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Littoral lichens as a novel source of potentially bioactive Actinobacteria

Delphine Parrot¹, Sanjay Antony-Babu²,³, Laurent Intertaglia³,⁴, Martin Grube⁵, Sophie Tomasi¹ & Marcelino T. Suzuki²,³

Cultivable Actinobacteria are the largest source of microbially derived bioactive molecules. The high demand for novel antibiotics highlights the need for exploring novel sources of these bacteria. Microbial symbioses with sessile macro-organisms, known to contain bioactive compounds likely of bacterial origin, represent an interesting and underexplored source of Actinobacteria. We studied the diversity and potential for bioactive-metabolite production of Actinobacteria associated with two marine lichens (*Lichina confinis* and *L. pygmaea*; from intertidal and subtidal zones) and one littoral lichen (*Roccella fuciformis*; from supratidal zone) from the Brittany coast (France), as well as the terrestrial lichen *Collema auriforme* (from a riparian zone, Austria). A total of 247 bacterial strains were isolated using two selective media. Isolates were identified and clustered into 101 OTUs (98% identity) including 51 actinobacterial OTUs. The actinobacterial families observed were: Brevibacteriaceae, Cellulomonadaceae, Gordoniaceae, Micrococcaceae, Mycobacteriaceae, Nocardioidaceae, Promicromonosporaceae, Pseudonocardiaceae, Sanguibacteraceae and Streptomycetaceae. Interestingly, the diversity was most influenced by the selective media rather than lichen species or the level of lichen thallus association. The potential for bioactive-metabolite biosynthesis of the isolates was confirmed by screening genes coding for polyketide synthases types I and II. These results show that littoral lichens are a source of diverse potentially bioactive Actinobacteria.

Bioprospecting has traditionally been more successful for some prokaryotic groups than others¹. Cultivable members of the Actinobacteria phylum in particular have an unrivalled record as a major source of bioactive molecules (about 45% of all microbial bioactive products discovered²). Since the discovery of streptomycin, these strains have been isolated from various environments³. However, as the number of novel leads from Actinobacteria dwindled in the 1990s⁴, the question was raised whether the “golden age” of actinobacterial bioprospecting was over. Increased research of environmental microorganisms from understudied ecosystems suggests this not be the case as new actinobacterial isolates continue to yield novel bioactive molecules.

So far most Actinobacteria have been isolated from terrestrial environments, especially soils⁴. However, marine environment might also be a promising source for bioprospecting⁵,⁶. Holding true to this promise, many novel chemical structures have been discovered from marine Actinobacteria⁷,⁸.
Coastal systems, nonetheless, are still understudied and despite an early promising study from Watson and Williams (1974) more comprehensive actinobacterial surveys are pretty recent. It should also be noted within this context, that these studies were mostly carried out on coastal sands and rhizosphere systems.

Among the underexplored coastal sources, marine lichens (referred to hereafter as those inhabiting the subtidal and intertidal zones) and littoral lichens (referred hereafter as those inhabiting the supratidal zone and subjected to sea spray) are unique. Like their terrestrial partners marine and littoral lichens are symbiotic associations between a photobiont (green algae and/or Cyanobacteria) and a mycobiont. Most lichens are outstanding producers of specific secondary metabolites that present biological activities e.g. antioxidant, cytotoxic, antimicrobial activities. Recent studies have demonstrated the prevalence of lichen-associated bacteria including Actinobacteria. While a number of publications reported the presence of cultivable bacterial associated with inland lichens, only few reported on cultivable bacteria from marine or littoral lichens (Caloplaca verruculifera, Lecanora helicopsis, Hydropunctaria maura and Verrucaria ceuthocarpa) whereas the study on another littoral lichen Roccella fuciformis reported a lack of cultivable bacteria. Thus, the diversity and the biological potential of lichen-associated bacterial communities are not yet sufficiently explored. In the study presented here, a culture-dependent approach was used to highlight marine and littoral lichens as a new source of cultivable bacteria and potential sources of Actinobacteria of interest.

Results

Overall diversity of isolates. After the lichen sampling (Fig. 1), serial dilutions of the washout and lichen homogenate (corresponding with bacteria on the surface and inside the thallus) were prepared. The bacterial inocula were plated on various media: marine agar (MA), actinomycete isolation agar (AIA) and International Streptomyces Project medium—2 (ISP 2) with nalidixic acid and cycloheximide (Table 1). Three representatives of each colony morphotype (whenever possible) on the triplicate plates were picked and transferred into the same media as they were isolated from. The partial 16S rRNA gene sequences of all strains were analyzed by comparison with the Eztaxon database using a global alignment algorithm. The length of the sequenced fragments and the result of BLASTn comparisons are presented in Supplemental Table S1 with the similarity scores to the closest match.

