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Bacterial Diversity across Individual Lichens▽†

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Symbioses are unique habitats for bacteria. We surveyed the spatial diversity of bacterial communities across multiple individuals of closely related lichens using terminal restriction fragment length polymorphism (T-RFLP) and pyrosequencing. Centers of lichens house richer, more consistent assemblages than species-poor and compositionally disparate lichen edges, suggesting that ecological succession plays a role in structuring these communities.

The community of organisms in any habitat is shaped by interactions among diverse influences over time. Influences include features of the abiotic environment, characteristics of colonizing species, and the successional trajectory of the community itself; understanding how different processes influence microbial diversity will be critical to translating ecological principles across the tree of life (13, 24). Community ecology often defines habitats according to parameters of the physical environment, for example, temperature or topography (8, 25). However, the task of parameterizing an organism's ecological niche changes when the habitat is another living organism (10, 14) or multiple species living symbiotically. Bacteria are commonly found within symbioses, for example, corals (18), mycorrhizas (5), and lichens (3, 7, 16). In these habitats, the changing dynamics of the symbiosis itself, including the growth, development, and interactions of symbionts, may affect community assemblage. We used lichens as a model to explore the diversity of bacterial communities housed across the living tissues of a symbiosis.

Lichens are structured associations of a fungus and algae and provide unique habitats for bacteria (4). The environment within the lichen thallus includes ongoing fluxes of nutrients, chemical signals, and secondary metabolites (11). Direct comparisons of the emerging literature on lichen-associated bacteria are difficult because experiments use a diverse set of methods, but evidence suggests that communities within lichens are distinct from the communities of adjacent substrates (4), and both microscopy and pyrosequencing suggest that lichen species with different morphologies house distinctive bacterial assemblages (3, 16). Communities of *Cladonia arbuscula* appear to be dominated by *Alphaproteobacteria* (7), while other lichens house culturable nonphotosynthetic nitrogen-fixing *Gammaproteobacteria* (19) or previously undescribed lineages of *Rhizobiales* (17). The metabolic activities of bacteria may provide a benefit to the lichen (16), and in fact the diversity of lichen actinomycetes has attracted attention specifically because these bacteria are a potential source of novel small molecules (15) that may benefit pharmaceutical research (9, 27).

We tested whether bacterial communities are also shaped by the different abiotic and biotic environments found across the thalli of individual lichens and whether bacterial communities of closely related and morphologically similar lichens growing on a single surface are distinct. Target species are in the genus *Xanthoparmelia*; *X. plittii* and *X. somloënsis* have different secondary chemistries (*X. plittii* contains stictic acid in its internal layer, while *X. somloënsis* contains salazinic acid) but share very similar foliose (or flat, leaf-like) morphologies (6). In these *Xanthoparmelia* lichens, the center of the thallus is the oldest part of the individual (Fig. 1) and appears to grow as a combination of original and regenerating tissues; the edges of the lichen are recent growth. Center modules have been exposed to ambient, colonizing bacteria for a longer period of time than edge modules. There may also be chemical and physical differences between the different locations; centers may be more likely to fragment, and data from, e.g., *Cetraria islandica* suggest that nitrogen concentrations are significantly lower in older fragments (21). In the *Xanthoparmelia* lichens we sampled, the centers housed dense numbers of column-shaped reproductive structures termed isidia, while the edges lacked isidia and were therefore flat.

In 2009, we cut 43 fragments from transects laid across nine lichen thalli found on a single gravestone in North Cemetery, Petersham, MA. Samples were characterized as "center," "intermediate," or "edge." We have been tracking the growth of these individuals since 2006 and can document that fragments now considered "intermediate" were "edge," or had not yet grown, in 2006 (Fig. 1). In contrast, growth rate data suggest that centers of the oldest lichens in our study are at least 10 years old (A. Pringle, unpublished data).

After DNA extraction from unwashed samples and amplification of the bacterial 16S rRNA gene, terminal restriction fragment length polymorphism (T-RFLP) analysis was used to obtain profiles of bacterial communities in each of the samples according to protocols previously described (22), except 35

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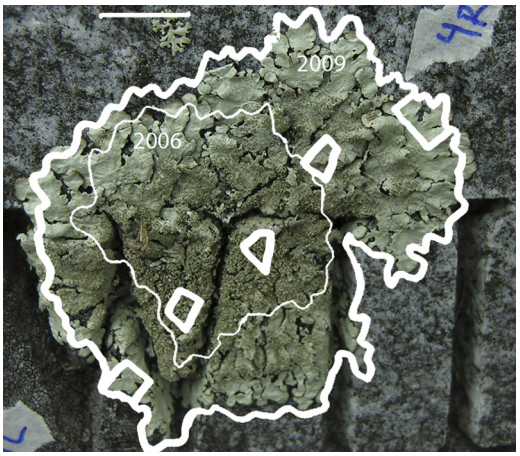

