Háskólinn á Akureyri
University of Akureyri

Can Lichens Serve as Hosts for *Pseudomonas syringae* in Icelandic Habitats?

Isolation, Plant-pathogenic Traits, and Metabolomic Insights into the Role of *P. syringae* in Icelandic *Peltigera* Lichens

Doctoral thesis

Natalia Ramírez

University of Akureyri
Natural Resource Sciences
2024

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Geta fléttur þjónað sem hýslar fyrir *Pseudomonas syringae* í íslenskum vistgerðum?

Einangrun *P. syringae* stofna úr íslenskri engjaskóf, greining á plöntusýkjandi virkni þeirra og áhrifum á hvarfefnamengi fléttnanna

Natalia Ramírez

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Acknowledgments

I am grateful for the opportunity to be a part of the Department of Natural Sciences at the University of Akureyri. It truly has been the right place for me both professionally and personally. I have appreciated the collaborative and supportive environment. I am especially grateful for my advisor Dr. Oddur Vilhelmsson who took a chance to bring me into the lab and train me. He has been both patient and supportive. That balance is hard to maintain and has been instrumental in my growth as a scientist and a person.

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Útdráttur

Bakterían *Pseudomonas syringae*, sem almennt er þekkt sem plöntusýkill, hefur víðtækara vistfræðilegt hlutverk í öðru umhverfi en ræktarlandi. Bakterían hefur verið einangruð úr ýmsum vistgerðum og er hlutverk hennar í umhverfinu oft tengt eiginleikum hennar til að mynda ískirnunga.

Eitt meginmarkmið verkefnisins var að einangra *P. syringae* úr fléttum. Í grein I er gert grein fyrir einangrunarferlinu frá ættkvíslinni *Peltigera*, eftir að hafa skimað 10 mismunandi fléttuættkvíslir. Sýnum var safnað á nokkum stöðum og í hvert sinn tekið sýni af *Peltigera* fléttu, mosa og öðrum lággróðri. Fjölmargir stofnar reyndust tilheyra *P. syringae* og í kjölfarið var vaxtarhæfni þeirra og meinvirkni könnuð í mismunandi plöntutegundum, þ.á.m. þekktum nytjaplöntum. Greint er frá niðurstöðum þeirra rannsókna í grein II, en þær benda til þess að íslensku stofnarnir búi yfir svipaðri sýkingarhæfni og þekktir faraldsstofnar.

Í síðasta hluta verkefnisins voru efnaskiptamengi *Peltigera* og annarra fléttna greind, og áhrif fléttuumhverfisins á *P. syringae* könnuð. Fjallað er um þær niðurstöður í grein III, en þær benda til þess að *Peltigera membranacea*, og aðrar fléttutegundir innan sömu ættkvíslar, hýsi *P. syringae* í meira mæli en aðrar fléttutegundir. Greining á vexti *P. syringae* stofnanna í æti, gerðu úr fléttuextrakti, bendir til lítilsháttar vaxtarörvunar *P. syringae* þegar fléttuextraktið er til staðar. Efnaskiptamengjagreiningin gefur til kynna að fléttur af *Peltigera* ættkvísl framleiði fá annars stigs efnaskiptaefni, en í fremur háum styrk. Meðal algengra örveruhemjandi fléttusýra, sem ekki fundust í *Peltigera* fléttunum, má nefna úbikvínón, sem hafa þekkta virkni gegn *P. syringae* og eru gjarnan hluti af vörnum plantna.

Jafnhliða ofangreindu voru erfðamörk *P. syringae* stofna, einangraðir á Íslandi, borin saman við stofna úr alþjóðlegum stofnasöfnum (Grein IV). Niðurstöður benda til þess að íslenskir *P. syringae* stofnar beri merki um langtíma einangrun frá þeim ferlum sem hafa ráðið dreifingu stofna sem safnað hefur verið erlendis og eru að mestu úr ræktarlandi.

Abstract

Pseudomonas syringae, traditionally recognized as a plant pathogen, has a broader ecological role beyond agriculture. In diverse non-agricultural environments, researchers have isolated the bacterium revealing non-pathogenic behavior and significant roles, often associated with its ice nucleation property.

The primary objective of this project was to achieve the first isolation of *P. syringae* from lichens. In Paper I, we detail the isolation process from a specific lichen genus, *Peltigera,* after screening 10 different lichen genera. The analysis extends to studying the *P. syringae* population within the same sampling points alongside *Peltigera*, moss, and tracheophytes when feasible. Following the isolation of this potential plant pathogen, the logical progression led to Paper II, where we explored the fitness and pathology of selected *P. syringae* strains, predominantly from more aggressive phylogroups, across ten different plant species, primarily crops. The results showed similar pathogenicity in some lichen strains compared with epidemic ones and a similar fitness in 8 out of 10 plant species tested.

The final project phase aimed to understand the exclusive isolation of *P. syringae* from a single genus. Paper III, adopting a metabolomics approach, analyzed differences in the profiles of *Peltigera* and non-*Peltigera* lichen genera that may influence *P. syringae* presence. *Peltigera's* overall profile shows a higher chemical investment, focusing on the production of certain compounds. Kinetics and inhibition analyses suggest a slightly increased growth of *P. syringae* in *Peltigera* media, though not considered decisive. The study also outlines specific compounds present or absent in *Peltigera*, including the absence of ubiquinones, recognized for their resistance role against *P. syringae* in various plant species.

Furthermore, experiments with *P. syringae* isolated in Iceland gain significance through collaboration with INRAE (Paper IV). Comparisons with a global database reveal that this Icelandic *P. syringae* population appears to have been isolated for at least 10,000 years.

Keywords: plant pathogenicity, Iceland, untargeted metabolomics, bacterial phytopathogen, non-agricultural habitat.

Contents

List of abbreviations

DNA – Deoxyribonucleic Acid
FISH – Fluorescence In Situ Hybridization
LC/MS – Liquid Chromatography Coupled To Mass Spectrometry
OD – Optical Density
PCA – Principal Component Analysis
PCR – Polymerase Chain Reaction
PG – Phylogroup
rpm – Revolutions Per Minute

List of figures

List of tables

Overview of original articles

This thesis is based on the following three papers. Hereafter they will be referred to by their numbers as follows:

Paper I: Ramírez, N., Sigurbjörnsdóttir, M.A., Monteil, C., Berge, O., Heiðmarsson, S., Jackson, R.W., Morris, C. and Vilhelmsson, O. (2023). *Pseudomonas syringae* isolated in lichens for the first time: Unveiling *Peltigera* genus as the exclusive host. Environmental Microbiology. https://doi.org/10.1111/1462-2920.16490 (Published)

Paper II: Ramírez, N., Caullireau, E., Sigurbjornsdottir, M.A., Vilhelmsson, O. and Morris, C.E. (2024). From lichens to crops: Pathogenic potential of *Pseudomonas syringae* from *Peltigera* lichens is similar to world-wide epidemic strains. Plant pathology.https://doi.org/10.1111/ppa.13915 (Published)