Several non-actinobacterial strains were able to survive the nalidixic acid treatment and the isolation process failed to isolate bacteria for L. pygmaea from the wash suspension using AIA medium. All 16S rRNA gene sequences matched with entries in Eztaxon-server with similarities ranging from 91 to 100% (over around 800 bp). Overall, a total of 116 unique 16S rRNA sequence types were recovered with 38/55, 24/37 and 54/94 (unique 16S rRNA/total strains) retrieved from L. confinis, L. pygmaea and R. fuciformis respectively. Non-actinobacterial sequences were members of phyla Proteobacteria, Firmicutes and Bacteroidetes. Interestingly some of these sequences were quite novel as they had low sequence similarity (<95%) to known described species. Eighteen strains showed a similarity percentage below or equal to 96% suggesting putative new genera, e.g. MOLA1416 which presents a 91% 16S rRNA gene similarity with Jannaschia pohangensis (present in L. confinis and the Arctic lichens C. verruculifera and H. maura) and Rhizorhapis suberifaciens (present in L. confinis and the C. verruculifera) 2) the Actinobacteria Streptomyces cyaneofuscatus (present in all Brittany lichens and L. helicopsis), Salinibacterium amurskyense (present in L. pygmaea and L. helicopsis) and Micrococcus luteus (isolated from all lichens except for L. helicopsis and H. maura) and 3) the Firmicute Bacillus aethus (isolated from L. pygmaea and H. maura).

Figure 1. The lichens species studied. (A) Lichina pygmaea (black fruticose gelatinous cyanolichen on rocky shores), (B) Lichina confinis (black fruticose gelatinous cyanolichen on rocky shores, white arrows) and (C) Roccella fuciformis (grey fruticose lichen on sheltered vertical rock faces).
### Table 1. Description of the lichen samples used as inocula.

| Lichen     | Symbiont       | Environment | Extract    | Medium| Description |
|------------|----------------|-------------|------------|-------|-------------|
| L. confinis| Cyanobacteria  | Marine      | Homogenate | MA    | LC_H_MA     |
| L. confinis| Cyanobacteria  | Marine      | Wash       | AIA   | LC_W_AIA    |
| L. confinis| Cyanobacteria  | Marine      | Homogenate | AIA   | LC_H_AIA    |
| L. confinis| Cyanobacteria  | Marine      | Wash       | MA    | LC_W_MA     |
| L. pygmaea | Cyanobacteria  | Marine      | Homogenate | MA    | LP_H_MA     |
| L. pygmaea | Cyanobacteria  | Marine      | Homogenate | AIA   | LP_H_AIA    |
| L. pygmaea | Cyanobacteria  | Marine      | Wash       | MA    | LP_W_MA     |
| R. fuciformis| Green algae  | Maritime    | Homogenate | MA    | RF_H_MA     |
| R. fuciformis| Green algae  | Maritime    | Wash       | AIA   | RF_W_AIA    |
| R. fuciformis| Green algae  | Maritime    | Homogenate | AIA   | RF_H_AIA    |
| R. fuciformis| Green algae  | Maritime    | Wash       | MA    | RF_W_MA     |
| C. auriforme| Cyanobacteria | Inland      | Wash       | ISP2  | CA_W_ISP2   |
| C. auriforme| Cyanobacteria | Inland      | Homogenate | ISP2  | CA_H_ISP2   |

The results of the analysis by BLASTn using the parameters and thresholds described showed no overlap between isolates of the marine and coastal lichens and the 2780 sequence in the queried database containing sequences from uncultured bacteria associated with lichens, while 8 strains from *Collema auriforme* (6 from wash water and 2 from homogenate) were similar (>98% identity over ca. 820 bp) to sequences belonging to Pseudomonadales (accession numbers JN023885, JN023697) previously retrieved from soils under moss crusts.

**Diversity of Actinobacteria.** In order to study the methodological and environmental factors that could influence the recoverability and diversity of Actinobacteria, a community analysis of sequences belonging to this phylum was performed by clustering sequences into OTUs at 98.5% identity (Supplemental Table S2). Since only a small fixed subset of representative strains from different morphotypes were recovered during isolation and subsequent subculture, presence and absence of OTUs were considered instead number of strains per OTU. In order to achieve a meaningful clustering using unifrac that utilizes phylogenetic distances, data from terrestrial lichen analyzed with a different medium was considered instead number of strains per OTU. In order to achieve a meaningful clustering using unifrac that utilizes phylogenetic distances, data from terrestrial lichen analyzed with a different medium was included as an outgroup. The clustering pattern is presented in Fig. 2.

Using the unweighted unifrac analysis (Fig. 2), bacterial communities could not be differentiated based on lichens species or sample treatment (e.g. homogenate or wash extract) but more reasonably by the media used. The communities associated with *Lichina* species showed a clustering depending on the isolation media whereas bacterial communities associated with *Roccella* species did not show such distinct clustering related to media. Overall, the three most evident clusters consisted of one corresponding to the communities isolated from MA medium, one from AIA medium and one specific from bacterial communities isolated from *C. auriforme* in ISP2 medium (Fig. 2).