FIG. 1. Diagram of a lichen transect. The inner line traces the lichen boundary in 2006, and the outer line marks the boundary of the same individual in 2009, at sampling. Polygons trace the approximate sizes and locations of sampled lichen pieces. Bar, 1 cm. The tracings and photograph are not aligned because tracings are taken directly from a plastic sheet taped over the surface of the lichen, while a photograph of the angled gravestone results in a skewed image of the thallus.

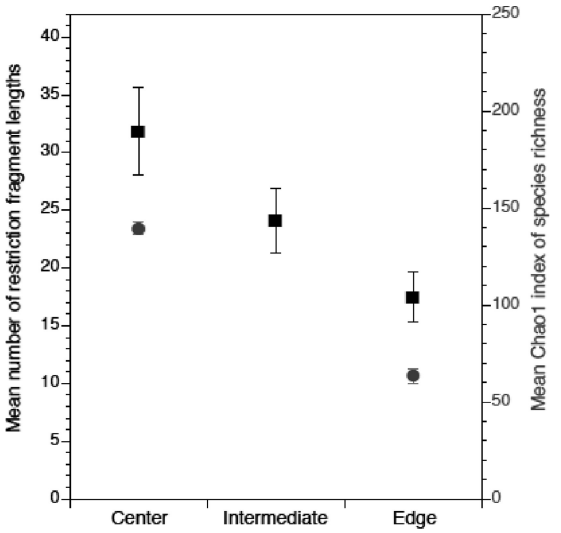

FIG. 2. Comparison of mean species richness of center, intermediate, and edge samples. The left axis shows species richness (squares) as defined by the number of different terminal restriction fragment lengths present (n = 7, 10, and 16 for center, intermediate, and edge, respectively). The right axis shows the mean Chao1 indices (circles) of species richness taken from 454 data, with species defined by 97% sequence similarity (n = 2 for center and edge, respectively). Error bars show ±1 standard error (SE) for each mean.

PCR cycles were used to obtain sufficient product for T-RFLP analysis. (Two edge, six intermediate, and two center samples did not provide enough data for analysis, most likely because of environmental PCR inhibitors; these samples were discarded.) We next used 454 sequencing of the 16S genes in four of the samples (two center samples and corresponding edge samples) to corroborate observed diversity trends and identify taxonomic affiliations of bacteria; bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) was performed by Research and Testing Laboratories (RTL; Lubbock, TX) according to RTL protocols previously described (23).

Data document declining bacterial species richness from the center to the edges of a lichen thallus (Fig. 2). We first used the number of T-RFLP fragments as a proxy for species richness; total fluorescence levels did not differ significantly among the three locations (mean total fluorescence levels: center samples, 176,627.9; intermediate samples, 190,822.3; edge samples, 235,716.6; t tests yielded P values of 0.15 for edge versus center, 0.26 for edge versus intermediate, and 0.55 for intermediate versus center). Individual center samples are significantly more diverse than individual edge samples (mean values: center, 31.9; intermediate, 24.1; edge, 17.5; $t_{21} = 3.48$, two-tailed $P = 0.002$ for edge samples versus center samples), a trend corroborated by 454 data (Fig. 2; see below). A two-way analysis of variance (ANOVA) (Stata release 10; StataCorp LP, College Station, TX) of the T-RFLP data confirms location as a significant predictor of bacterial species richness, even when controlling for lichen species; the reverse is not true (Table 1).

Bacterial communities from lichen centers cluster together, while samples from edges are more disparate (Fig. 3). Nonmetric multidimensional scaling (NMS) ordination is a technique designed to visualize differences in community composition within a two-dimensional space: pairwise distances among the T-RFLP profiles of the 33 samples were calculated as distance matrix D with elements according to the equation $D_{ij} = (1 - QS_{ij})$, where QS_{ij} is the Sørensen similarity quotient for the ith and jth samples (26), and a multiresponse permutation procedure (MRPP with weight for each group i equal to $w_i = n_i/\Sigma n_i$, where n_i is the number of samples in group i) was used to evaluate degree of clustering (PC-Ord version 5; MJM Software). When samples were grouped by location on the lichen, chance-corrected within-group agreement (A) was 0.039 ($P = 0.008$), where positive values of A indicate more clustering by group than expected by chance, up to a maximum A value of 1 if all communities were identical within each group. When bacterial communities were grouped by lichen species, A was 0.02 ($P = 0.015$). Small values of A are common for ecological data, and the interpretation of these statistics is subject to the permutation distribution of A (20). MRPP may give a low P value even when the clustering has limited ecological significance, although this bias is more commonly associated with larger sample sizes. In our data, visual inspection of the NMS results (Fig. 3) suggests that intermediate and center samples occupy a different ecological space than edge points but that the differences between the distributions of samples from different lichen species may be less ecologically relevant.