Paper III: Ramírez, N., Vinchira-Villarraga, D., Rabiey, M., Sigurbjörnsdóttir, M.A., Heidmarsson, S., Vilhelmsson, O., Jackson, RW. Simplicity in Complexity: Deciphering the Metabolic Factors Guiding *Pseudomonas syringae* preference for *Peltigera* lichen among Icelandic lichen host (Submitted)

Other papers published during this study:

Paper IV: Morris, C.E., Ramírez, N., Berge, O., Lacroix, C., Monteil, C., Chandeysson, C., Guilbaud, C., Blischke, A., Sigurbjörnsdóttir, M.A. and Vilhelmsson, O.Þ., 2022. *Pseudomonas syringae* on plants in Iceland has likely evolved for several million years outside the reach of processes that mix this bacterial complex across Earth's temperate zones. Pathogens, 11(3). https://doi.org/10.3390/pathogens11030357 (Published)

Declaration of Contribution to the thesis

The following authors have contributed to the main papers and manuscripts for this thesis: Natalia Ramírez (RM), Dr. Oddur Vilhelmsson (OV), Dr. M. Auður Sigurbjörnsdóttir (MAS), Dr. Cindy E. Morris (CEM), Dr. Starri Heiðmarsson (SH), Dr. Odile Berge (OB), Dr. Robert W. Jackson (RWJ), Cecile Monteil (CM), Emma Caullireau (EC), Dr. Mogjan Rabiey (MR), Dr. Diana Vinchira-Villaraga (DV) and Dr. Élodie Vandelle (EV).

Paper I:

The study was designed by OV, MAS, CEM, RWJ, and OB as well as the initial sampling trip in which it also participates CM. Subsequent sampling trips were led by NR, in collaboration with OV, MAS, or SH. NR handled the laboratory processing after receiving guidance on the procedures from CM. The first draft of the paper was composed by NR and subjected to revision by all supervisors.

Paper II:

CEM, NR, and EC were the study's designers. NR, in collaboration with EC, conducted all laboratory work and data analysis under CEM's guidance. NR took the lead in drafting the manuscript, which was subsequently reviewed and approved by CEM, OV, EV, and MAS.

Paper III:

DV, MR, RWJ, and NR collaborated on the study's design. NR conducted the lichen sampling, while all laboratory work was performed by NR and DV. SH identified the lichen species. NR authored the manuscript, with supervision and contributions from all experts involved in the study's design, as well as OV and MAS.

The following authors have contributed to the collaboration papers and manuscripts for this dissertation: Dr. Christelle Lacroix (CL); Charlotte Chandeysson (CC), and Dr. Caroline Guilbaud (CG).

Paper IV:

The conceptualization of the study was a collaborative effort by CEM, OB, MAS, and OV. The methodology was developed collectively by all authors, and the validation process involved contributions from CEM, OB, CL, and CG. Formal analysis was conducted by CEM, NR, OB, CL, and CG. The investigation phase was a team effort involving CEM, NR, OB, CM, CC, and CG. Resources for the study were provided by CEM and OV. Data curation was managed by CEM, NR, OB, and CL. The original draft of the manuscript was prepared by CEM, and all authors contributed to the review and editing process. Project administration was overseen by CEM, MAS, and OV, while funding acquisition was led by CEM and OV.

List of published Sanger sequences

The sequence data, comprising 220 *P. syringae* Sanger sequences isolated in Iceland from *Peltigera* lichens, tracheophytes, and mosses, have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers ON092835 to ON093054.

List of published metabolites

The metabolic profile of seven species belonging to *Cladonia*, *Stereocaulon* and *Peltigera* lichens will be deposited in the NCBI database.

1 Introduction

1.1 *Pseudomonas syringae* description

Pseudomonas syringae, a well-known species complex within the Gammaproteobacteria, is primarily recognized for its capacity to induce a wide array of diseases in various agriculturally significant crop species. Presently, the *P. syringae* species complex is categorized into thirteen evolutionarily distinct phylogenetic groups, referred to as phylogroups (PGs), a classification based on both multilocus sequence analysis and whole-genome sequencing (Berge et al., 2014; Dillon et al., 2019). Of these, seven PGs are designated as primary PGs, sharing a more recent common ancestor, and encompassing most recognized type and pathotype strains, as well as the majority of strains responsible for infecting agriculturally significant crops. In contrast, secondary PGs exhibit considerably greater divergence, are more frequently encountered in environmental samples, and may lack some of the essential virulence factors that are conserved in primary PG pathogens (Dillon et al., 2019).

Many ecological investigations concerning plant-pathogenic bacteria have primarily concentrated on agricultural settings which might have biased the *P. syringae* population and overrepresented the epidemic strains in certain situations over the typical not aggressive nature of *P. syringae*. There is a growing body of research focused on isolates obtained from wild plant species and non-agricultural settings, including aquatic environments, rain, ice, and soil (Karasov et al., 2018; Kritzman and Zutra, 1983; Lamichhane et al., 2015; Mansfield et al., 2012; Monteil et al., 2013; Morris et al., 2013, 2008, 2007). Furthermore, it has become evident that characteristics related to adaptation to both biotic and abiotic stress in non-agricultural environments can also serve as virulence factors in plants, as demonstrated by Morris et al. (2008). This adaptation to non-host environments has been proposed to have played a significant role in the evolution of *P. syringae*'s ability to infect plants and has had an impact on its

epidemic potential, as indicated by Bartoli et al. (2015, 2014). Over recent decades, the continuous exploration of habitats hosting *P. syringae* has revealed its presence in environments previously considered unsuitable for its survival.

Nevertheless, the extent of spread and exchange between diverse environments of *P. syringae* remains largely unknown. Studies like that of Upper et al. (2003) have field-tested the dispersion of *P. syringae* among different crop plants, demonstrating how environmental factors influence spread, while distance barriers play a less determinant role. Although various studies have identified different sources of *P. syringae* dispersal, such as rain or irrigation splash, aerosols from dry leaves, insects, and human dispersion, particularly through farming equipment (Pattemore et al., 2014; Roberts, 1997), these spread studies often focus on plants and neglect other environments like lichens, mosses, or debris that could potentially aid in *P. syringae* dispersal or serve as hosts.

Interestingly, we have a more comprehensive understanding of *P. syringae* dispersion on a global scale than in minor distances. A study comparing *P. syringae* isolates from rivers in North America, Europe, and New Zealand revealed that the genetic structure was unaffected by geographical location. In the research conducted by Monteil et al. (2016), they conclude that *P. syringae* population isolated from crops during a disease outbreak was the same than the strains from various water sources isolated in the same period. A similar conclusion was drawn in Morris et al., (2008) when comparing *P. syringae* populations in the water cycle and wild plants. The results showed minimal genomic differences between these strains, and a significant portion of virulence genes was found to be present in both types of strains, finding the same population in the water cycle as in crops during epidemic cases. These findings indicate a mixing of the populations from both substrates.