We analyzed the two main clusters from marine lichens at a higher phylogenetic resolution. The heat-map at family level shows that Brevibacteriaceae, Nocardioidaceae and Promicromonosporaceae were mostly/primarily isolated in marine agar (MA medium) while Microbacteriaceae, Gordoniaceae, Pseudonocardiaceae and Streptomycetaceae were mostly frequent in actinomycetes isolation agar (AIA medium). Cellulomonadaceae and Mycobacteriaceae were isolated from both media. In just one case the most similar communities within a particular medium was from the same lichen genus (i.e. Microbacteriaceae and Streptomycetaceae in AIA medium for *Lichina* spp.; Fig. 2). It is important to point out that the heat-map is a reflection of the diversity (i.e. number of distinct taxa) within a family rather than a reflection of the number of strains. In addition we analyzed the environmental origin of nearest relatives of all Actinobacteria strains from marine/littoral lichens. Among the 55 top 16S rRNA sequences, 16 were from strains previously isolated from the marine environment (Table 2). In addition, except for strains related to *Paraöerskovia* and *Marmorica* a clear relationship was not found between the high salinity medium utilized and the marine origin of nearest relatives, as many supposedly marine species were isolated in AIA and some supposedly non-marine organisms were isolated exclusively in MA. Nonetheless, it should be pointed out that among the 16 supposedly marine species retrieved 10 were exclusively isolated in MA.

The comparison of lichen bacterial communities using the same medium isolated from homogenate and washwater allowed a better view of the relationships between these communities and to infer...
a putative life style (epi- and/or endolichenic). Some bacterial families were mostly isolated from homogenate extract (Cellulomonadaceae and Streptomycetaceae) while Pseudonocardiaceae was only isolated from wash extract. Brevibacteriaceae, Micrococcaceae, Mycobacteriaceae, Nocardiaceae and Promicromonosporaceae were isolated from both sample types. Thereby bacterial communities associated with lichens might show a specific distribution in the lichen thallus (on the surface and/or inside; Fig. 2).

Figure 3 shows the overall Actinobacteria diversity observed in each lichen species irrespective of the sample kind (wash/homogenate) or the isolation media. It can be observed that *R. fuciformis* contained the most diverse communities, followed by *L. pygmaea* and *L. confinis*. This observation was however not true when four families of particular interest to bioprospecting, Nocardioidaceae, Promicromonosporaceae, Pseudonocardiaceae and Streptomycetaceae were considered. When comparing the Actinobacteria OTU richness among the two media a majority of bacterial communities was isolated from marine agar medium (54 versus 28) again indicating that salinity might be at play for the isolation of these bacteria. When a similar comparison of total OTU richness between homogenate and wash was done, a slight dominance of lichen-associated bacterial communities isolated from homogenate extract (48 versus 40) was recorded.

At a higher phylogenetic resolution, each marine lichen species harbored specific actinobacterial communities. Two actinobacterial OTUs (operational taxonomic units) were common (OTU ID 1 and 18) (Supplemental Table S2) to *L. pygmaea* and *L. confinis* (Fig. 4) (similarity ≥98.5%). Between marine and littoral lichens, six actinobacterial OTUs were common between *L. confinis* and *R. fuciformis* (OTU ID 5, 11, 17, 25, 72 and 98) while no actinobacterial OTUs was present only in *L. pygmaea* and *R. fuciformis*. Interestingly more similar actinobacterial strains were observed from lichens found spatially closer in the environment: *L. pygmaea* with *L. confinis* and *L. confinis* with *R. fuciformis*. Finally, two actinobacterial OTUs (OTU ID 21 and 83) corresponding to *Streptomyces* and *Micrococcus* genera were common to all three lichens (Fig. 4) and might represent eventual contaminants.

