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SHORT COMMUNICATIONS

Prediction of the Metabolic Functions of Nitrogen, Phosphorus, and Sulfur Cycling Bacteria Associated with the Lichen *Peltigera frigida*

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Abstract—Lichens are currently interpreted as complex self-sustaining ecosystems formed by the interaction of the primary symbionts and other microorganisms. These microorganisms, which colonize the surface of lichen thalli, could be crucial actors in nutrient cycling. Here, we used PICRUSt2 to predict and compare the potential functions of bacteria closely associated with *Peltigera frigida* thalli and their substrates. We found that these bacteria could potentially transform organic and inorganic molecules related to nitrogen, phosphorus, and sulfur cycles. Although further experiments to verify these potential contributions are required, these results reinforce the proposal of the nutrient-cycling role of bacteria associated with *P. frigida*.

Keywords: lichen microbiome, nutrient cycling, functional prediction, Southern Chile

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Lichens have traditionally been defined as a mutualistic symbiosis between a mycelial fungus (mycobiont) and an extracellular photobiont (a green alga and/or a cyanobacterium). The mycobiont provides structural support to the symbiosis, while the photobiont fixes carbon via photosynthesis and, if it is a cyanobacterium, it also has the ability to fix nitrogen (Hawksworth and Honegger, 1994). However, since the discovery of various other microorganisms associated with lichens, they were re-described as multispecies symbioses (Aschenbrenner et al., 2016), and currently, they have even been considered complex self-sustaining ecosystems (Hawksworth and Grube, 2020).

Lichens may carry part of their microbiome in their vegetative propagules (Hodkinson et al., 2012; Aschenbrenner et al., 2016) or recruit it from the substrates where they grow (Aschenbrenner et al., 2016; Leiva et al., 2021). Moreover, metagenomic and proteomic studies indicate that bacteria in the lichen microbiome may contribute to lichen symbiosis by providing nutrients (Grube et al., 2015), controlling the establishment of potential pathogens, and stimulating the vegetative growth of lichen thalli (Sigurbjörnsdóttir et al., 2016), among other functions.

Here, we assessed the potential functions related to phosphorus, nitrogen, and sulfur cycling of bacteria closely associated with thalli and substrates of *Peltigera frigida* lichens. *Peltigera frigida* has been reported only in the southern extreme of South America (Martínez et al., 2003; Magain et al., 2018), and in Chile, this species inhabits mainly the Patagonian *Nothofagus* forests (Quilhot et al., 2012; Zúñiga et al., 2015; Orlando et al., 2021). Despite its low abundance in other regions of Southern Chile, *P. frigida* is highly abundant in the Coyhaique National reserve in the Aysén Region (Zúñiga et al., 2015; Leiva et al., 2021).

The dataset used in this work consists of 75982 bacterial amplicon sequence variants (ASVs) previously obtained by Leiva et al. (2021) from 10 samples of *P. frigida* thalli and their substrates collected in a *Nothofagus pumilio* forest in the Coyhaique National Reserve, Chile. This dataset, deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the accession number PRJNA561460, was used to perform a functional inference using PICRUSt2 (Douglas et al., 2020). From the output dataset after "Hidden-state prediction," functions of interest related to nitrogen, phosphorus, and sulfur

cycles were filtered according to their Enzyme Code or their KEGG Orthology number following the R script available at https://doi.org/10.6084/m9.figshare.14312861. Predicted genes for each function were treated, in this work, as genetic markers for the relevant function. Also, some gene markers were grouped to visualize the parts of the cycle involving multiple gene markers. Finally, the "Metagenome prediction" section of the PICRUSt2 procedure was completed with this modified input. A total of 83 gene markers were assessed, of which 51 were found in the samples (Table S1). The relative abundance of molecular markers with at least 3% frequency of occurrence in the thalli or substrates was compared between these sample types using a dependent parametric Welch's *t*-test or a dependent non-parametric Mann–Whitney–Wilcoxon (Table 1). ASVs predicted to each function were taxonomically assigned using the DADA2 R package 1.20.0 (Callahan et al., 2016) with reference to the GTDB genomic taxonomic database (release 86) (Parks et al., 2018) (Table S2).