The diversity among samples, as measured by multivariate

TABLE 1. ANOVA results

Parameter	df	F^a	P
Location	2, 27	5.16	0.013
Lichen species	1, 27	1.30	0.265
Location-lichen species interaction	2, 27	0.17	0.844
Model	5, 27	2.96	0.030

[a] F statistics reflect type III (marginal) sums of squares.

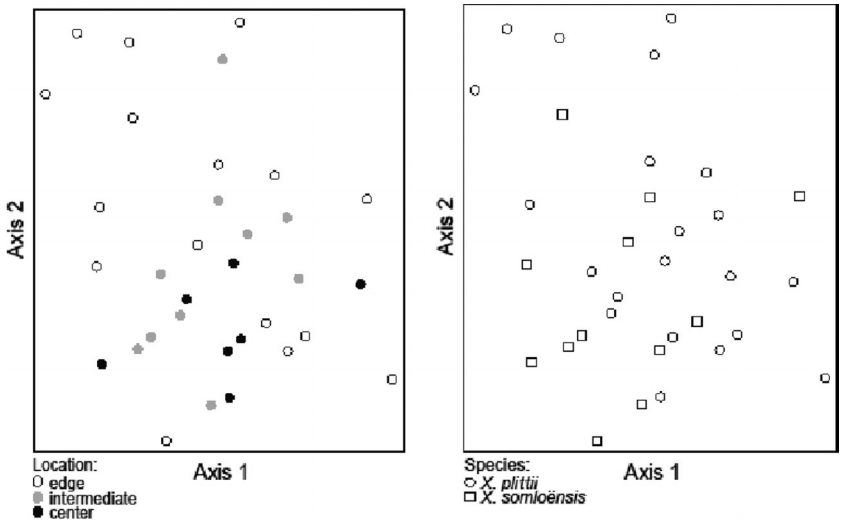

FIG. 3. Nonmetric multidimensional scaling (NMS) of samples' T-RFLP profiles, grouped by location on lichen thallus (left) and lichen species (right).

dispersions (2), was significantly higher within the group of edge samples than within the groups of either intermediate or center samples. Beta diversity was described by once again calculating D and then following the method described by Anderson (1). We assigned each sample to a point in a multidimensional Euclidean space and performed a one-way ANOVA on the distances from each point to its group centroid. Tests were implemented in R (version 2.12.0; R Foundation for Statistical Computing [http://www.R-project.org/]) using vegan (modified by us from version 1.17-4 [http://CRAN.R-project.org/package=vegan]). Distances were significantly different across the three groups ($F_{2,30} = 8.29$, $P = 0.002$); pairwise two-tailed t tests confirm significant differences between dispersions of edge and intermediate groups ($t_{24} = 3.02$, $P = 0.006$) and edge and center groups ($t_{21} = 3.67$, $P = 0.001$), but not between intermediate and center groups ($t_{15} = 0.90$, $P = 0.38$). Communities at lichen edges appear to be more variable than the more uniform assemblages of the lichen center.

Trends were further parsed using 454 sequencing of single center and edge fragments taken from each of two *X. plittii* lichens. After discarding all sequences found only once within the global database, removing chimeras with ChimeraSlayer (Broad Institute, Cambridge, MA [http://microbiomeutil.sourceforge.net/#A_CS]), and removing sequences classified as chloroplasts by the Ribosomal Database Project (RDP) classifier (Michigan State University [http://rdp.cme.msu.edu/]), taxon richness was estimated by calculating the Chao1 index of each of the four samples in RDP. When operational taxonomic units (OTUs) were defined by 97% similarity (roughly equivalent to "species"), center samples appeared significantly richer in species than edge samples (mean Chao1 indices: center samples, 139.4; edge samples, 63.5; $P = 0.002$) (Fig. 2), corroborating the T-RFLP analysis. The difference between edge and center samples was also significant when OTUs were defined by any level of similarity between 85 and 100%. The Shannon index of species diversity measures the evenness, or relative abundance, of species, as well as richness, and was also calculated in RDP; the difference between the means of center and edge samples approached significance (mean Shannon indices: across center samples, 3.7; across edge samples, 2.9; $P = 0.076$).