Regarding Actinobacteria from marine and littoral lichens, 8 strains (MOLA1448, MOLA1441, MOLA1513, MOLA1522, MOLA1523, MOLA1521, MOLA1599 and MOLA1557) representing 3 potential novel species were identified, based on a 16S rRNA gene similarity percentage less than 97% to type strains of described species (Supplemental Table S1, Table 2). These novel strains likely belong to the genera *Agromyces*, *Aeromicrobium*, and *Nocardioides*. Among these, 7 were isolated from marine agar media even though these are most closely related to bacterial strains isolated from terrestrial environments (Table 2). Five of the strains (MOLA1448 from *L. confinis*, MOLA1521-23 and MOLA1599 from *R. fuciformis*) belong to family Nocardioidaceae of interest for bioprospecting. Finally, as no previous chemical studies were conducted with the nearest relative of these five strains that potentially represent new species (Table 2), they would be of particular interest for the discovery of novel bioactive compounds.
| Strains     | Media | Top Hits NCBI             | Identity (%) | Strains and Origin                                                                 | Description                                                                 | Production of metabolites |
|------------|-------|---------------------------|--------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------------------|---------------------------|
| MOLA1487   | AIA   | NR116119.1                | 1548         | Strain YIM60513<sup>†</sup> Endophyte of Gloriosa superba                           | Saccharopolyspora gloriosae                                                  |                           |
| MOLA1503   | AIA   | HQ113380.1, NR033048.1, AB019290.1 | 1493         | Strains FCM1027<sup>¥</sup>; Kitami A1<sup>†</sup>, DD4 digestive tract of Daphnia magna; waste-water of a sugar-beet factory | *Exopolysaccharides<sup>62</sup>                                               |                           |
| MOLA1515   | AIA   | EF672652.1                | 1391         | Strain AR3 rhizosphere of heavy metal accumulating willow trees                     | Agromyces sp. (Agromyces terreus)                                            |                           |
| MOLA1526   | AIA   | AB695377.1, NR114322.1    | 1448–1511    | Strains H97-3<sup>†</sup>, H93-5, isolated from sea sediment                       | Sediminihabitans lutes                                                        |                           |
| MOLA1538   | MA    | KJ627769.1, KC768764.1, FR744933.1, AB330407.1 | 1531         | Strains JI2, Iso-74, RAM276, CCO128 oil filed water, water treatment sand filter, soil, Baltic sea | *Microbacterium oxydans Microbacterium sp. (Microbacterium foliorum)<sup>†</sup> |                           |
| MOLA1549   | MA    | HG965212.1, KJ676478.1    | 1489–1543    | Strains M27<sup>†</sup>, NEAE-42, s3j2 Intertidal seaweed Fucus spiralis, soil       | Streptomyces cyaneofuscatus Streptomyces caurovensis Streptomyces flavolimosum (Streptomyces cyaneofuscatus) | *daunomycin, *cosmoycin B *galtamycin B *malphulin<sup>64</sup> |
| MOLA1554   | MA    | JX173791.1                | 1522         | Strain TRM46621 "salty beach", Xinjiang                                            | Streptomyces sp. (Streptomyces carpaticus)                                   |                           |
| MOLA1450   | AIA   | KM978822.1                | 1515         | Strain AHT-1 River sediment                                                        | Kocuria rhizophila                                                            |                           |
| MOLA1464   | AIA   | JQ924405.1                | 1500         | Strain CGMCC 4.1782<sup>†</sup> Potato spot                                       | Streptomyces praecox (Streptomyces setonit)<sup>†</sup>                        |                           |
| MOLA1467   | MA    | NR_114323.1               | 1472         | Strain H25-14<sup>†</sup> Sea sediment                                            | Paramycolabacter sedimincola                                                  |                           |
| MOLA1451   | MA    | JQ716239.1                | 1430–1452    | Strain BGMG-B34 Soil samples from the Yellow River Delta                            | Cellulosimicrobium sp. (Cellulosimicrobium terreum)                          |                           |
| MOLA1452   | MA    | AB461094.1                | 1380–1404    | Strain IK2, S6P soybean stem                                                       | Nocardiodaceae bacterium (Nocardioidea mesophilus)                            |                           |
| MOLA1448   | MA    | DQ448721.1                | 1454–1461    | Strain CNNJ872 PL04 marine sediment                                                | Marmoricola sp. (Marmoricola aquoreus)                                        |                           |
| MOLA1449   | MA    | KI843153.1                | 1504–1548    | Strain VL-80 onion waste                                                           | Micrococcus luteus<sup>†</sup> (Micrococcus aloeverae)<sup>†</sup>            | *Lutoside (unnamed strain)<sup>20</sup> * [1H]-Indole acetic acid (unnamed strain)<sup>20</sup> |
| MOLA1443   | MA    | KM817772.1                | 1450–1489    | Strain SKCB-14 sorghum                                                            | Brevibacterium sp. (Brevibacterium epidermidis)                               |                           |
| MOLA1441   | AIA   | NR_116877.1               | 1341         | Strain BZ41<sup>†</sup> isolated from hydrocarbon-contaminated soil               | Agromyces baumannensis (Agromyces terreus)                                    |                           |