1.2 *Bacillus velezensis* as competitor example

Lichens microbiota is distributed in a biofilm (Grimm et al., 2021). The highly dynamic nature of biofilms renders them extremely robust to environmental fluctuation and often very efficient in the production or degradation of a variety

of compounds. It is known that a shift in the nutrient source can influence the relative growth rate and thus the relative abundance of the species. This congregation of different bacteria in different amounts creates a high number of complex interactions. Interactions can be positive, increasing the fitness of all partners, a phenomenon known as synergism. Alternatively, interactions can be negative for at least one of the bacteria, such as in antagonism. Antagonism involves competitive interactions where one bacterium inhibits the growth or survival of another through the production of inhibitory substances like antibiotics, metabolites that prevent adhesion of the competitor, pH changes that affect the other bacteria, or even predation, where one organism feeds on another (Moons et al., 2009).

In this study, *Bacillus velezensis* serves as an example for *P. syringae* antagonist. We examine its ability to adapt to various lichens, aiming to determine whether it could potentially contribute to the reduction of *P. syringae* populations in certain lichens. *B. velezensis* has been used in agriculture due to the antifungal activity against plant pathogenic fungi (Zhang et al., 2022), the plant promoting capacity of some of the strains (Torres et al., 2020) and the ability to inhibit different bacteria, including *P. syringae*, making it a valuable antibacterial agent in agriculture (Wang et al., 2023) due to the production of antimicrobial compounds of bacterial cyclic lipopeptides and different surfactins (Grady et al., 2019). However, in a study conducted by Grady et al. (2019) , it was observed that *B. velezensis* did not inhibit *P. syringae* DC3000. Instead, there was a significant reduction in root colonization success, suggesting that *B. velezensis* can also employ alternative methods beyond antimicrobial activity.

B. velezensis was isolated for the first time in 2005 in Spain from brackish water from a river that gives the name to the bacterium (Ruiz-Garcia et al., 2005). Afterwards, it has been commonly isolated from soil (Grady et al., 2019). However, it's important to note that even if *B. velezensis* has been isolated from some lichens (Zou et al., 2023) many lichen species have been observed to inhibit the growth of *Bacillus sp.* (Kosanić and Ranković, 2019; Mitrović et al., 2011). This mechanism likely aims to prevent potential harm from *Bacillus sp.* to the lichen mycobiont, given the well-documented antifungal activity of this bacterium,

which extends to the *Ascomycete* division (Grady et al., 2019) which constitutes approximately 98% of lichen mycobionts (Lutzoni et al., 2001).

1.3 Lichen description

Lichen thalli, the principal structure of lichens, consists of a complex network of fungal hyphae and photosynthetic cells. Lichens are formed by a fungal partner which in the 98% of known lichens is Ascomycota (Lutzoni et al., 2001). Lichens are also classified based on the symbiont. The most common symbiont is unicellular green algae (85% of the lichens) mainly from Chlorophyta division such as *Trebouxia*, Chlorococcoid or *Coccomyxa*, occurring in about 40% of all lichens., while those associated with cyanobacteria are around 10% mostly *Nostoc*. Only a 3% of the lichens are associated with both and are called tripartite (Honegger, 2001; Rikkinen, 1997).

Notably, lichens also host internal bacterial communities (Cardinale et al., 2006; Leiva et al., 2021), in addition to other fungi -sometimes parasitic- (Bates et al., 2012; Spribille et al., 2016), archaea (Bjelland et al., 2011; Garg et al., 2016), microinvertebrates (Satkauskiene, 2012) and viruses (Eymann et al., 2017). All these organisms produce various secondary metabolites, especially the mycobiont, which are crucial for the survival of the lichen (Kalra et al., 2023)(Figure 1). Despite their heavy reliance on atmospheric moisture for water absorption, making them poikilohydric, lichens exhibit remarkable resilience in harsh environments. They are highly responsive to fluctuations in water availability, temperature, and sunlight exposure, which can lead to significant changes in their internal environment (Kranner et al., 2008; Øvstedal and Smith, 2001; Selbmann et al., 2010). In response to these conditions, lichens may enter a dormant state, enhancing their resistance to oxidative stress (Grube et al., 2015; Kranner et al., 2008). Some of these metabolites are responsible for antagonistic interactions which are thought to help these long-living organisms to survive against pathogens that attack their microflora (Cernava et al., 2015).

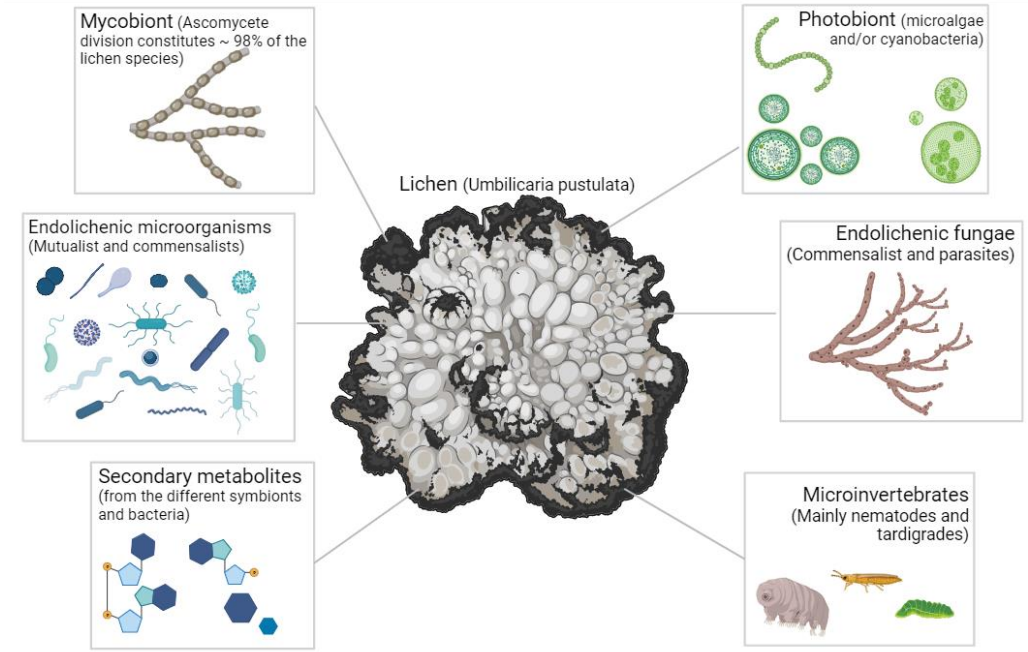


Figure 1. Scheme illustrating the components of the lichen thallus that constitute the organism.

Lichens display a wide range of morphological forms, including crustose (crust-like), foliose (leaf-like), or fruticose (branching), with the fungal partner primarily influencing these variations. The photobiont contributes photosynthetically derived sugars to the fungus (Hill and Smith, 1972). In lichens involving both an alga (the phycobiont) and a cyanobacterium, the cyanobacterium (or cyanobiont) plays a significant role in nitrogen fixation (Klarenberg, 2021; Klarenberg et al., 2020) and supplies the fungal partner with ammonia (Rowell et al., 1985).

Lichens demonstrate the capacity to endure the challenges presented by deserts, polar regions, and chemically rich environments. This resilience involves a combination of morphological and physiological adaptations, as well as alterations in ecological behavior, allowing species to acclimate to relatively sheltered niches within extreme environments (Armstrong, 2017).The ability of lichen thalli to withstand extreme conditions has even prompted investigations into their survival under outer space radiation (Sancho et al., 2007) and Mars-like conditions (de la Torre Noetzel et al., 2018).