The bacteria closely associated with *Peltigera frigida* lichens that are predicted to be potentially related to phosphorus cycling were more abundant than those related to nitrogen and sulfur cycles. In thalli, the more abundant phylum related to these predicted functions was *Proteobacteria*, while in the substrates it was *Acidobacteriota*, which is in line with the relative abundance of these bacterial groups in each microenvironment (Leiva et al. 2021) (Fig. 1).

Among the molecular markers related to the nitrogen cycle (Table 1 and Fig. 2a), the relative abundance of nitrification *nxrAB* group highlights (point a in Fig. 2a); however, the codes used for this group (K00370 and K00371) also include the genes of denitrification (*narGH*). The fact that other nitrification markers were barely detected, and that in this set most bacteria with *nxrAB*/*narGH* genes also harbored *narI* (point 3 in Fig. 2a), suggests that these markers, mainly from the order *Betaproteobacteriales* (phylum *Proteobacteria*) (Table S2), were most probably involved in the denitrifying process. Also related to denitrification, gene markers *nirK/nirS* and *nosZ* (points 4 and 6 in Fig. 2a, respectively) appeared significantly higher (1.4 ± 0.6 and 4.4 ± 2.0 times (mean ± SD)) in thalli than in substrates. They were mostly matching to the class *Gammaproteobacteria* (54%, phylum *Proteobacteria*) and the family *Chitinophagaceae* (77%, phylum *Bacteroidetes*), respectively (Table S2). The sequential reduction of nitrogen oxides during denitrification may be crucial since these oxides could decrease the photosynthetic rates in lichens (Hauck, 2010; Gadsdon et al., 2010), so bacteria in the thalli removing these damaging compounds could be beneficial for the lichen symbiosis. Predicted markers related to denitrification mainly matched the phyla *Proteobacteria* and *Bacteroidota* (Fig. S1), which were two of the most abundant phyla in the bacterial microbiome of *P. frigida* lichens (Leiva et al., 2021). Another marker showing an abundance above the 3% threshold was *nirBD* (point 7 in Fig. 2a), related to assimilatory/dissimilatory nitrate reduction. However, the ammonium produced by this pathway would not be available to the rest of the symbionts since the other markers related to the dissimilatory pathway (*napAB*) had low abundance (Table S1). Regarding organic nitrogen mineralization, the relative abundance of the markers when grouped (point c in Fig. 2a) was significantly (1.2 ± 0.1 times) higher in the thalli than in the substrates, and most matched with the phylum *Proteobacteria* in the thalli (56%) and *Acidobacteriota* in the substrates (48%) (Table S2 and Fig. S1). Bacteria possessing these genes could be recycling old parts of the lichen thalli, obtaining nitrogen at a lower energetic cost for the lichen community than by nitrogen fixation (Grube et al., 2015).

Among the molecular markers related to the phosphorus cycle (Table 1 and Fig. 1b), the relative abundance of the marker related to inorganic phosphorus solubilization (point 1 in Fig. 2b) was significantly (1.6 ± 0.4 times) higher in the substrates than in the thalli, being in the former mostly related to the phylum *Acidobacteriota* (Fig. S1), specifically to the class *Luteitalea* (40%) (Table S2). Bacteria carrying this gene could be solubilizing phosphorus from the soil since it is highly abundant but poorly bioavailable in the volcanic soils from the region of study (Borie and Rubio, 2003). Conversely, the gene markers related to organic phosphorus mineralization (point d in Fig. 2b) showed a relative abundance of 1.3 ± 0.1 times higher in the thalli than in the substrates, being in the former mostly from the phylum *Proteobacteria* (50%) (Table S2). As in the case of nitrogen compounds, lichen-associated bacteria performing this function could be mineralizing phosphorus from decaying old parts of the thalli (Grube et al., 2015).

