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BRIEF REPORT

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Pseudomonas syringae isolated in lichens for the first time: Unveiling *Peltigera* genus as the exclusive host

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Abstract

Pseudomonas syringae is a bacterial complex that is widespread through a range of environments, typically associated with plants where it can be pathogenic, but also found in non-plant environments such as clouds, precipitation, and surface waters. Understanding its distribution within the environment, and the habitats it occupies, is important for examining its evolution and understanding behaviours. After a recent study found *P. syringae* living among a range of vascular plant species in Iceland, we questioned whether lichens could harbour *P. syringae*. Sixteen different species of lichens were sampled all over Iceland, but only one lichen genus, *Peltigera*, was found to consistently harbour *P. syringae*. Phylogenetic analyses of *P. syringae* from 10 sampling points where lichen, tracheophyte, and/or moss were simultaneously collected showed significant differences between sampling points, but not between different plants and lichens from the same point. Furthermore, while there were similarities in the *P. syringae* population in tracheophytes and *Peltigera*, the densities in *Peltigera* thalli were lower than in moss and tracheophyte samples. This discovery suggests *P. syringae* strains can localize and survive in organisms beyond higher plants, and thus reveals opportunities for studying their influence on *P. syringae* evolution.

INTRODUCTION

The last decade has seen increased research on the *Pseudomonas syringae* complex outside the context of crops. This has led to new insights on the adaptations of microbiota associated with plants (Rosier et al., 2018; Werner et al., 2014; Zilber-Rosenberg & Rosenberg, 2008), highlighting the correlation between the polyvalence of virulence and adaptations to a greater number of habitats (Morris et al., 2013; Passera et al., 2019). The expansion of virulence among strains from non-agricultural habitats is explained by Menard et al. (2007) according to three factors, (1) the advantage of not depending only on one host for survival, (2) horizontal gene transfer of DNA, which could play an important role in improving aggressiveness in such a way that the bacterial pathogen becomes more efficient at infecting crops or improving resistance to different stress conditions, and (3) competition with other microorganisms from different habitats might reinforce traits of self-defence (Leiva et al., 2021).

The surface of land masses dedicated to crops is surpassing other types of vegetated lands across the planet (Ellis et al., 2010). Iceland is markedly different from agricultural regions in terms of the paucity of cropped lands, thus providing an opportunity to study the adaptation of *P. syringae* without the strong influence of local agriculture. Iceland is an isolated and

pristine island in the North Atlantic Ocean with ample vegetation, a limited area dedicated to agriculture, and with climate conditions favourable for *P. syringae*. The Icelandic geographical characteristics minimize the risk of microbial spill-overs from agronomic practices such as grafting or transportation of plant materials that can contribute to localized and/or long-distance spread. Furthermore, the arable land farmed in Iceland represents only 1% out of 10^5 km^2 of land surface whereas vegetation covers about 25% of the total land surface (Denk et al., 2011). The Icelandic climate is classified as subpolar oceanic, with temperatures varying between 2°C and 14°C on average along the year, extreme variability in day length, and high solar UV radiation exposure, based on the data provided by Ogilvie and Jónsson (2001). In previous work, we reported the widespread occurrence of *P. syringae* in Iceland on wild vascular plants including tracheophytes and moss (Ogilvie & Jónsson, 2001). This led us to question if there was an even greater ubiquity of *P. syringae* across all types of vegetation in Iceland, and in particular on lichens.

Lichens found on the ground (called terricolous lichens) are a significant constituent of Iceland's vegetation, accounting for approximately 3.64% of vegetation coverage (Table S2). The thallus, the lichen's body, is composed of interwoven fungal hyphae and photosynthetic cells. However, they also contain internal bacterial communities (Cardinale et al., 2006; Leiva et al., 2021), as well as fungi (Bates et al., 2012; Spribille et al., 2016), archaea (Bjelland et al., 2011; Garg et al., 2016), and viruses (Eymann et al., 2017). Lichens thrive in hostile habitats despite their almost complete dependency on the atmosphere for water intake, that is, they are poikilohydric and respond markedly to changes in water availability, temperature, and exposure to sunlight. This leads to considerable fluctuations in the endolichenic environment (Kranner et al., 2008; Øvstedal & Smith, 2001; Selbmann et al., 2010) and the possibility of entering a dormant stage where they are more resistant to oxidative stress (Grube et al., 2015; Kranner et al., 2005). The hardiness of lichen thalli has led researchers to study their survival when exposed to radiation in outer space (Sancho et al., 2007) as well as in Mars-like conditions (de la Torre et al., 2018). However, to what extent the lichen-associated microbiome contributes to their survival is still unclear (Leiva et al., 2021; Pisani et al., 2011). Recent research suggests an essential role of lichen-associated microbes in their survival (Bates et al., 2011; Bjelland et al., 2011; Cardinale et al., 2006; Cardinale et al., 2008; Cardinale et al., 2012; Grube & Berg, 2009; Hodkinson & Lutzoni, 2009; Mushegian et al., 2011; Selbmann et al., 2010). Sigurbjörnsdóttir et al. (2015) suggested that the microbiome of seashore lichens is associated with nutrient scavenging, nitrogen fixation, and mobilization of iron and phosphate among other activities of the lichen metabolism.