While numerous species are present in tropical regions, they only emerge as a substantial component of the vegetation in more severe environments (Armstrong, 2017). The extent to which the lichen-associated microbiome contributes to their survival remains a subject of ongoing research (Leiva et al., 2021; Pisani et al., 2011).

1.4 Description of *Peltigera*, *Cladonia* and *Stereocaulon* genera

The lichen genus *Peltigera* (L.) Willd., introduced by Willdenow (1787), is one of the earliest established names in lichenology. *Peltigera* species are typically foliose, characterized by broad lobes. This genus is usually considered terricolous and muscicolous (Burgaz et al., 2003) but it can also be found in tree bark and rocks (Jüriado et al., 2017). *Peltigera* includes around 66 lichen species, some of which, like *P. leucophlebia* and *P. aphthosa*, are tripartite, while others, like *P. membranacea*, exclusively harbor cyanobacteria (Martínez et al., 2003). The difference between *P. membranacea* with the other two species can be easily seen with metagenomic and metabolomic studies, which, for example, a much lower terpenoid production in *P. membranacea* compared with the tripartite ones (Miadlikowska and Lutzoni, 2000).

Stereocaulon species typically display a dimorphic thallus configuration consisting of a crustose primary thallus and a fruticose secondary thallus (Högnabba, 2006). The cosmopolitan lichen genus *Stereocaulon* comprises approximately 125 fruticose lichen species (Coppins and Smith, 2009). It is usually attached to soil or rock (Smith and Øvstedal, 1991). *S. alpinum* and *S. vesuvianum*, for example, exhibit a tripartite structure, participating in a three-part symbiotic relationship (Torres et al., 2023).

Among *Cladonia* species, the primary thallus is often crustose or squamulose, followed by a secondary fruticose thallus, constituting approximately 475 species (Santiago et al., 2010). However, the identification of the species is usually a

challenge even when using a genetic approach (Pino-Bodas et al., 2013) The preferred substrates are soil and rocks (Martin et al., 2011; Tolpysheva and Timofeeva, 2008). The species used in this thesis, *C. arbuscula* and *C. chlorophaea*, follow a bipartite arrangement and engage an algal partner (Pisani et al., 2011).

1.5 Bacteria in lichen thalli

The mycobiont forms the structural framework of the lichen thallus, while the photobiont exists as colonies or layers sheltered beneath the fungal outer layers. In numerous lichen species, one or both partners produce secondary metabolites endowed with antibacterial properties, creating a somewhat selective environment conducive to the flourishing of intricate bacterial communities. Some of these communities are found on the lichen's surface, forming biofilm-like communities (Grube et al., 2015), while others reside endothallically, below the surface within extracellular polysaccharides (Cardinale et al., 2008; Grube and Berg, 2009). Consequently, lichens serve as hosts for substantial bacterial populations, and recent years have witnessed the gradual unveiling of the identity and roles of these bacteria in the lichen symbiotic association (Hodkinson and Lutzoni, 2010; Sigurbjörnsdóttir et al., 2015).

The predominant functions attributed to bacteria associated with lichens include nitrogen fixation (Cardinale et al., 2006; Liba et al., 2006), antifungal or antibacterial activity (González et al., 2005), and nutrient transfer from the rock surface (Banfield et al., 1999) or attachment to the thallus in saxicolous lichen (de los Ríos et al., 2002). The bacterial microbiome exhibits adaptability to the diverse environmental requirements of the lichen (Sigurbjörnsdóttir et al., 2014). While there is notable variability between lichen species and studies, it is evident that most lichens harbor extensive, intricate microbiota predominantly consisting of Alphaproteobacteria. Other lineages may also be detected at considerable relative abundances, including Betaproteobacteria, Acidobacteria, Actinobacteria, and Eubacteria among others (Figure 2). Notably, taxa more commonly associated with phytopathogens, such as members of the Xanthomonadaceae, Pseudomonadaceae, and Enterobacteriaceae, are also

present, albeit in smaller numbers. In between the bacteria detected in lichens, recent studies isolated plant pathogens such as *Burkholderia glathei* (Cardinale et al., 2006) or *Xanthomonas sp.* and its close relative *Xylella sp.* in the membranous dog lichen (*P. membranacea*) microbiome (Sigurbjörnsdóttir et al., 2015). While many of the bacteria residing in lichen thalli remain uncultured, members of a substantial portion of the dominant lichen-associated taxa have been successfully isolated and cultivated in pure culture (Vilhelmsson et al., 2016).

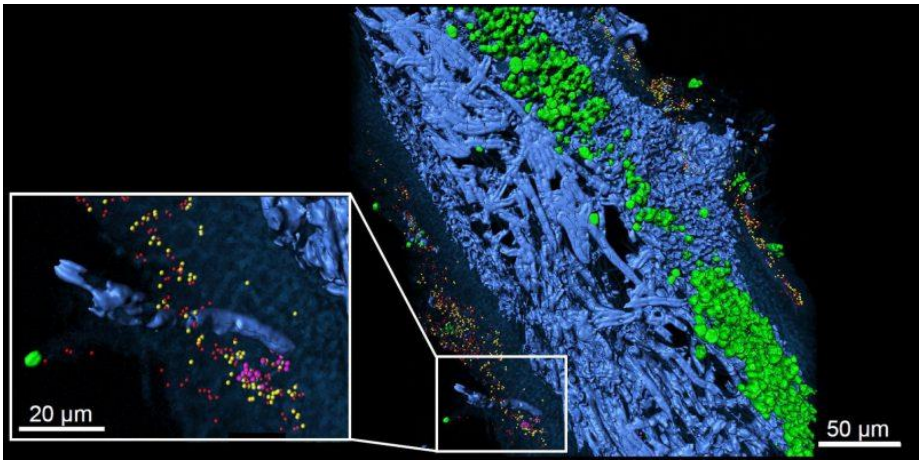


Figure 2. Cross-sectional analysis of the lichen thallus from *Lobaria pulmonaria.* The reconstruction of Fluorescence In Situ Hybridization (FISH) image reveals the wide distribution of Eubacteria (in red) and Alphaproteobacteria (in yellow), Betaproteobacteria (in pink), fungal hyphae (in blue), and algae in green (adapted from Grube et al., 2015).

Nitrogen fixation is usually a limiting factor in lichens that lacks a cyanobacterial symbiont, that is why these lichen species usually host bacteria that are nitrogen fixators. Bacteria found on internal and external surfaces of lichens may also help lichen symbionts to fulfill other nutritional requirements, including the acquisition of phosphorus and amino acids (Grube et al., 2009; Liba et al., 2006).