Continued
| Strains     | Media | Top HitsNCBI | Scores | Identity (%) | Strains and Origin Description | Production of metabolites |
|-------------|-------|--------------|--------|--------------|---------------------------------|--------------------------|
| MOLA1435    | MA    | HQ677231.1   | 1504   | 99           | Strain A-08 in larval culture of Argopecten purpuratus | Brevibacterium sp. (Brevibacterium pustulatum) |
| MOLA1425    | AIA   | LN626361.1   | 1531   | 100          | Strain M-26 Fucus spiralis (host) | Streptomyces allidoflavus† |
| MOLA1408    | AIA   | KF848947.1   | 1522   | 100          | Strain FZ3 hazelnut husk waste | Streptomyces sp. (Streptomyces radioginosohelvolus)† |
| MOLA1407    | AIA   | HM584291.1   | 1535   | 100          | Strain CJ-G-TSA6 isolated from internal organs of edible snow crabs (Chionoecetes japonicus) | Ageria sp. (Salbacterium amor-skyense) |
| MOLA1406    | AIA   | KM678243.1   | 1522   | 100          | Strain B035 sediment from Lake Michigan | Streptomyces flavogriseus† (Streptomyces anulatus§) |
| MOLA1548    | AIA   | AJ296094.1   | 1395   | 97           | Strain OS-6 isolated from coastal marsh | Microbacterium sp. (Microbacterium marvale) |
| MOLA1545    | MA    | AB376081.1   | 1531   | 100          | Strain YT0066 “environmental samples” | Microbacterium sp. (Microbacterium pumilum) |
| MOLA1538    | MA    | JF274912.1   | 1404   | 97           | Strain PL34a1_S1 olive mill wastewater | Aeromicrobium sp. (Aeromicrobium tamlense) |
| MOLA1530    | MA    | AF544638.1   | 1509   | 99           | Strain VM0587 PAH-contaminated soil | Mycobacterium vaccae§ (Mycobacterium vanbaalenii) |
| MOLA1524    | MA    | FR682685.1   | 1504   | 99           | Strain R-36360 soil | Microbacterium sp. (Microbacterium invictum) |
| MOLA1516    | MA    | NR_112842.1  | 1502   | 99           | Strain Sp080513C-307 marine sponge Halicola sp. | Streptomyces tatryxannensis*JBIR-107944 |
| MOLA1597    | AIA   | NR_043931.1  | 1489   | 99           | Strain UMS-62 rhizospheric soil of Alium victoriales var. platyphyllum | Agromyces allii (Agromyces terreus) |
| MOLA1593    | AIA   | JX949623.1   | 1312   | 97           | Strain MDT1-31-2 glacier | Gordonia sp. (Gordonia hankookensis) |
| MOLA1590    | AIA   | KF561998.1   | 1539   | 100          | Strain M7ER1 Sunflower | Microbacterium sp. (Microbacterium paraoxydans) |
| MOLA1589    | AIA   | HE716916.1   | 1537   | 100          | Strain/SM-04 Micanthus sp. | Cartobacterium flaccumfaciens |
| MOLA1588    | AIA   | KJ855063.1   | 1275   | 98           | Strain QA-38 (Bos taurus coreanae) | Microbacterium sp. (Microbacterium flavescent) |
| MOLA1584    | AIA   | EU584521.1   | 1487   | 100          | Strain Everest-gws-50 glacial meltwater (Mount Everest) | Microbacterium sp. (Microbacterium kitamiense)* Exopolysaccharides |
| MOLA1583    | AIA   | NR_104507.1  | 1517   | 99           | Strain ON-333 soil around a wastewater treatment | Gordonia hankookensis |
| MOLA1582    | AIA   | MA EU741242.1| 1454   | 99           | Strain 13679F Marine beach sand | Gordonia sp. (Gordonia defluiti) |
| MOLA1580    | AIA   | KC355272.1   | 1524   | 99           | Strain KUDC7176 Rhizosphere Elymus tsukahimensis | Isoptericola variabilis (Isoptericola nanjingensis) |
| MOLA1579    | AIA   | FR837628.1   | 1507   | 99           | Strain 5-4-1 oligotrophic peat soil | Streptomyces beijiangensis (Streptomyces brevisporus) |
| MOLA1537    | MA    | MA NR_112793.1 | 1499 | 98           | Strain CTT-337=NBRC 1043527 sea sediment | Paraorskovia marina (Sediminibacter luteus Paraorskovia sediminicola) |
| MOLA1575    | MA    | MA AY159888.1 | 1535   | 99           | Strain 9TTH11321 biofilm forming marine bacteria on glass surface in Dae-Ho Dike (Korea) | Micrococcus sp. (Micrococcus yunnanensis) |

Continued
Table 2. List of cultivable Actinobacteria isolated from marine and littoral lichens. Strains: strains from the MOLA collection of microorganisms (WDCM911); Media: MA (marine agar) and AIA (Actinomycete Isolation Agar); Top Hits NCBI: accession number of NCBI sequences with top blastn scores; Scores (NCBI blastn scores); description: NCBI description and in parenthesis top EZTaxon type species hit whenever different (see also table S1); Production of metabolites. Description based on literature searches (Web of Science, http://apps.webofknowledge.com) or queries against the Natural Products in the REAXYS (http://www.reaxys.com) databases. Strains shaded in grey had highest hits to strains previously recovered from marine environments. ND: not determined and NF: not found. *correspondence between metabolite and strain †found in a strain without a 16S rRNA sequence; § found in a strain with a different 16S rRNA sequence than the top NCBI hit; ¶ found in a different strain with identical 16S rRNA sequence than the top NCBI hit.

Genetic screening: PKS type I and II systems. The biotechnological potential of the isolates was examined using a PCR-based screening for PKS system types I and II. Type I was found to be more common found in 45.8% of the actinobacterial OTUs when compared to 34.7% that were positive for type II. However, the ratio was highly impacted by strains that belonged to the family Streptomycetaceae in which 92.8% were positive for PKS I and only 35% of those showed evidence of PKS II. One of the other three families of interest, Nocardioidaceae had more amplification of PKS II (50%) than type I (30%). Interestingly, Promicromonosporaceae strains were not positive for any of the genes tested (Fig. 3).