In light of the widespread abundance of lichens in Iceland and the recent isolation of *P. syringae* from tracheophytes and moss in this country, we wondered if *P. syringae* might also be present in lichen thallus. Therefore, the objective of this study was to determine if lichens host *P. syringae* and if so, to characterize the abundance and diversity of the populations of this bacterial complex. Here we demonstrate that populations of bacteria in the *P. syringae* complex are widespread in a single genus of lichen and that the genetic diversity of the populations on lichens resembles that of nearby tracheophytes and moss.

EXPERIMENTAL PROCEDURES

Sampling

A total of 68 samples (Table S1) from 34 sites across Iceland were chosen based on habitat types and geography (Figure 1). Samples were collected across 17 sub-habitats that belong to 6 habitat types based on the Icelandic Institute of Natural History (NI) and the EUNIS habitat classification system (Davies et al., 2004). The sampling points were located from 64.3° N to 65.7° N latitude and 14.3° W to 22.2° W longitude. Of these samples, 38 were lichens, representing 16 species, including lichens with green algae, cyanobacteria, or both as photobionts (Table S1). Plants and lichens collected at the same site were no farther than 15 cm from each other. This allowed us to assess the spillover of strains between different vegetation types at the same site. A voucher for each sample was deposited to the herbarium of the Icelandic Institute of Natural History (NI).

Isolation, characterization, and quantification of bacteria

All samples were ground with a sterile pestle in a sterile mortar within 5 h of sampling or, when that was not possible, they were kept in darkness at 4°C and processed within 2 days as described by Morris et al. (2007).

Suspensions of ground tissue were plated on 10% Trypticase soy agar (TSA) as described previously to estimate the total culturable bacteria associated with each sample (Guilbaud et al., 2016). Hence, we will refer to populations estimated from counts on TSA as "total culturable bacteria". These suspensions were also plated on the semi-selective medium KBC (Berge et al., 2014) for viable counts of *P. syringae*-like colonies following the procedure described by Morris et al. (2022). *P. syringae*-like colonies were then streaked onto a fresh KB medium (King et al., 1954) and the morphology and fluorescence were noted.