Among the molecular markers related to the sulfur cycle (Table 1 and Fig. 2c), the marker related to sulfate adenylyl-transferase (point 1 in Fig. 2c) had a higher relative abundance in the substrates than in the thalli. This marker can be related to both assimilatory and dissimilatory sulfate reduction; however, it would be more related to the former since other sulfate dissimilatory reduction markers were depreciated. Therefore, the sulfur produced by this pathway would not be available to other microorganisms of the symbiosis. The sulfide:quinone oxidoreductase and sulfite dehydrogenase markers (points 2 and 3 in Fig. 2c, respectively) were highly abundant, but only the latter was significantly (14 ± 12 times) more abundant in the thalli than in the substrates and was mainly related to

Table 1. Molecular markers assessed as part of the nitrogen, phosphorus, and sulfur cycles with a relative abundance ≥3%. The metabolic pathways, summarized reactions, enzymes or groups, and genes are shown. In the column ID, numbers refer to the markers related to a specific reaction, while letters refer to groups comprising different markers carrying out complementary or equivalent reactions. In the last column, if a significant difference was encountered ($p < 0.05$) when comparing the relative abundances of markers in *P. frigida* thalli and substrates, the microenvironment where the abundance was higher is indicated

Metabolic pathway	ID	Summarized reaction	Enzyme or Group[1]	Gene(s)[2]	Higher in[4]
Nitrogen cycle					
Nitrification/denitrification	a	$NO_2^- \rightarrow NO_3^- / NO_3^- \rightarrow NO_2^-$	Nitrite oxidoreductase (Group)	*nxrAB/narGH*	–
Denitrification	b	$NO_3^- \rightarrow NO_2^-$	Nitrate reductase (Group)	*narGHI*	–
Denitrification	4	$NO_2^- \rightarrow NO$	Nitrite reductase, NO-forming	*nirK/nirS*	Thalli ($p = 2.18$E-02)
Denitrification	5	$NO \rightarrow N_2O$	Nitric oxide reductase, subunit B	*norB*	–
Denitrification	6	$N_2O \rightarrow N_2$	Nitrous oxide reductase	*nosZ*	Thalli ($p = 4.19$E-06)
Assimilatory/dissimilatory nitrate reduction	7	$NO_2^- \rightarrow NH_4^+ / NH_4^+ \rightarrow NO_2^-$	Nitrite reductase, NADH	*nirBD*	–
Mineralization	c	$NO \rightarrow NH_4^+$	Nitrogen mineralization (Group)	*E3.5.1.4/amiE/ure/glsA/aspA/E3.5.1.1/ansA/ansB*	Thalli ($p = 5.93$E-05)
Phosphorus cycle					
Solubilization–organic acid excretion	1	$Pi \rightarrow PO_4H_3$	Quinoprotein glucose dehydrogenase	*gcd, mGDH*	Substrates ($p = 7.58$E-05)
Mineralization–phytases	a	Phytic acid $\rightarrow PO_4^{3-}$	Phytases (Group)	*E3.1.3.8/phy/phyA/appA*	Substrates ($p = 1.46$E-02)
Mineralization–phosphatases	b	$R{-}PO_4{-}R \rightarrow PO_4{-}R$	Phospho-di-esterases (Group)	*qplQ/acpA*	Substrates ($p = 1.63$E-04)
Mineralization–phosphatases	c	$PO_4H_2R \rightarrow PO_4H_3$	Phospho-monoesterases (Group)	*phoD/phoA/phoB/phoN/aphA/cppA/ushA*	Thalli ($p = 3.91$E-03)
Mineralization–phosphonatases	8	$NH_2{-}(CH_2)_2{-}PO_3 \rightarrow CHO{-}CH_2{-}PO_3$	2-Aminoethyl phosphonate-pyruvate aminotransferase	*phnW*	Thalli ($p = 6.28$E-06)

Table 1. (Contd.)