Discussion
Several cultivable bacterial strains were isolated from marine and littoral lichens including members of the Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. These cultivable strains belong to 30 different genera (Supplemental Table S1) some of which are known to produce bioactive compounds (Nocardiaceae, Promicromonosporaceae, Pseudonocardiaceae and Streptomycetaceae). The results of the BLASTn analysis against sequences of uncultured bacteria of lichen origin in the NCBI database suggests that, as is the case in the most other environments, the microorganisms isolated in our study did...
Streptomyces uncialis associated with isolated from coli have been reported from this taxa in the first half of 2014 alone. Additionally, Takagi and Shin-ya (2011) evaluated a "spent force" in terms of biodiscovery, one has to note that at least 13 novel biomolecules have been shown to be valuable bioresources for pharmacological prospecting. Furthermore, a large numbers of taxa (for example Nocardiaceae, Promicromonosporaceae, and Pseudonocardiaceae) have been already shown to be producible bioactive compounds. Uncialamycin, an enediyne with antibacterial properties against human pathogens, has been isolated from L. confinis (MA) medium allowed the isolation of a wider diversity of bacteria in the Proteobacteria Firmicutes and Actinobacteria that could be isolated. In addition even with the addition of nalidixic acid, marine agar medium the isolation of a wider diversity of bacteria in the Proteobacteria Firmicutes and Bacteroidetes and almost the 2/3 of the unique bacterial strains from L. confinis were isolated using MA medium (Supplemental Table S1). The choice of growth media influenced the bacterial diversity associated with lichens more than the sample type indicating that it is an important factor to consider in experiments. Our results also indicate that many of the bacterial families could be more strongly associated with the lichen thallus. For instance bacteria belonging to the family Cellulomonadaceae, Microbacteriaceae, Gordoniaceae and Actinomycetes were more predominant in homogenate samples, thus indicating a putatively closer association or a more occasional relationship.

Interestingly many of the actinobacterial strains from marine and littoral lichens were not streptomycetes (Table 2). Not until recent times have the widespread occurrence and relative common presence of "non-streptomycete" actinobacteria have only recently come to light. The work presented here adds to the literature that rare actinomycetes is a misnomer. These taxa (for example Nocardiaceae, Promicromonosporaceae, and Pseudonocardiaceae) have been shown to be producible bioactive compounds. Uncialamycin, an enediyne with antibacterial properties against human pathogens, has been isolated from L. confinis (MA) medium allowed the isolation of a wider diversity of bacteria in the Proteobacteria Firmicutes and Actinobacteria that could be isolated. In addition even with the addition of nalidixic acid, marine agar medium the isolation of a wider diversity of bacteria in the Proteobacteria Firmicutes and Bacteroidetes and almost the 2/3 of the unique bacterial strains from L. confinis were isolated using MA medium (Supplemental Table S1). The choice of growth media influenced the bacterial diversity associated with lichens more than the sample type indicating that it is an important factor to consider in experiments. Our results also indicate that many of the bacterial families could be more strongly associated with the lichen thallus. For instance bacteria belonging to the family Cellulomonadaceae, Microbacteriaceae, Gordoniaceae and Actinomycetes were more predominant in homogenate samples, thus indicating a putatively closer association of these taxa. Whereas presence of Pseudonocardiaceae strains in only one of the wash samples might indicate an episymbiotic association or a more occasional relationship.

Some of the bacterial strains associated with lichens have been previously investigated for the production of secondary metabolites and some interesting compounds have been described. Uncialamycin, an enediyne with antibacterial properties against human pathogens (Staphylococcus aureus, Escherichia coli and Burkholderia cepacia), and cytotoxic properties, as well as cytotoxic cladamidamides A-G, were isolated from Streptomyces uncialis associated with Cladonia uncialis. Angucycline with cytotoxic and antibacterial properties against Micrococcus luteus and a butenolide (inactive) were isolated from a Streptomyces sp. associated with an unidentified lichen collected in Japan. Six aminocoumarins (coumarinocines A-E) showing antibacterial properties were also isolated from a Streptomyces sp. associated with Cladonia gracilis. Thus bacteria associated with lichens show interesting biological properties in terms of producing antibacterial or DNA damaging molecules. The study presented here adds to these observations and shows that the lichens and in particular marine/littoral lichens are not only a source of Streptomyces strains but can also be a source of more Actinobacteria with high potential for the exploitation of bioactive compounds. Marine and littoral lichen-associated bacteria represent thus a promising yet under explored to discover new natural products.

A major aspect of bacterial symbionts is their possible interactions with their hosts. Lichens are unique models to study interactions between fungi, algae and bacterial symbionts. One important approach to understand such complex and yet interesting interactions is to focus on interactions from isolates retrieved from the lichen holobiont. This is particularly true for chemical interactions since currently access of specific member of the symbiosis is not possible without cultivation. Actinobacteria are especially known to influence secondary metabolism of fungi and thus these bacteria are good model organisms to study metabolic interactions. Although, as we showed here, culture-dependent methods do not reflect the prevalent organisms, in the context of chemical interactions these "rare" microorganisms do not represent a majority of the bacterial communities associated with lichens. However, to our knowledge to date only two studies have analyzed the microbial community of marine and littoral lichens (Hydropunctaria mauro, Asphodelium nodosum, Caloplaca verruculifera, Verrucaria ceuthocarpa and Lecanora helicopis) via cultivation independent methods, the latter of which used community fingerprinting and did not report any 16S rRNA sequences from uncultured organisms. Hence, we cannot completely rule out that some of our isolates might represent dominant organisms in the marine and littoral lichens studied here, even though this is unlikely. On the other hand we were able to identify certain bacterial strains in at least three marine lichens from two distinct regions (J. pohangensis, S. cyanosepecatus and M. luteus) indicating that these species might be frequently in association with marine lichens.