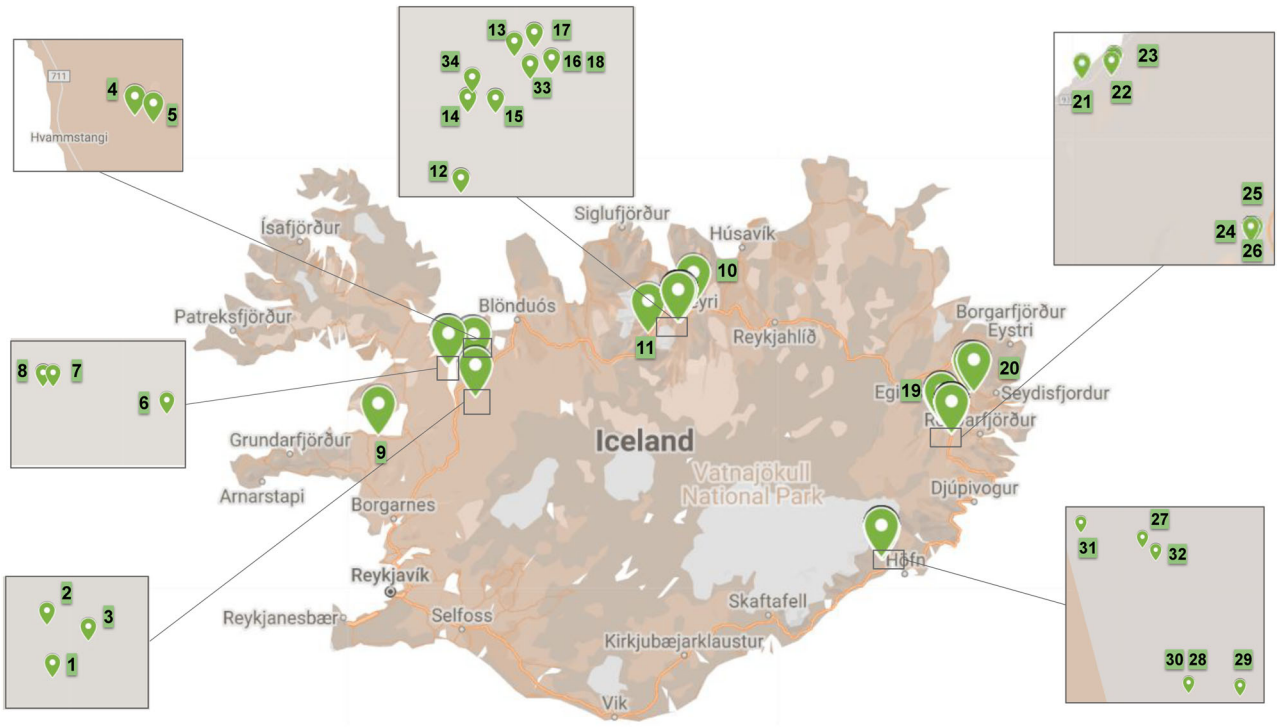

FIGURE 1 Sampling sites in Iceland. The 68 samples analysed in this work were collected in 34 locations and based on prevailing habitat types to represent a wide range of the sub-Arctic heathlands characteristic of northern and eastern Iceland.

Storage of the strains

After 2 days of incubation on KB in darkness at room temperature (ca. 20–25°C), bacteria were purified and put into short-term storage by suspending a loopful of bacterial growth into phosphate buffer (autoclaved-sterilized TP1 buffer: 8.75 g of K_2HPO_4 and 6.75 g of KH_2PO_4 diluted in 1 L of distilled water) and then stored at 4°C. For long-term storage, equal volumes of the bacterial suspension in TP1 and of sterile glycerol (42.5% vol/vol) were mixed in sterile screw-cap tubes. These were stored at −80°C in the culture collection of the University of Akureyri. Bacterial strains are listed in Table S1.

PCR and sequencing

From the colonies that grew on KBC for each sample, up to thirty colonies were selected from a single dilution for purification and characterization. Isolates from these colony-forming units (CFUs) were tested with PCR using the primer pair Psy.F and Psy.R following the protocol from Guilbaud et al (Guilbaud et al., 2016) with the SimpliAmp thermal cycler (Applied Biosystems, Darmstadt, Germany). Isolates that tested positive were purified on KB media and then a conserved region of the citrate synthase (*cts*) gene was amplified for sequencing by PCR using the primers Fcb43-fwd and Rcb43-Rev described previously (Xin et al., 2018). The *cts* region can accurately predict the phylogenetic affiliation for more than 97% of strains as demonstrated in Berge et al. (2014).

The *cts* region extracted previously was amplified using the primer pair Fcb43-fwd and Rcb43-Rev. Subsequently, the first PCR-positive samples were subjected to a second PCR using the *cts*-Fp and *cts*-Rp primer pair. Finally, Macrogen Europe BV (Amsterdam, The Netherlands) performed Sanger sequencing of the *cts* domain in all samples that tested positive in both PCR rounds, using the primers *cts*-Fseq and *cts*-Rseq (Guilbaud et al., 2016).

Phylogenetic characterization

The sequences were cleaned and trimmed with Seq Scanner Software and sequences with insufficient quality were eliminated from the analyses. Cleaned and trimmed sequences from both ends of the amplicons (forward and reverse) were overlapped. After overlapping they were aligned by ClustalW and cut to a uniform length of 307 bp using Molecular Evolutionary Genetics Analysis 11 (Mega11). Non-redundant sequences were used to obtain genetic distance matrices and neighbour-joining phylogenetic trees with a bootstrap value of 1000. Phylogenetic trees in this study were referenced with (i) a data set that compiles 933 strains representing a dozen habitat types (wild and cultivated plants, surface freshwaters, irrigation water, groundwater, epilithic biofilms, leaf litter, cloud

water, rain and snowfall, snowpack, and soil) from 27 countries from Northern and Southern Hemispheres; and (ii) 609 *P. syringae* haplotypes isolated from Iceland (Morris et al., 2022).

The genetic distance between *P. syringae* isolated from 10 different sites was assessed by aligning strain sequences isolated from lichens, tracheophytes, and/or moss from the same site and across sites and computing pairwise distances at Mega 11.

Hypersensitive reaction test—(HR)

The HR test was done on tobacco according to Morris et al. (2007) with phylogroup 1 and 2 strains isolated from *Peltigera* lichens and with strain CC0094 as a positive control (Table S3).

Data analyses

Statistical analyses (t-tests, graphs, ANOVA) were carried out in Microsoft Excel and with R studio® package ggplot2.

