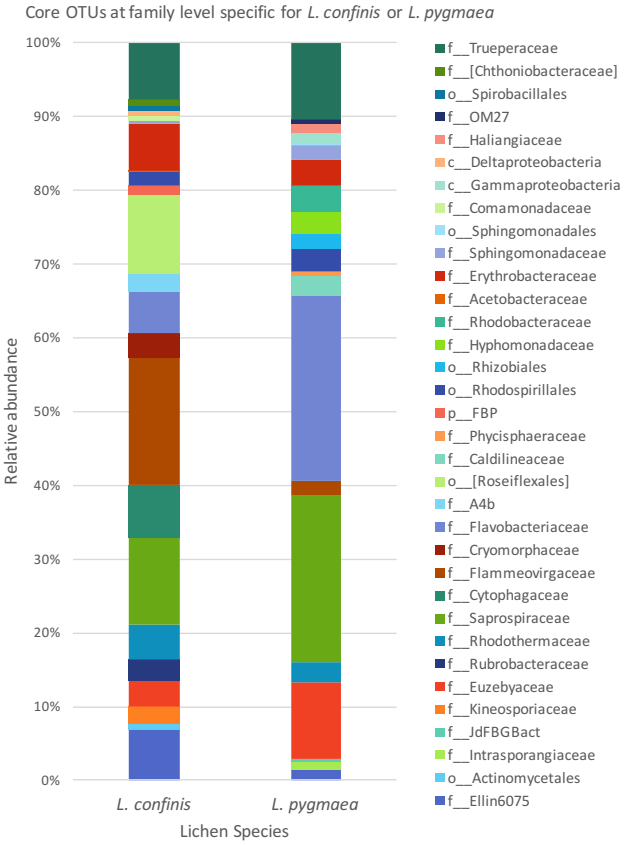
A


Core OTUs at family level for the *Lichina* genus
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
L. confinis
L. pygmaea
Lichen species
f__Trueperaceae
c__Gammaproteobacteria
o__Myxococcales
f__Erythrobacteraceae
f__Rhodobacteraceae
f__Pirellulaceae
f__Phycisphaeraceae
f__Caldilineaceae
f__Flammeovirgaceae
f__Saprospiraceae
f__Rhodothermaceae
f__Intrasporangiaceae
f__C111

C

L. confinis
38
33
42
L. pygmaea

B


Core OTUs at family level specific for *L. confinis* or *L. pygmaea*
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
L. confinis
L. pygmaea
Lichen Species
f__Trueperaceae
f__[Chthoniobacteraceae]
o__Spirobacillales
f__OM27
f__Haliangiaceae
c__Deltaproteobacteria
c__Gammaproteobacteria
f__Comamonadaceae
o__Sphingomonadales
f__Sphingomonadaceae
f__Erythrobacteraceae
f__Acetobacteraceae
f__Rhodobacteraceae
f__Hyphomonadaceae
o__Rhizobiales
o__Rhodospirillales
p__FBP
f__Phycisphaeraceae
f__Caldilineaceae
o__[Roseiflexales]
f__A4b
f__Flavobacteriaceae
f__Cryomorphaceae
f__Flammeovirgaceae
f__Cytophagaceae
f__Saprospiraceae
f__Rhodothermaceae
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f__Kineosporiaceae
f__JdFBGBact
f__Intrasporangiaceae
o__Actinomycetales
f__Ellin6075