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could in fact be more relevant than their abundance alone might indicate. Culture based approaches such as the one used in this work are therefore necessary to advance the overall understanding of interactions among different organisms in these symbiosis.

**Methods**

**Lichen sampling.** Lichen samples were collected from France at Erquy (48°37′47.9″ N and 2°28′31.55″ W) in April 2012 and from Austria at Kesselfallkamm (47°12′21.26″ N and 15°23′57.27″ E) near Graz in November 2012. Three marine/littoral species were collected on seashore rocks on Brittany coasts (France): *Lichina pygmaea* (Müll.) Agardh (marine lichen), *L. confinis* (Lightf.) Agardh., (marine lichen) and *Roccella fuciformis* (L.) DC., (littoral lichen) and, one inland species was collected on rocks (Austria):
Collema auriforme (With.). Lichens were identified based on their morphological characters such as size, color and chemical reaction (potassium hydroxide, para-phenylenediamine, sodium hypochlorite).

Isolation of cultivable bacteria. Marine and littoral lichens were briefly washed with sterile water in sampling sites (to remove non-symbiotic bacteria) then kept on ice through transit to the lab. On arrival to the lab the samples were aseptically divided into small pieces (1–2 g) using sterile scalpels. The pieces were washed three times with 20 ml of sterile seawater. The wash suspensions were stored and the washed lichen pieces were ground using a blender. The wash suspensions and lichen homogenates were used as separate inocula. Serial dilutions (N to N⁻³) in sterile seawater of wash and homogenate were performed and 100 μL were plated in Marine Agar (MA, Difco™ Marine Agar, BD Le Pont de Claix, France) and Actinomycete Isolation Agar (AIA, Difco™ Actinomycete Isolation Agar, BD) both media supplemented with nalidixic acid [(NA382-1G) Sigma-Aldrich, Lyon, France] and cycloheximide [(C7698-5G) Sigma-Aldrich] in triplicate. Plates were incubated at 25°C until the growth of the colonies on the Petri dishes and until the no new colonies appeared (up to 21 days). Colonies were isolated and purified using morphological characteristics [color, diameter, surface (smooth and/or rough), relief (convex or flat) and edge format (regular or irregular)], on their respective media (MA or AIA). Bacterial strains were stored in a 50% glycerol solution and 5% DMSO in Marine Broth (MB, Difco™, BD) at −80°C. The strains were deposited in MOLA culture collection of the Observatoire Océanologique de Banyuls/Mer. Bacteria from C. auriforme, which was used as an outgroup for the analysis of marine/littoral lichens were isolated using a similar protocol, except that lichens were washed with NaCl (0.85%)/Peptone from casein (1%) solution and plated into Difco™ ISP 2 agar (BD).

Genomic DNA extraction. Colonies were picked using sterilized inoculating loop and transferred in their respective liquid media depending on the original isolation media. That is, strains isolated from marine agar were cultured in marine broth (MB) and those from actinomycetes isolation agar were enriched in Luria-Bertani broth (LB) for 72 hours at 25°C. Suspensions of 950 μL of these cultures were dispensed in microcentrifuge tubes. The tubes were centrifuged at 12500 g for 5 min and supernatants were discarded. The pelleted biomass was used to isolate genomic DNA using Wizard® Genomic DNA Purification Kit (Promega, Lyon, France) following the manufacturer’s protocol.

PCR amplification of 16S rRNA genes. Aliquots (1 to 2 μL) of DNA samples were used as templates to amplify the 16S rRNA gene in a 10 μL PCR mixture with 0.4 μL (10 μM) universal bacterial primers 27Fmod (5′-AGG GTT TGA T CM TGG CTC AG-3′) and 1492Rmod (5′-TAC GGY TAC CTT GTT ACY ACT T-3′), 1X buffer, 1 μL MgCl₂ (25 mM), 0.4 μL dNTPs (20 mM) and 0.05 μL of Platinum Taq Polymerase (5 U/μL; Life Technologies, St Aubin, France). PCRs were conducted in a Veriti Thermal cycler (Life Technologies) with initial denaturing step (95°C for 5 min) followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 50°C for 30 sec and primer extension step at 72°C for 1.30 min, and a final extension step at 72°C for 10 min. Amplicons were separated by agarose gel electrophoresis (1%, 15 min at 100 V) in TAE buffer stained with ethidium bromide and visualized under UV.

Sequencing and phylogenetic analysis of 16S rRNA gene products. The amplicons were purified using the Agencourt® AMPure® XP Kit (Beckman Coulter, Villepinte, France). Aliquots (1 μL) of these samples were used as templates in a 10 μL sequencing reaction mixture with 0.5 μL Big Dye Terminator (V3.1; Life Technologies), 1.75 μL of Buffer BDT (5X; Life Technologies) and 1 μL (3.2 μM) universal bacterial primer 907r (5′-AGR GTT TGA TCM TGG CTC AG-3′). The dye terminator reactions were conducted in a Veriti Thermal cycler (Life Technologies) with 40 cycles of 95°C for 10 sec, 50°C for 5 sec and 55°C for 2.30 sec. The post-reaction mixes were cleaned using Agencourt® CleanSeq® Kit (Beckman Coulter) and sequenced using ABI 3130xl genetic Analyser Sequencer (Life Technologies). The partial 16S rRNA gene sequences obtained were aligned using the Staden Package (Gap4). However, 19 samples of bacteria from C. auriforme were purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel Duren, Germany) and sequenced by Macrogen (Amsterdam, The Netherlands) using universal bacterial primer 907r also. All sequences were compared using the BLAST algorithm with the sequences of EzTaxon server (http://eztaxon-e.ezbiocloud.net) database. Sequences were deposited in Genbank under accession numbers KM273865—KM274111.