RESULTS

Peltigera was the only lichen genus found to harbour detectable populations of *P. syringae*

Bacteria were isolated from 16 different lichen species residing on rocks, soil, and tree bark substrates at 34 sites in northern, eastern, and southeast Iceland (Figure 1). Among these, only *Peltigera* spp. (*P. membranacea*, *P. leucophlebia*, *P. aphthosa*, and *P. canina*) harboured *P. syringae* at detectable population sizes (Table S1).

Total culturable bacterial populations (determined on TSA) were found to be higher in *Peltigera* than the non-*Peltigera* lichen samples (*t*-test *p*-value = 0.036479, df = 33.) In contrast, the total culturable bacterial population densities on *Peltigera* were not significantly different than those found on moss and vascular plants (*p*-values = 0.454656745 and. 0.217576389 for the respective pair-wise comparisons) (Figure 2).

Classification of *P. syringae* isolated from *Peltigera*

P. syringae strains from *Peltigera* thalli were identified as belonging to phylogroups (PG) PG01a, PG01b, PG02b, PG10a, PG10b, PG10d, PG13a based on the classification scheme of Berge et al. (2014). Strains belonging to PG10 were abundantly found associated with *Peltigera* thalli (60%). PG10 was dominant in 11 out of 17 *Peltigera* thalli, being absent in only 3 samples. PG13 represented 22% of the total *P. syringae* isolates found in *Peltigera*. These two phylogroups are usually associated with environmental habitats thus it may not be unexpected that they were the most common types found in *Peltigera* samples. Strains from PG01 and/or PG02 were detected on half of the lichen thalli. PG02 represented 14% of the *P. syringae* isolates, while only 4% of the total *P. syringae* from

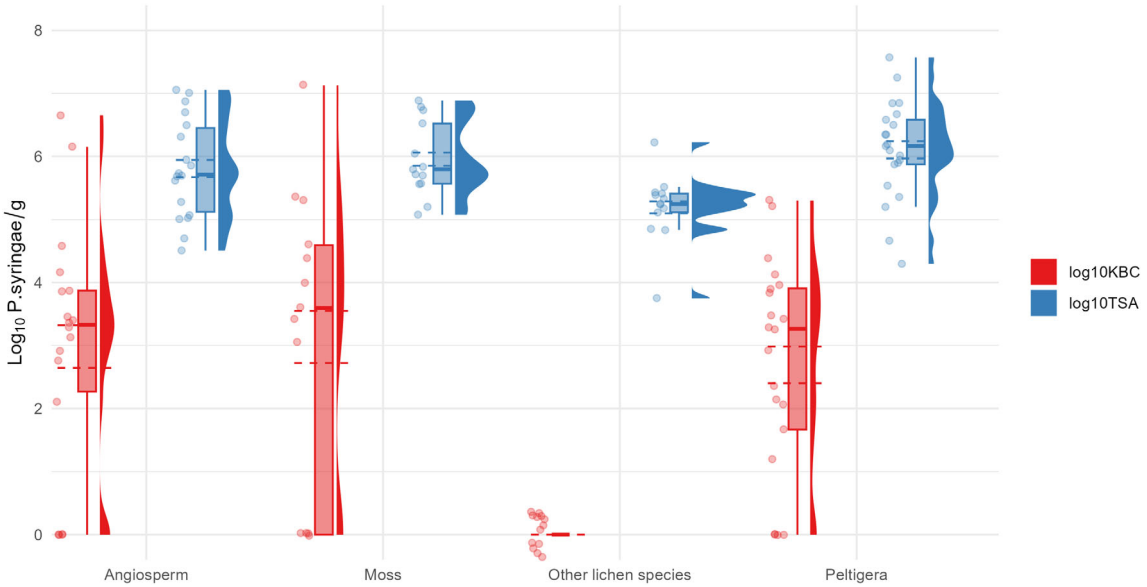

FIGURE 2 Total (TSA) and *Pseudomonas syringae*-like (KBC) populations on different plants and lichens. Bacterial numbers are shown as Log_{10} per g of fresh tissue. Dots indicate the individual samples for the summary statistics that are represented by the box and violin plots. Thick solid lines indicate the mean while dashed lines represent the 96% confidence intervals. The detection threshold is 5.00×10^{-1} *P. syringae* g^{-1}. Description of the sample characteristics is in Table S1.

Peltigera was identified as PG01. Interestingly some strains from PG01 had the same *cts* sequence as strain DC3000 pathogenic on tomatoes and isolated in the UK in 1960 (Xin et al., 2018) (see Figure 3).