1.6 Iceland as a study site

The extent of land designated for crop cultivation is now surpassing that of other vegetated land types worldwide (Ellis et al., 2010). Iceland stands out significantly from agricultural regions due to its limited cropland, presenting a unique opportunity to investigate *P. syringae* adaptation without the strong influence of local agriculture. Situated as an isolated and pristine island in the North Atlantic Ocean, Iceland boasts abundant vegetation, a small proportion of land allocated to agriculture, and favorable climate conditions for *P. syringae* (Figure 3). Its distinct geographical features help minimize the risk of microbial spill-overs resulting from agricultural practices like grafting or the transportation of plant materials, which can contribute to localized and long-distance dissemination. Additionally, in Iceland, arable land accounts for a mere 1% of the total land area of 10^5 km^2, while vegetation covers approximately 25% (Denk et al., 2011). The Icelandic climate is categorized as subpolar oceanic, featuring average temperatures ranging from 2 to 14°C throughout the year, significant variations in day length, and intense solar UV radiation exposure, as reported by Ogilvie and Jónsson (2001). As anticipated in an extreme environment, one of the prevailing vegetation types comprises lichens, constituting approximately 3.64% of the surface cover, solely composed of terricolous lichens (Ramírez et al., 2023). In a prior study, we documented the widespread presence of *P. syringae* in Iceland on wild vascular plants, including tracheophytes and moss (Morris et al., 2022). This discovery prompted us to inquire whether *P. syringae*'s ubiquity extended across all vegetation types in Iceland, with a particular focus on lichens.

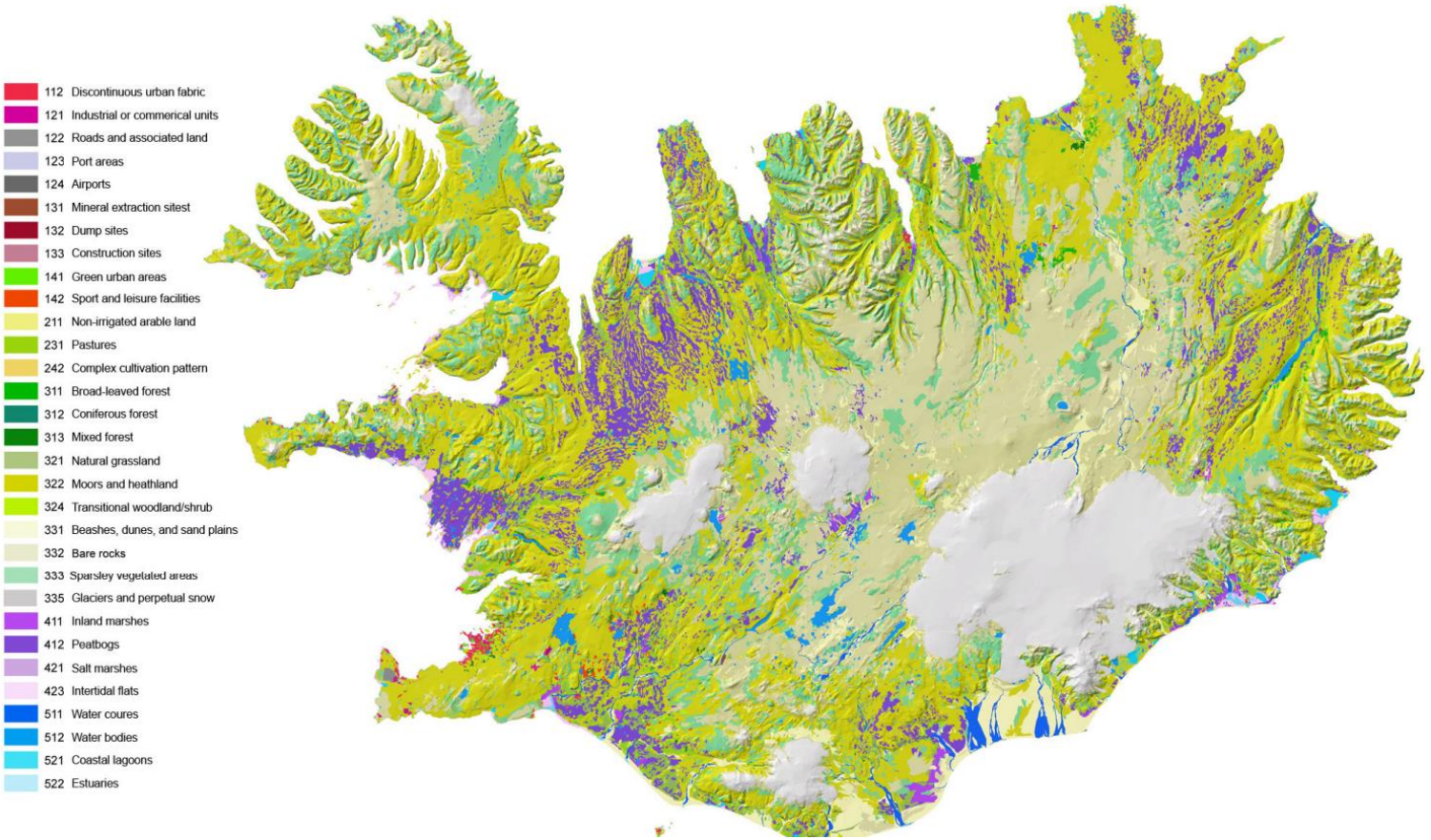

112 Discontinuous urban fabric
121 Industrial or commerical units
122 Roads and associated land
123 Port areas
124 Airports
131 Mineral extraction sitest
132 Dump sites
133 Construction sites
141 Green urban areas
142 Sport and leisure facilities
211 Non-irrigated arable land
231 Pastures
242 Complex cultivation pattern
311 Broad-leaved forest
312 Coniferous forest
313 Mixed forest
321 Natural grassland
322 Moors and heathland
324 Transitional woodland/shrub
331 Beashes, dunes, and sand plains
332 Bare rocks
333 Sparsley vegetated areas
335 Glaciers and perpetual snow
411 Inland marshes
412 Peatbogs
421 Salt marshes
423 Intertidal flats
511 Water coures
512 Water bodies
521 Coastal lagoons
522 Estuaries

Figure 3. Vegetation map of Iceland from 2008 made by the CORINE project named CLC2006 (source: https://www.lmi.is/static/files/corine/corineskyrsla-enska.pdf).

1.7 Metabolites in lichens

In the field of lichen biology, metabolites can be broadly categorized into primary and secondary groups. Primary metabolites, such as proteins, lipids, and carbohydrates, are essential for lichen metabolism and structure (Huneck and Yoshimura, 1996). In contrast, secondary metabolites, often referred to as 'lichen acids', are complex yet non-essential small molecules. While several of these compounds are already found in plants or other fungi, approximately 80% exhibit the highest diversity in lichens (Huneck and Yoshimura, 1996). The straightforward and logical explanation for this phenomenon is that they result from the symbiotic relationship (Lawrey, 1986).