Community analysis of cultured Actinobacteria. The sequences of all isolates were subject to an analysis pipeline using QIIME v1.5.0β. All sequences were clustered into at 98.5% identity level using the uclust algorithm in usearch 5.2 (http://drive5.com/usearch/). OTUs were classified using the rdpg Classifier and a modified database based on the Green genes October 2012 taxonomy (http://greengenes.secondgenome.com). An OTU table considering sampling units of unique combinations between lichen species, inoculum (homogenate or wash water) and isolation medium was created. Since by the addition of nalidixic acid the study selected for Actinobacteria, the subsequent analysis was performed only with organisms in this phylum. The OTU table and sequence files were parsed using shell scripts to select Actinobacteria. Sequences were aligned and masked to enable reconstruction of phylogenetic tree using
QIIME's default parameters (a pyram alignment and a fast tree tree). The placement of all sequences in the tree was calculated using an unweighted unifrac dissimilarity matrix which was finally used for UPGMA clustering and Cytoscape (v.3.1.0) visualization.

**BLASTn analysis.** In order to compare isolate sequences to those most frequently found in lichens via cultivation independent methods we performed a NCBI Genbank search using with [“lichen OR lichens” AND 16S AND uncultured NOT photobiont”] as query. All the resultant 2780 sequences were downloaded and used to create a database for blast queries. Sequences of all isolates were used as queries to the database using BLASTn v. 2.2.29+ with the following parameters: -e 1 -b 10 -v 10 -n T -r 1 -q -2 -G 0 -E 0 -m 8. Sequences with identities of above 98% over 200 bp were identified by parsing using awk and shell scripts. In addition in order to identify we performed a megablast analysis against the NCBI Genbank database (using default parameters) and retrieved the top scores for from cultured microorganisms. The environmental origin of the organisms was determined based on a search in the databases of the culture collections where these strains were deposited or the metadata associated with the NCBI entry. Data regarding the production of biomolecules was obtained by searches in the Web of Science, (http://apps.webofknowledge.com) or the REAXYS (http://www.reaxys.com) Natural Products databases. Finally in order to compare our strains to the strains isolated from those isolated by Sirgubjörnsdóttir and colleagues from other marine lichens 29, sequences from that study were retrieved and used to create a local blast database which was queried using megablast.

**Screening for presence of PKS type I and II systems.** In the lookout for evidence of biosynthetic potential, PCR based screening was carried out on genomic DNA isolated from the strains. PKS-I amplifications were carried out using the primers set-2 F (5′-CCG CAG CGG CTS TTS CTS GA-3′) and set-2 R (5′-GTS CCS GTS CCG TGS TGS TCS A-3′) as described by Courtois et al., (2003)30. The original conditions described by the authors were modified so as to accommodate use of fast PCR reaction mix KAPA2G (Clinisciences, Nanterre France). Briefly, the amplifications were performed using 4 min initial denaturation at 95°C followed by 4 touch-down cycles with annealing temperature of 65–62°C for 15 sec. The touch-down cycles were followed by 36 amplification cycles of constant annealing temperature of 61°C for 15 sec. Both the touch-down and the following standard cycles used a denaturation temperature of 95°C for 15 sec and amplification at 72°C for 30 sec. Cycling was followed by a final amplification step at 72°C for one minute.

PKS type II screens were carried out using the primers KSαF (5′-TSG CST GCT TCG AYG CSA TC-3′) and KSαR (5′-TCG CCB AAG CCA AAG GT-3′) as described by Metsä-Ketelä et al., (1999)31. The modified PCR conditions were as follows: the initial denaturation was carried out at 95°C for 4 minutes followed by 40 cycles of denaturation at 95°C for 15 sec, annealing from 56 to 64°C for 15 sec and amplification at 72°C for 30 sec. The PCR reaction concluded with a 1 min extension at 72°C. The potential presence of the polyketide synthase systems were inferred based on production of amplicons of desired sizes (ca. 600 bp).

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Author Contributions
The work was carried out as part of D.P’s doctoral thesis at University of Rennes 1. The experiment was designed by D.P., S.T., M.G., M.T.S. and L.I. The lichen samples were collected by D.P., S.T. and M.G. Actinobacterial isolation was carried out by D.P. and L.I. Strain sequencing was carried out by D.P. and L.I. Sequence analysis was performed by D.P., L.I. and M.T.S. S.A.-B. screened the isolates for PKS genes. The manuscript was written by D.P., S.A.-B. and M.T.S.

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