P. syringae from lichens are not specific lineages distinct from those on tracheophytes and mosses

Ten sampling points where lichen, tracheophyte, and/or moss were collected simultaneously were used for genetic distance analyses. We tested the hypothesis that *P. syringae* populations on lichens were genetically more distant from those on tracheophytes or mosses on the same site than from those on lichens at other sites. As a simple test of this hypothesis, we calculated the pair-wise distances between lichen and non-lichen populations at each site and the pair-wise distances between lichen populations at all sites. The statistical differences among these sets of pair-wise distances were compared with t-tests. The statistical analyses showed significant differences between sampling points, but not between different plants and lichens from the same sampling point (*p*-value: 0.0008) (Figure 4).

Despite the similarities in genetic diversity between *P. syringae* populations on tracheophytes, moss, and *Peltigera*, *P. syringae* densities in *Peltigera* thalli showed lower average values (1.28×10^4 *P. syringae* g^{-1}) than moss and tracheophyte samples with values of 1.07×10^6 and 2.31×10^6 *P. syringae* g^{-1}, respectively (Figure 3). The same tendency was observed in PG01 presence, which was higher in plants than in *Peltigera* (*p*-value: 0.0215). Furthermore, PG04 and PG07 were absent in lichens, although PG04 was only isolated in one tracheophyte (Figure 5).

Almost all isolates from PG01 and PG02 are capable of inducing a hypersensitive response in tobacco

Forty *P. syringae* isolated from *Peltigera* thallus identified as PG01a, PG01b, and PG02b were tested for the ability to induce an HR in tobacco. The selection of these PGs for the HR test was based on their common association with epidemics. HR is a rapid response of localized programmed cell death normally indicative of the potential to cause pathogenicity in plants and underpinned by the presence of *hrp*/*hrc* genes in the type III protein secretion system (Collmer et al., 2000). A total of 33 out of 40 strains were HR-positive.

DISCUSSION

Here we show that the full range of Icelandic vegetation—including lichens—are habitats for *P. syringae*. However, it is surprising that only one out of 10 different lichen genera consistently harboured *P. syringae* while it was not detected in the other genera.

The finding of *P. syringae* in lichens also shows once again the great adaptive capacity of this bacterial complex. To live on lichens suggests that *P. syringae* has some potential to tolerate dryness. We noted that even the *Peltigera* samples in an apparent anhydrobiotic state harboured *P. syringae*. It has been proposed that the resilience of *P. syringae* under dry conditions relies on the ice nucleation property (INA) (Weng et al., 2017). The ice nucleation property in microorganisms, which allows them to initiate ice formation at relatively warm temperatures, has been linked to a higher resilience to dry conditions. This ability enables microorganisms to survive by forming a protective layer of

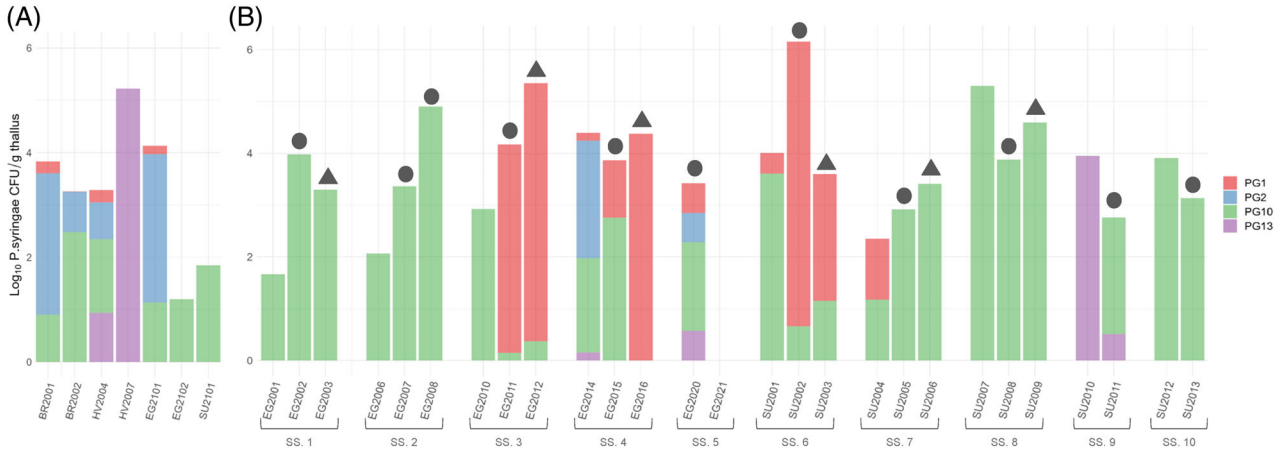

FIGURE 3 *Pseudomonas syringae* population size and proportion of the four dominant phylogroups. Different colours in the bars correspond to the proportions of *P. syringae* PG01, PG02, PG10, and PG13 whereas the height of the bar represents the population size. (A) Data from seven sites where only *Peltigera* lichens were sampled. (B) Ten sampling sites where lichen, tracheophytes, and/or moss were sampled. Circles on the top of the bars correspond to tracheophyte samples while triangles represent moss specimens. Further description of samples can be found in Table S1. The geographic location of the sampling sites is indicated in Figure 1.