Metabolic pathway	ID	Summarized reaction	Enzyme or Group[1]	Gene(s)[2]	Higher in[4]
Mineralization–phosphonatases	9	$CHO{-}CH_2{-}PO_3 \rightarrow PO_4$	Phosphono-acetaldehyde hydrolase	*phnX*	Thalli ($p = 4.09E-04$)
Mineralization–phosphonatases	10	$CO_2H{-}CH_2{-}PO_3 \rightarrow PO_4$	Phosphono-acetate hydrolase	*phnA*	Thalli ($p = 2.18E-03$)
Mineralization–phosphonatases	11	$CH_3{-}PO_3 \rightarrow PRP_n$	Alpha-D-ribose 1-methyl phosphonate 5-triphosphate synthase	*phnIGHL*	Thalli ($p = 9.28E-08$)
Mineralization–phosphonatases	12	$PRP_n \rightarrow PO_4^{-3}$	Alpha-D-ribose 1-methyl-phosphonate 5-triphosphate diphosphatase	*phnM*	Thalli ($p = 1.46E-02$)
Mineralization	d	$PO \rightarrow PO_4^{-3}$	Phosphorus mineralization (Group)	*E3.1.3.8/phy/phyA/appA/phoD/phoA/phoB/phoN/aphA/cppA/phnX/phnA/palA/pphA*	Thalli ($p = 3.03E-06$)
Sulfur cycle					
Assimilatory/dissimilatory sulfate reduction	1	$SO_4^{-2} \rightarrow SO_3^{-2} / SO_3^{-2} \rightarrow SO_4^{-2}$	Sulfate adenylyltransferase	*cysND/sat/met3*	Substrates ($p = 1.33E-04$)
Hydrogen sulfide oxidation[3]	2	$H_2S \rightarrow S_n^{-2}$	Sulfide:quinone oxidoreductase	*sqr*	–
Hydrogen sulfide oxidation[3]	3	$SO_3^{-2} \rightarrow SO_4^{-2}$	Sulfite dehydrogenase	*E1.8.2.1*	Thalli ($p = 2.20E-04$)
Thiosulfate oxidation	a	$S_2O_3^{-2} \rightarrow SO_4^{-2}$	Sulfur-oxidizing protein (Group)	*soxABCXYZ*	Thalli ($p = 2.59E-06$)
Thiosulfate oxidation	10	$S_2O_3^{-2} \rightarrow S_4O_6^{-2}$	Thiosulfate dehydrogenase	*tsdA*	Thalli ($p = 1.47E-06$)
Mineralization	b	$So \rightarrow Si$	Sulfur mineralization (Group)	*alsA/pisa1/gns/arsb/ids/EC 3.1.6.18/betC/sts*	Substrates ($p = 1.95E-03$)

[1] A "Group" includes markers according to "Gene(s)" column to give better evidence of some parts of the cycles.
[2] When a code involves more than one gene, they are separated by slashes; when various genes are needed to carry out a part of the metabolic pathway, names are indicated without a separator. Besides, when the enzyme could not be associated with any gene, the enzyme code (EC) was used instead.
[3] The markers for "Hydrogen sulfide oxidation" are part of a more complex process that is shown in Table S1.
[4] A hyphen in the "Higher in" column indicates no significant difference in relative abundance between thalli and substrates.