Patterns of bacterial abundance were analyzed by classifying the sequences with the RDP classifier (Fig. 4). Many sequences remained unidentified; any sequence not identified to order at the 60% confidence level was assigned to the "unclassifiable" category. All samples were overwhelmingly dominated by *Proteobacteria* and *Acidobacteria*. The *Acidobacteria* were members of *Acidobacteria* Gp1 and made up a significantly higher

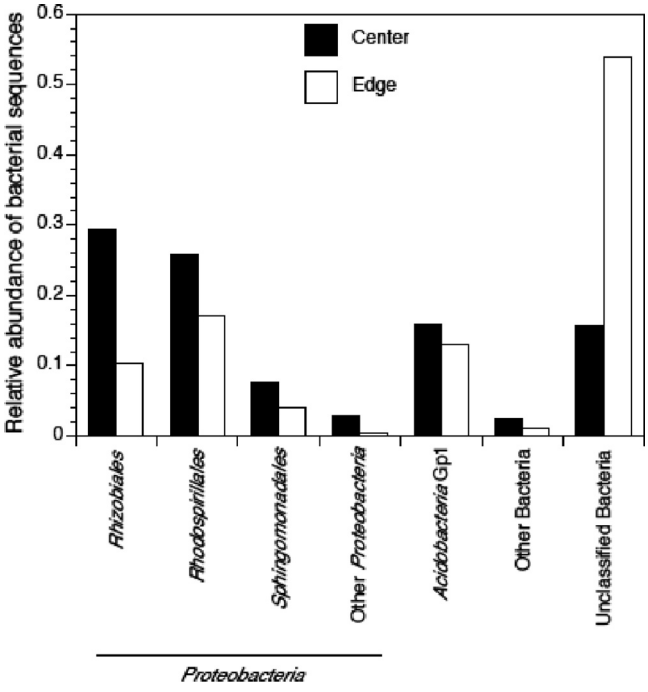

FIG. 4. Relative abundance of dominant taxa in center and edge samples, according to pyrosequencing data. *Rhizobiales*, *Rhodospirillales*, and *Sphingomonadales* are orders within the class *Alphaproteobacteria*.

proportion of center versus edge communities. The *Proteobacteria* were dominated by *Alphaproteobacteria*. Almost all of the sequences within the *Alphaproteobacteria* belonged to the orders *Sphingomonadales*, *Rhodospirillales*, and *Rhizobiales*. Although it is clear that different groups of bacteria are not evenly distributed across center and edge habitats, the large proportion of unclassified bacteria precludes definitive conclusions about differences in dominant proteobacterial taxa between centers and edges (see the supplemental material for a table showing BLASTN results for representative unclassifiable sequences). Whether these unclassified bacteria represent novel lineages remains to be determined.

In the aggregate, data suggest that the centers of individual lichens house both greater numbers of bacterial species and more-consistent communities. In contrast, edges are species poor and the bacterial communities of different edges are more variable. Lichen centers have been exposed to dispersing bacteria for longer periods of time, perhaps allowing for a greater subset of ambient colonizers to establish. Patterns may also indicate something like a climax community forming in the center parts of individual thalli, while the edges house more-random assemblages. The mechanism for this effect is unclear; however, the similarity between center and intermediate samples in both species richness and composition suggests that the physical contrast of the isidiate lichen center, compared to the flat edges, may also be important in determining bacterial community composition. The thicker center of a lichen has more biomass per unit area and thus more available habitat; the vertical isidia may also afford more ecological niches by creating gradients in light and moisture availability. Differences in the secondary chemistry of center and edge modules may also affect bacterial communities, although it is not clear whether bacteria come into direct contact with the secondary metabolites.

The precise differences in abiotic and biotic parameters across a lichen and the relative influence of these parameters, dispersal limitation, and succession on the community must be elucidated with additional research on both lichen physiology and bacterial functions and adaptations. However, as our data make clear, bacterial communities within a lichen are not static and instead are subject to the effects of ecological processes operating on small scales. Lichens have been conceptualized as self-contained ecosystems (12). The organisms growing within these habitats form communities that, like plants undergoing succession on the sand dunes of Lake Michigan (8), appear to assemble predictably in response to a confluence of variables, including features of the symbioses in which they are housed.

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