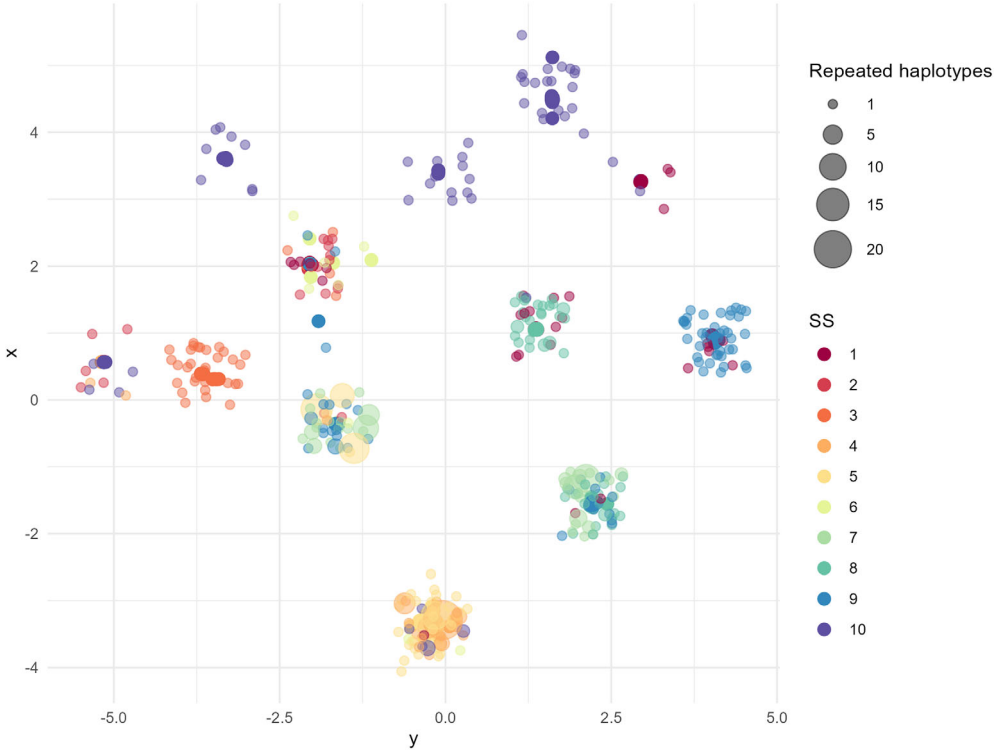

FIGURE 4 Genetic distance between *Pseudomonas syringae* from 10 distinct sampling sites isolated from *Peltigera* lichen, tracheophyte, and/or moss. *t*-Distributed Stochastic Neighbour Embedding graph (t-SNE) created to display 487 *P. syringae* strains that were collected from *Peltigera* lichen thalli, along with other organisms such as tracheophytes and/or moss, from 10 different sampling sites. The graph depicts identical isolates as larger dots that group 5, 10, 15, or 20 isolates, as indicated in the legend. Each isolate from the 10 sampling sites is assigned a different colour for easy identification. Alignments were performed using 307 bp sequences to calculate the genetic distance between isolates.

ice crystals around their cells, which helps to prevent cellular dehydration and provides a physical barrier against environmental stresses (Christner et al., 2008; Pummer et al., 2012). Nevertheless, *P. syringae* isolation in *Peltigera* does not necessarily mean permanent cohabitation.

P. syringae exhibits adaptability and resilience to endure a variable environment, much like lichens which rely heavily on atmospheric conditions that cause rapid fluctuations in the thallus' water content. Adapting to living in lichens could be an advantage for plant pathogenic Gammaproteobacteria that need to occasionally face harsh conditions on leaf surfaces. Lichens could provide an extra niche to survive where other plant pathogenic bacteria might not have this capacity (Ahmadjian, 1995; Erlacher et al., 2015). Furthermore, lichens are considered very long-living, stable microenvironments for bacterial colonization (Selbmann et al., 2010) giving the possibility for long-term survival in environments that they could not attain on other substrates. Although lichens cover just 8% of the Earth's terrestrial surface (Ahmadjian, 1995), significantly less than the estimated 30% covered by plants, they provide an extensive habitat for bacteria to inhabit (Field et al., 1998). The potential advantage for *P. syringae* to inhabit lichens is not known. However, recent research suggests the essential role of lichen-associated microbes for lichen survival.