Over 1050 recognized lichen metabolites are cataloged in the Lichen DataBase (LDB). Lichen compounds are predominantly characterized by their acidic nature, leading to the commonly employed term "lichen acids." (Vartia, 1973). These secondary metabolites serve various functions within the lichen symbiosis and their ecological niche. They can make up to 30% as is the case of *Pentagenella fragillima Darb*. which produce massive amounts of the depsidone psoromic acid (Huneck, 1973) but in most lichens the amount varies from 5 to 10% of the lichen thallus' dry weight (Molnár and Farkas, 2010). The prevalent types include mononuclear aromatic compounds, depsides, depsidones, diphenyl ethers, and dibenzofurans (Yousuf et al., 2014) (Figure 4). Depsides and depsidones are known by their antioxidant and antibiotic properties (Calcott et al., 2018; Shukla et al., 2010). Diphenyl ethers have been identified with a role of antiherbicide (Scalla et al., 1990), antibacterial (Ji et al., 2020) and antifungal (Zhu et al., 2021). The most common lichen compound belonging to Dibenzofurans is usnic acid and it is cytotoxic and antibacterial activities (Millot et al., 2016). The majority of bioactive compounds are primarily produced by the mycobiont partner. However, in certain instances, the photobiont, has been observed to participate in the production of specific key secondary metabolites (Crittenden and Porter,

1991; Kalra et al., 2023). Some function as allelopathic agents, influencing the growth and survival of neighboring organisms, providing lichens with a competitive advantage (Paukov et al., 2019). Others impact lichen palatability, acting as a form of defense against herbivores. Additionally, specific secondary metabolites enhance the permeability of the cell membranes of phycobionts, the algal partners within lichens, potentially aiding in nutrient exchange and survival in challenging conditions. Moreover, some of these metabolites can act as shields against excessive ultraviolet (UV)-B radiation, protecting the photosynthetic components of lichens from harmful UV rays (Paukov et al., 2019).

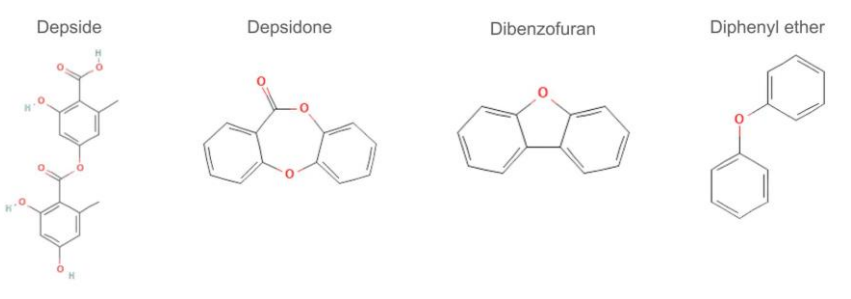


Figure 4. Molecular structure of some of the most common compounds isolated from lichens.

Several prior studies have highlighted metabolic variations among species within the Peltigera genus. For instance, Rowell et al. (1985) examined the distinctions in nitrogen metabolism between P. canina and P. aphthosa. Additionally, Laufer et al. (2009) investigated how different Peltigera species exhibit varying laccase profiles. Laccases are enzymes that, according to current knowledge, play a role in the decomposition of organic compounds, particularly lignin, and potentially contribute to pathogen defense (Beckett et al. 2005). Nevertheless, the significant diversity observed in extracellular lichen laccases suggests that they may have more than one role in lichen biology (Laufer et al., 2009).

It's noteworthy that a substantial portion of studied lichen species synthesizes substances with varying degrees of antimicrobial activity (Romagni and Dayan, 2002), which could have implications for their interactions with other microorganisms in their environment. Intriguingly, closely related secondary metabolites often exhibit diverse biological effects, adding complexity to their analysis and suggesting their adaptability to different ecological contexts (Hager

et al., 2008; Molnár and Farkas, 2010). Some studies of lichen secondary metabolites showed antibiotic effects against different *Pseudomonas* species such as *P. aeruginosa* from different bacterial antagonist (Çobanoğlu et al., 2010) as well as inhibition of *P. syringae* from some lichen species from the genus *Cladonia* (Aydin et al., 2017).

Overall, these protective compounds collectively contribute to the unique and enduring conditions for lichen existence, resulting in remarkable longevity, with certain lichens living for several millennia (Denton and Karlén, 1973) and the spectacular adaptability of lichens to extreme environments including alpine and polar regions where usually dominate the landscape (Boustie et al., 2011).

1.8 Metabolomics for the study of host-bacteria interaction

Metabolomics is the systematic exploration of cellular reaction-derived metabolite compositions. In the past decade, significant progress has been made in both instrumental technology and the necessary software tools for conducting multivariate analysis. This methodology involves the quantitative assessment of low molecular weight compounds using techniques like liquid and gas chromatography, often coupled with mass spectrometry or NMR spectroscopy (Baker, 2011).

A significant portion of these bioactive metabolites probably remains undiscovered, primarily because of the slow growth rate of lichens. The slow growth of lichens hinders the comprehensive study of their metabolites and poses challenges in extracting these compounds in substantial quantities. Consequently, recent advancements in metabolomics have increased interest in conducting metabolic analyses on lichens (Kalra et al., 2023).

1.9 Hypotheses regarding the exclusive isolation of *P. syringae* from *Peltigera*

After exclusively isolating *P. syringae* from lichens of the *Peltigera* genus, we embarked on investigating the potential differences between *Peltigera* and other lichen genera. This study was prompted by the intriguing observation that suborder Peltigerineae lichens seemed to prioritize the production of high levels of superoxide for defense, rather than diversifying their defenses with multiple secondary metabolites, as is typical in most lichens (Beckett et al., 2003). Conveniently, *P. syringae* is not susceptible to superoxide (Minardi and Mazzucchi, 1988). This might be the case of other metabolites that conveniently are or are not present in *Peltigera* influencing *P. syringae* presence. Additionally, we considered that the absence of *P. syringae* in other lichen species, as detected in our study, might be due to the higher water content in *Peltigera* compared with other lichens. Finally, we speculated that the differences in bacterial populations, particularly the higher culturable bacteria levels in *Peltigera* compared to other lichen genera, could be another explanatory factor (Ramírez et al., 2023).

In the metabolomics approach, our primary hypothesis proposes the presence of a unique metabolite within *Peltigera* lichen, possibly enhancing *P. syringae*'s survival. An alternative hypothesis suggests that non-*Peltigera* lichen genera may contain metabolites inhibiting *P. syringae* growth. A third theory speculates on indirect interactions, suggesting that lichens might influence the population of *B. velezensis*, which shares an ecological niche with *P. syringae* and is known to negatively affect *P. syringae* abundance. Specifically, the *Peltigera* lichen might inhibit *B. velezensis*, creating an environment conducive to *P. syringae*, or conversely, non-*Peltigera* lichen genera might enhance the population of *B. velezensis* on their thallus, thereby inhibiting *P. syringae* growth.

In this research, we pursued a multifaceted investigation into the presence of *P. syringae* in lichens, the characterization of the strains isolated, the analyses of the pathogenicity capacity and the interactions of *P. syringae* with lichens, employing diverse methodologies and objectives.

Our initial objective centered on the isolation of *P. syringae* from lichens, a pioneering effort that aimed to expand our knowledge of potential plant pathogens within these symbiotic structures, with *P. syringae* as a case of study. Subsequently, we conducted a comprehensive characterization of the isolated *P. syringae* strains. This involved a detailed exploration of their bacterial lineage, PGs, and distribution patterns. This foundational work laid the groundwork for understanding the diversity and presence of *P. syringae* within the lichen microbiome.