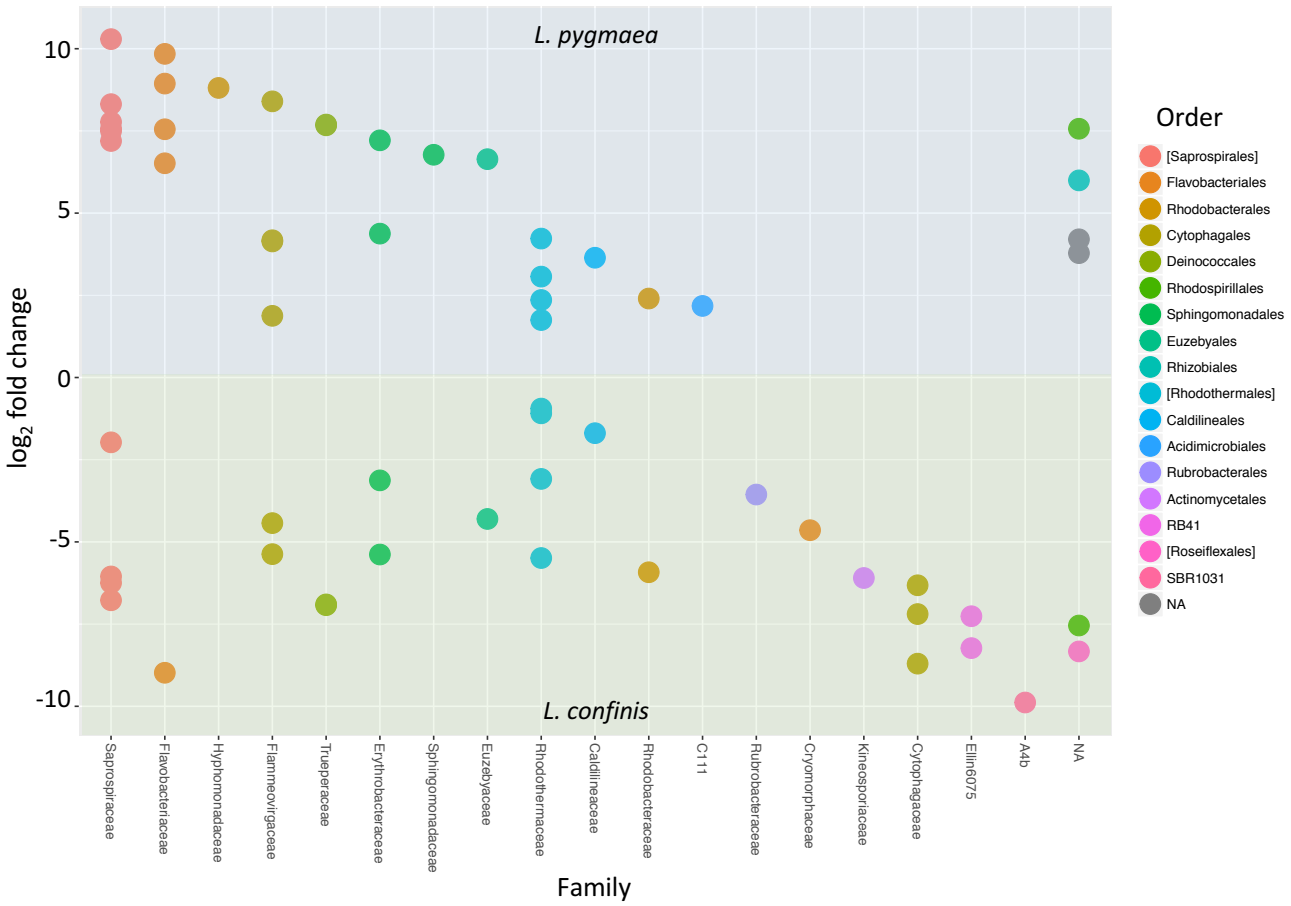
L. pygmaea
L. confinis
10
5
0
-5
-10
log2 fold change
Saprospiraceae
Flavobacteriaceae
Hyphomonadaceae
Flammeovirgaceae
Trueperaceae
Erythrobacteraceae
Sphingomonadaceae
Euzebyaceae
Rhodothermaceae
Caldilineaceae
Rhodobacteraceae
C111
Rubrobacteraceae
Cryomorphaceae
Kineosporiaceae
Cytophagaceae
Ellin6075
A4b
NA
Family
Order
[Saprospirales]
Flavobacteriales
Rhodobacterales
Cytophagales
Deinococcales
Rhodospirillales
Sphingomonadales
Euzebyales
Rhizobiales
[Rhodothermales]
Caldilineales
Acidimicrobiales
Rubrobacterales
Actinomycetales
RB41
[Roseiflexales]
SBR1031
NA