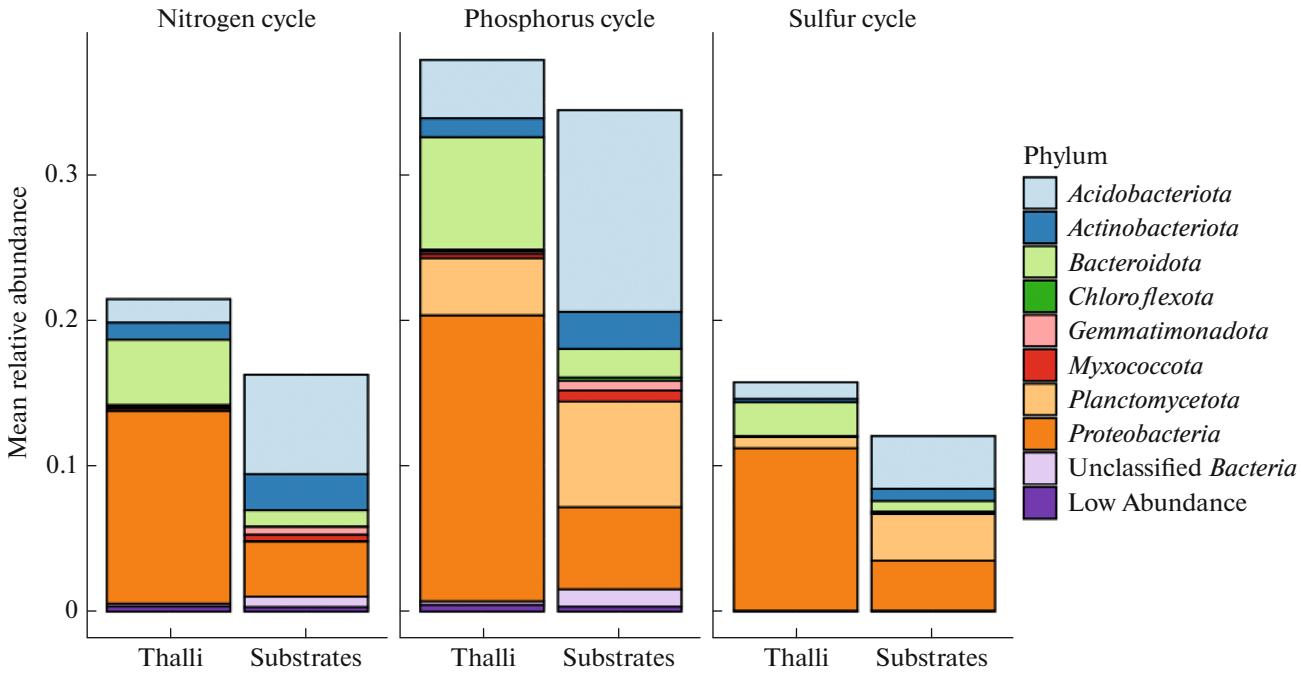

Fig. 1. Mean relative abundance and taxonomic composition of molecular markers related to each nutrient cycle. Mean relative abundance was calculated by averaging all markers encountered for each cycle and dividing by the predicted number of bacteria.

the genus *Rhizobacter* (94%, phylum *Proteobacteria*) (Fig. S1, Table S2), which was one of the most abundant genera in thallus samples (Leiva et al., 2021). Bacteria carrying these markers could contribute to the fitness of lichens by degrading hydrogen sulfide and sulfite, since these compounds have been described as atmospheric pollutants (Mizalski and Niewadomska, 1993; Barton et al., 2014), and sulfite has a negative effect on photosynthesis in lichens (Hill, 1974). Besides, markers related to thiosulfate oxidation, sulfur-oxidizing protein and thiosulfate dehydrogenase, (points a and 10 in Fig. 2c, respectively) had a significantly (7.3 ± 3.9 and 4.1 ± 1.7 times) higher relative abundance in the thalli than in the substrates, and they were mainly from the family *Burkholderiaceae* (90%, phylum *Proteobacteria*) and the class *Bacteroidia* (64%, phylum *Bacteroidota*), respectively (Table S2 and Fig. S1). These processes may be crucial for sulfur cycling in lichens since the Sox complex generates sulfate, the main form in which sulfur is available to lichens and most organisms (Barton et al., 2014), and the TsdA complex may complement it with the reverse reaction generating thiosulfate (Liu et al., 2013). Finally, the markers related to the mineralization of organic sulfur (point b in Fig. 2c), of which 84% matched with the phylum *Acidobacteriota* in the substrates (Table S2 and Fig. S1), also had significantly higher relative abundance in the substrates than in the thalli, which could be related to the fact that sulfur in the soil is mainly associated with organic molecules (Hillel et al., 2004).