In a subsequent phase, our focus shifted to assessing the fitness of four *P. syringae* strains from *Peltigera* lichens belonging to PG1 and PG2 across a range of ten plant species. This objective sought to elucidate the adaptability and interaction dynamics of these strains with various host plants. Additionally, we conducted pathogenicity tests on 17 *P. syringae* strains from *Peltigera* (PG1 and PG2) in three selected plant species. Epidemic strains from other parts of the world were used in both experiments as positive controls. This dual approach allowed us to examine not only the fitness of the strains but also their potential impact on plant health, providing a more comprehensive understanding of their pathogenic potential.

In an integrative approach, we employed LC/MS untargeted metabolomics to gain deeper insights into the isolation of *P. syringae* from the lichen genus *Peltigera*. This involved analyzing the exo-metabolites of *P. syringae* cultured in lichen-enriched broth, as well as extending our investigation to the metabolic profile of three different lichen genera. By utilizing metabolomics, we aimed to decipher the intricate chemical interplay between *P. syringae* and its lichen hosts. This holistic approach provided valuable information on the ecological and functional implications of the interactions between bacteria and lichens.

1.10 Project objectives

In summary, our research journey encompassed the isolation, characterization, fitness assessment, and pathogenicity testing of *P. syringae* strains from lichens, coupled with a metabolomics-driven exploration to understand the nature of *P.*

syringae-lichen interactions. This comprehensive approach aimed to unravel the complex relationships between *P. syringae* and its diverse hosts, contributing to a deeper understanding of microbial ecology and host-pathogen interactions. In summary, this thesis endeavors to achieve the following objectives:

Paper I

1.1. Screen *P. syringae presence* among different lichen species sample in Iceland.

1.2. Comprehensive characterization of the isolated *P. syringae* strains to gain insights into their bacterial lineage, PGs, and distribution.

1.3. Examine the genetic and quantitative variations in the *P. syringae* population between angiosperm, moss, and lichen within the same sampling point to decipher potential exchange of this bacterium among these different organisms.

Paper II

2.1. Assess the fitness of the *P. syringae* strains isolated from *Peltigera* lichen from PG1 and PG2 in ten plant species.

2.2. Pathogenicity test of seventeen *P. syringae* strains from *Peltigera* belonging to PG1 and PG2 in three plant species.

2.3. Comparison of fitness and pathogenicity results with epidemic strains from other parts of the globe.

Paper III

3.1. Examine the untargeted metabolomics profiles of three distinct genera—*Peltigera, Cladonia,* and *Stereocaulon*—to elucidate overall variations in metabolite abundance and predominant types of metabolites.

3.2. Identify metabolites that are solely absent/ present in *Peltigera* or which difference in abundance is very high and decipher if those might enhance *P. syringae* population.

3.3. Identify metabolites that are solely absent/ present in non-*Peltigera* lichens or which difference in abundance is very high and decipher if those might inihibit *P. syringae* population.

Paper IV

4.1. Isolate P. syringae from Icelandic plants.

4.2. Assess P. syringae abundance, affinity for different plant types and distribution across habitats.

4.3. Compare P. syringae population from Icelandic plants with the strains known worldwide.

2 Methodology

The methodologies employed in this project encompassed the initial isolation of *P. syringae* from lichens, moss, and tracheophytes using KBC media. Bacteria exhibiting the expected *P. syringae* phenotype were subsequently verified through two different PCR methods, and those positive in both were subjected to Sanger sequencing (further details in Paper I). Following this, seventeen *P. syringae* strains from *Peltigera* thallus were vacuum infiltrated into three distinct plant species for a pathogenicity test. Symptom observation and measurement were conducted over a 14-day period post-infiltration. Additionally, fitness analyses of *P. syringae* isolated from *Peltigera* lichen involved inoculating four different strains into 10 plant species, measuring bacterial growth after 1- and 8-days post-inoculation. Known pathogenic strains were included in both experiments for comparative purposes (additional information in Paper II). The project concluded with an untargeted metabolomics approach, examining the metabolic profiles of seven species from three lichen genera isolated in Iceland. This analysis aimed to understand variations in specific compounds and explore whether such differences could account for or contribute to the exclusive isolation of *P. syringae* from *Peltigera* lichen (additional specifics Paper III).

This section will address various methods that have not been covered in the articles or were not extensively explained, including unsuccessful approaches.

2.1 Sampling criteria and screening of *P. syringae* in lichens

A total of 68 samples were collected from 34 sites across Iceland, chosen based on habitat types and geographical distribution (See methodology scheme Figure 5). These samples, including 38 lichens representing 16 species, covered 17 sub-habitats within 6 habitat types. The sampling locations ranged from 64.3º N to

65.7º N latitude and 14.3º W to 22.2º W longitude. Proximity between plants and lichens at each site, within 15 cm, allowed for assessing strain spill-over between different vegetation types. Voucher specimens for all samples were deposited in the herbarium of the Icelandic Institute of Natural History. Further details can be found in Paper I.

The initial rationale for field site selection and sampling methods included collecting diverse lichen specimens from various accessible areas in Iceland. The primary objectives were to obtain a general understanding of the lichen species exhibiting *P. syringae* presence and to determine if there was any preference by the bacterium for specific areas or habitat types.

Following preliminary sampling and culturing of *P. syringae*-like isolates on KBC plates, we adapted our sampling strategy to control the potential issue of cross-contamination of *P. syringae* to lichens from surrounding flora. This was deemed necessary because of the high abundance of *P. syringae* in angiosperms and moss surrounding the lichen. To address this concern, we modified our sampling approach, collecting samples of lichens, tracheophytes, and moss from the same location whenever those organisms were present.

Subsequent sampling revealed, however, that cross-contamination was likely not a major issue, as we observed that non-*Peltigera* lichens at the same sampling sites did not exhibit detectable levels of *P. syringae*. Nevertheless, the revised sampling procedure was maintained. The goal was to characterize *P. syringae* populations across lichen, tracheophytes and moss within and between sampling points, aiming to unravel the dynamics of lichen *P. syringae* populations.

In the process of identifying *P. syringae* strains, we utilized the citrate synthase (cts) housekeeping gene (409 bp), as probed by Berge et al. (2014), to predict the phylogenetic affiliation for over 97% of the tested strains within the *P. syringae* complex. This was achieved using a distance threshold of 4.0% for PG determination and 1.8% for clade affiliations.