Table 1. Lichen species analysed in this study

Lichen species	**Mycobiont**	**Photobiont**	**Habitat**	**Date**	**Location**
Lichina confinis	*Lichinomycetes, Lichinales, Lichinaceae*	Cyanobacteria: *Rivularia* sp	Upper eulittoral	16.10.13	Erquy, France (Atlantic Ocean)
Lichina pygmaea	*Lichinomycetes, Lichinales, Lichinaceae*	Cyanobacteria: *Rivularia* sp	Lower eulittoral	16.10.13	Erquy, France (Atlantic Ocean)
Xanthoria sp.	*Lecanoromycetes, Teloschistales, Teloschistaceae*	Green alga: *Trebouxia* sp	Mesic-supralittoral	16.10.13	Erquy, France (Atlantic Ocean)
Xanthoria sp.	*Lecanoromycetes, Teloschistales, Teloschistaceae*	Green alga: *Trebouxia* sp	Xeric-supralittoral	06.03.14	Banyuls sur mer, France (Mediterranean Sea)
Collema auriforme	*Lecanoromycetes, Peltigerales Collemataceae*	Cyanobacteria: *Nostoc* sp.	Terrestrial	26.11.13	Kesselfalklamm, Austria
Collema crispum	*Lecanoromycetes, Peltigerales Collemataceae*	Cyanobacteria: *Nostoc* sp.	Terrestrial	26.11.13	Kesselfalklamm, Austria
Collema flaccidum	*Lecanoromycetes, Peltigerales Collemataceae*	Cyanobacteria: *Nostoc* sp.	Terrestrial	26.11.13	Kesselfalklamm, Austria
Leptogium lichenoides	*Lecanoromycetes, Peltigerales Collemataceae*	Cyanobacteria: *Nostoc* sp.	Terrestrial	26.11.13	Kesselfalklamm, Austria

Table 2. Summary of alpha diversity metrics of the non-cyanobacterial bacterial sequences associated with different lichen species and seawater. The mean number of the cyanobacterial sequences are also listed for comparison.

Lichen species	**N° samples**	**mean N° bact seqs**	**mean N° cyano seqs**	**Observed OTUs**	**ACE**	**Shannon**	**Inv Simpson**
L.confinis	9	5686.6	5805.7	317.4	495.0	4.3	42.4
L.pygmaea	9	10111.6	7020.6	493.3	723.0	4.2	24.9
Seawater	2	9539.5	962.5	517.5	704.3	4.6	43.6
Xanthoria sp. Atl.	3	13074.7	103.7	423.3	596.8	4.3	37.7
Xanthoria sp. Med.	3	9295.0	378.0	383.0	618.6	4.0	24.2
L.lichenoides	5	5405.8	8279.2	717.0	998.8	5.4	69.6
C. auriforme	3	3554.3	13618.3	620.0	909.4	5.2	84.0
C. crispum	2	6853.0	6707.0	574.0	799.3	4.7	30.6
C.flaccidum	1	6763.0	8653.0	602.0	828.8	4.9	45.8

bact seqs, non-cyanobacterial bacterial sequences
cyano seqs, cyanobacterial sequences
Inv Simpson, Inverse Simpson

Table 3. Levels of phylogenetic clustering of the lichen associated bacterial communities as measured by the Nearest Taxon Index (NTI) and the Net Relatedness Index (NRI).

Lichen species	**N° taxa**	**NRI**	**runs > null**	**NTI**	**runs > null**
Coastal Brittany lichens					
L. confinis	271	1.42	905	2.30**	991
L. pygmaea	281	3.49**	998	1.68*	951
Xanthoria sp. Atlantic	224	3.13**	997	2.15*	978
Inland Austrian lichens					
L. Lichenoides	294	2.15*	983	0.12	552
C. auriforme	260	2.53**	992	1.73	944
C. crispum	226	1.32	902	0.92	825
C. flaccidum	205	1.05	859	1.61	938

* $p < 0.05$, ** $p < 0.01$