The abundance of PG10 over other phylogroups in lichens was expected due to the high prevalence of this phylogroup in environmental habitats (Morris et al., 2022). The reason for the low occurrence of Icelandic PG02 strains in lichens may be due to their apparent inclination towards graminaceous plants in Icelandic tracheophytes, rather than dicots (Morris et al., 2022). Furthermore, INA could offer an advantage in the Icelandic climate. The quasi-absence of PG07, commonly considered to have important saprophytic capacity (Bartoli et al., 2014) could be explained by the subpolar oceanic clime of Iceland (Lohmann et al., 1993). The cold weather at these latitudes constrains the decomposition of organic material and slows nutrient cycles (McGuire et al., 2009; Sigurdsson et al., 2016) which might be why PG07 is less fit in these conditions.

The abundance of *P. syringae* as part of the microbiome of *Peltigera* lichens might be surprising. The lichen thalli microbiome is dominated by Alphaproteobacterial (Sigurbjörnsdóttir et al., 2015) while angiosperms commonly harbour Actinobacteria and

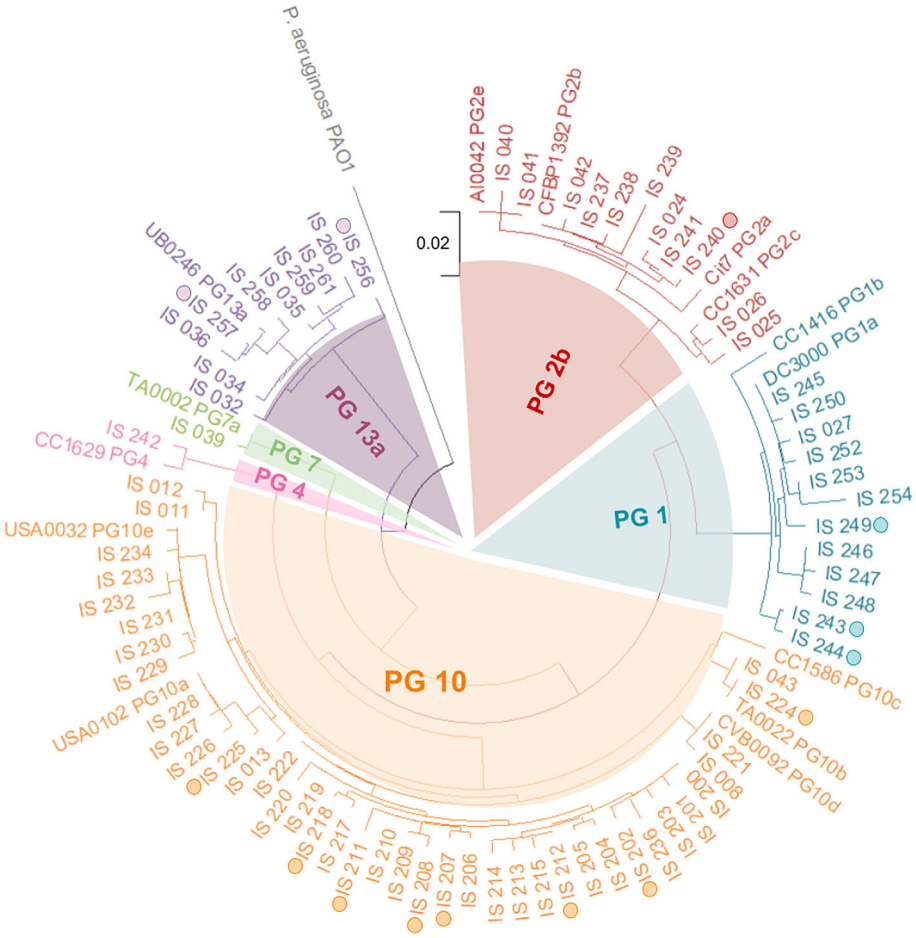

FIGURE 5 Neighbour-joining phylogenetic tree constructed using 101 concatenated cts sequences of *Pseudomonas syringae* isolated in Iceland (names starting with "IS"), with a bootstrap value of 1000. The tree includes haplotypes from lichens, tracheophytes, and moss that were sampled for this study, as well as tracheophytes from Morris et al. (2022). The sequences used were 295 bp long, including gaps. Identical sequences were removed, leaving only one representative sequence for each haplotype, resulting in a total of 89 haplotypes represented by 461 isolates. Phylogroups were classified according to Berge et al. (2014), with an example of each included as a reference. The different colours of the branches indicate the phylogroups named in the inner circle. Fourteen haplotypes isolated from *Peltigera* lichens are denoted by dots on the labels of the branches of the tree.