Overall, our results show that metagenome prediction is a cost-effective way to assess potential functions of bacteria in lichen thalli and a valuable tool to propose hypotheses to be tested by further experiments in the future. In this context, our *in silico* predictions suggest that bacteria closely associated with *P. frigida*, mainly those of the phylum *Proteobacteria*, have the potential to play an essential role in obtaining nutrients that are essential for the symbiosis. Among these, nitrogen and phosphorus may be obtained from recycling organic compounds of old parts of the thalli. Furthermore, these bacteria could also contribute to obtaining phosphorus and sulfur from the main inorganic and organic forms in the soil, respectively. Finally, some bacteria associated with *P. frigida* could contribute to the degradation of nitrogen and sulfur compounds, which are environmental pollutants and interfere with photosynthesis, a crucial function carried out by the cyanobacterial photobiont.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interest.

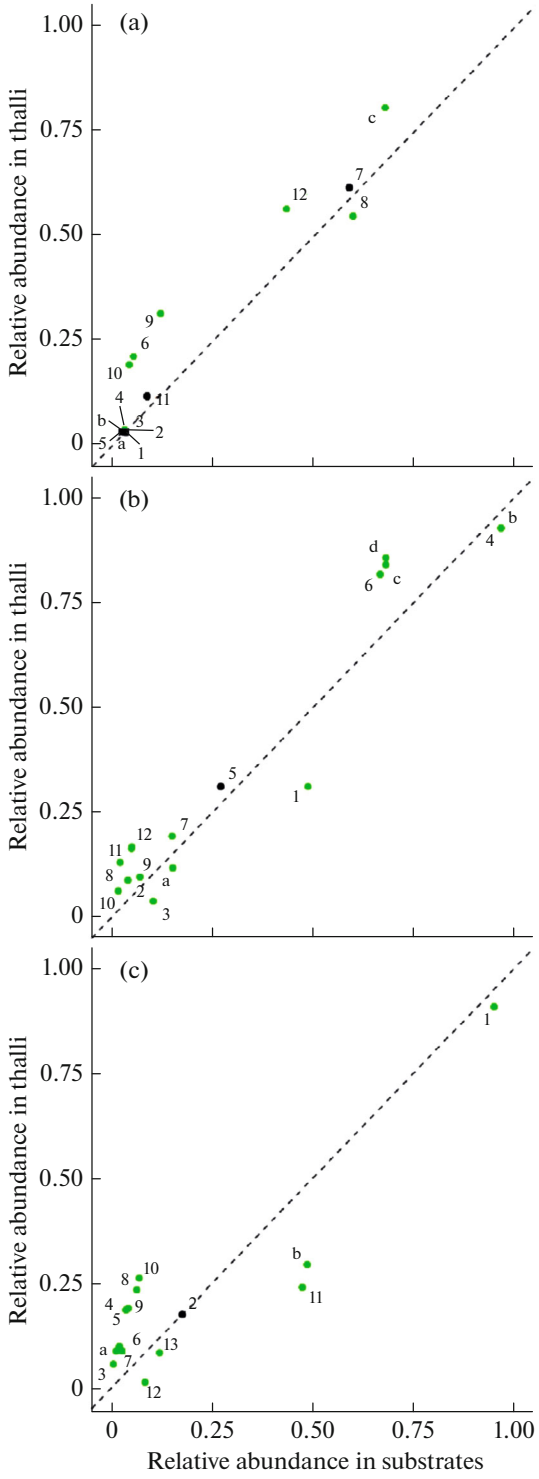

Fig. 2. Comparison of the relative abundance of molecular markers related to nutrient cycling between substrates (*X*-axis) and thalli (*Y*-axis) of *P. frigida* lichens. Numbers and lowercase letters indicate the molecular markers included in the column ID in Table 1. In green, markers showing a relative abundance significantly different between thalli and substrates ($p < 0.05$). The dotted line shows the theoretical zone where the relative abundance in thalli and substrates is identical. (a) Molecular markers related to the nitrogen cycle. (b) Molecular markers related to the phosphorus cycle. (c) Molecular markers related to the sulfur cycle.

The authors declare that animals were not used in the experiments.

SUPPLEMENTARY INFORMATION

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