Gammaproteobacteria (Bashir et al., 2022). Our results point to a genetically similar *P. syringae* population in lichens, tracheophytes and moss from the same sampling site.

All lichens and plant samples were apparently healthy. Indeed, lichens do not have any known bacterial pathogens. Nevertheless, almost all *P. syringae* from PG01 and PG02 isolated from *Peltigera* tested positive to induce a hypersensitive response in tobacco indicating that they have some phytopathogenic potential. This finding is expected given that previous research has demonstrated that environmental strains of *P. syringae* retain their Type 3 Secretion Systems (T3S), which could also be the case for the *P. syringae* from lichens. Currently, there are no reports of diseases caused by *P. syringae* in Iceland. Nevertheless, temperatures in the Iceland-Greenland area are predicted to increase by about 2–3°C by the end of this century (Arnason, 2007). This increase in temperature could favour the expansion of field crops (vs. greenhouse crops) in Iceland. If this happens, newly converted croplands could harbour *P. syringae*. In this light, our report of the widespread occurrence of *P. syringae* on vegetation in Iceland opens the door to an opportunity to witness the beginning of the transition to agriculture and the possible emergence of diseases caused by *P. syringae*—and perhaps other plant pathogens.

Overall, *Peltigera* seems to favour higher populations of total cultural bacteria than the other lichens we sampled. The genus *Peltigera* comprises some of the largest terricolous lichens of Europe (Nardini et al., 2013). Its growth habitat—on the ground among grasses rather than exposed on rocks or branches of trees—might contribute to its favourability as a microbial habitat. Twenty-three species of this foliose lichen have been found in Iceland (Manoharan-Basil

et al., 2016; Vitikainen, 2007). A factor that might influence *Peltigera* success as a *P. syringae* host could be related to the ability of *Peltigera* species to adapt to arid environments due to their relatively lower osmotic potential and turgor loss point (Nardini et al., 2013) which help to maintain cell turgor pressure during drought periods (Zhu et al., 2018) being a more stable environment for *P. syringae* than other lichens without these characteristics. We hypothesize that *Peltigera* are colonized by *P. syringae* due to their characteristic defence mechanism which lacks secondary metabolites with antibiotic or antimicrobial properties such as usnic acid or montagnetol production (Huneck & Yoshimura, 1996). The lack of secondary metabolites is compensated by the production of superoxide in the external part of the lichen in high quantities even during non-stressed periods (Zorrilla et al., 2022). Superoxide is a bactericide used by several plants as a defence mechanism. Interestingly, *P. syringae* survival in tobacco leaves is not affected by superoxide presence (Minardi & Mazzucchi, 1988). This apparent property might be the reason why we can observe this association.

P. syringae seems to be more fit on *Peltigera* lichens as a host compared to other lichens. The genetic similarity of *P. syringae* on *Peltigera* lichens and on plants collected at the same sampling site suggests that there is a spillover of populations between plants and lichens. Our work does not reveal the direction of this spill-over. Hence, our findings indicate an overlapping *P. syringae* population between plants and lichens and the absence of lichen-specific lineages. The majority of *P. syringae* from lichens belonged to PG10. The majority of those in PG01 and PG02 were able to induce a hypersensitive reaction in tobacco. Further work is needed to demonstrate that *P. syringae* from *Peltigera* lichen has the potential to be pathogenic to crops.

AUTHOR CONTRIBUTIONS

Natalia Ramírez: Methodology (lead); investigation (lead); formal analysis (lead); writing – review and editing (lead). **Margrét Auður Sigurbjörnsdóttir:** Conceptualization (lead); project administration (supporting); supervision (lead); writing – review and editing (supporting). **Cecile Monteil:** Methodology (supporting). **Odile Berge:** Formal analysis (supporting); investigation (supporting); supervision (supporting); writing – review and editing (supporting). **Starri Heiðmarsson:** Formal analysis (supporting); supervision (supporting); writing – review and editing (supporting). **Robert Jackson:** Conceptualization (supporting); supervision (supporting); writing – review and editing (supporting). **Cindy E. Morris:** Conceptualization (lead); investigation (lead); supervision (lead); writing – review and editing (lead). **Oddur Vilhelmsson:** Conceptualization (lead); investigation (supporting); project administration (lead); supervision (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequence data are available in the DDBJ/EMBL/GenBank databases under accession number ON092835-ON093054.

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