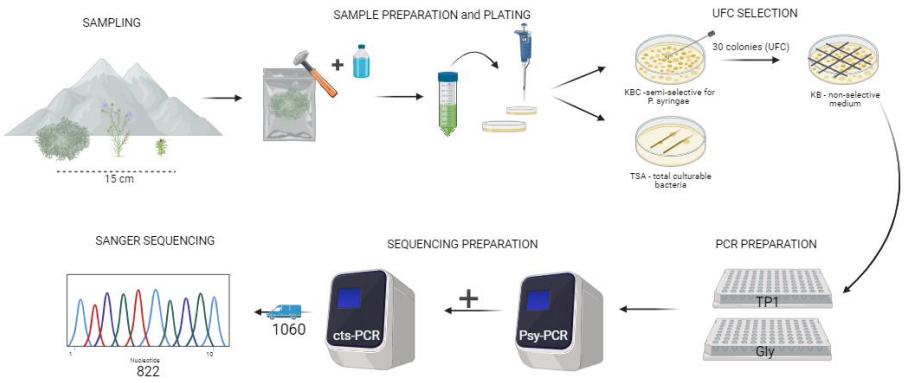


Figure 5. Outline of the methodology used in sample processing.

2.2 Surface sterilization of *Peltigera* lichen proved unfeasible

In order to enable differentiation of endo- and ectothallic bacteria, surface sterilization of the lichen thalli was attempted. *Peltigera* lichen samples were divided in two halves, having one half treated conventionally, while another set underwent treatment adapted from that of Biosca (2016). Each flask containing lichen samples was supplemented with 30 ml of Ringer solution mixed with T20. The flasks were agitated at a speed of 250 revolutions per minute (rpm) for a duration of one and a half hours. Subsequently, a 6 ml aliquot of the resulting extract was collected, and an equivalent amount of 42.5% glycerol solution was added to preserve it before storing in a freezer. The remaining liquid was discarded. Concurrently, the lichen thalli were treated with 10 mL of TP1 solution for plating onto KBC and TSA media. The subsequent steps mirrored the procedure used for non-sterilized samples.

An alternative sterilization approach involved immersing the thalli in 17% hydrogen peroxide (H_2O_2) for a duration of 60 seconds. Afterward, the thalli were rinsed twice with distilled water, following a modified protocol inspired by Kinkel et al. (1989).

The results obtained by both sterilization methods were inconsistent, giving in some cases a higher number of bacteria in the sterilize thallus than the non-sterile (Figure 6). There are several potential explanations for the inconsistent results. For example, the intricate architecture of the lichen thallus, with fungal hyphae interconnecting with cyanobacteria, and sometimes algae, presented significant challenges. The complexity of this symbiotic combination made it difficult, firstly, to distinguish the boundaries between the inner and outer regions of the lichen, and secondly, to adequately access and treat all the intricate crevices within the thallus. Additionally, the first sterilization method was originally designed for use in different lichen genera, and the second method was originally described for plant leaves, which could potentially impact the success of the sterilization process.

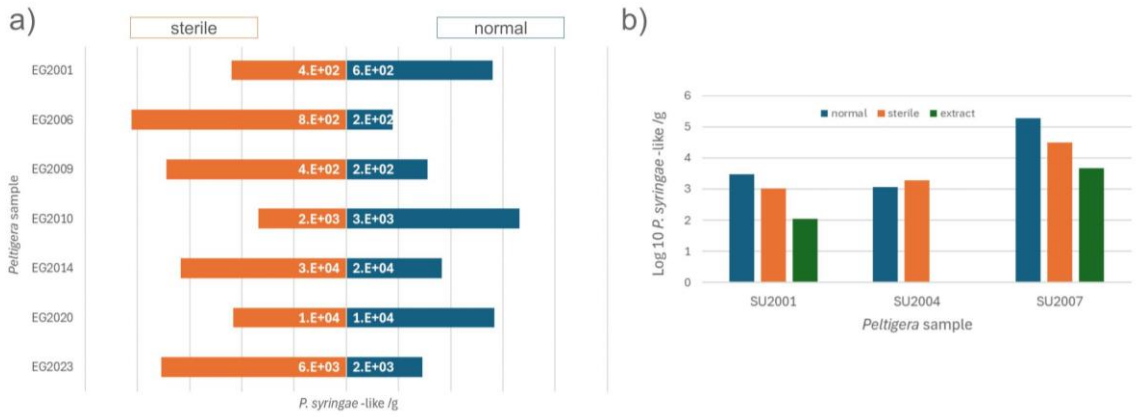


Figure 6. Bacterial count of isolates identified as *P. syringae*-like. a) Comparison of *P. syringae*-like count in normal treated thalli of *Peltigera* with those in sterilized thalli, as described in the method outlined in (Kinkel et al., 1989); b) Comparison of *P. syringae*-like count in normal treated thalli of *Peltigera* with those in both sterilized thalli and the extract from the thallus that underwent sterilization. This followed a modified method as per (Kinkel et al., 1989).

2.3 Selection of plant species, *P. syringae* strains, and incubation conditions for fitness and pathogenicity testing

In the process of selecting plant species, various criteria were considered. For the pathogenicity test, three plant species were chosen based on their potential

utility for Icelandic agriculture, being currently cultivated either indoors or outdoors: kale (*Brassica oleracea*), cucumber (*Cucumis sativus*) and barley (*Hordeum vulgare*). The 10 plant species selected for fitness analyses included model plants like tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and thale cress (*Arabidopsis thaliana*), along with plants of evolutionary research interest, such as rice (*Oryza sativa*), which has never been cultivated in Iceland. The remaining plants provide a diverse range such as spinach (*Spinacia oleracea*) or garlic chives (*Allium tuberosum*), encompassing wild plants like annual mugwort (*Artemisia annua*) and those used in the pathogenicity tests for consistency.

For the bacterial selection, we focused this study on phylogroup PG1 and PG2 strains isolated from *Peltigera* lichens. Even though these are among the least abundant PGs found in *Peltigera* where the most common was PG10 followed by PG13. We consider that PG1 and PG2 were the most appropriate for these experiments as they are the most common PGs of epidemic strains (Berge et al., 2014). Following the construction of a phylogenetic tree using the PG1 and PG2 strains isolated from *Peltigera* lichen, the selection process involved choosing two distinct strains from each PG for fitness analyses and 17 strains for the pathogenicity test aiming to represent the varied strains from these PGs isolated from *Peltigera*.

The incubation conditions and the inoculation/infiltration volumes were selected with the aim of creating a favorable situation for the bacteria to grow and develop symptoms on the plants (Grimstad and Frimanslund, 1993). However, this is not easy to reach for all strains because *P. syringae* exhibits a broad temperature range, and various strains are tailored to different temperature conditions. For instance, *P. syringae* pv. actinidiae shows heightened invasiveness at relatively low temperatures (10-20°C; optimum 15±3°C). Nonetheless, symptoms have been observed at temperatures exceeding 25°C in regions like France, Italy, and Portugal (Loreti, 2019). Hence, we consider this variability in our interpretation of the results.

2.4 Approaches to solve the plate contamination problems with *A. thaliana*

For the *P. syringae* fitness test in *A. thaliana*, the experiment had to be repeated up to four times due to plate contamination issues. *A. thaliana* leaves, situated close to the soil, encounter numerous microbes. The higher bacterial population and/or diversity compared to other plant species tested posed challenges in identifying and quantifying *P. syringae* on KB media, which is not species-specific. Additionally, the short life cycle of *A. thaliana* led to a rapid leaf deterioration within 7 days post-inoculation. To minimize non-*P. syringae* microbes, we modified our approach by watering plants without splashing onto the leaves and selecting the top leaves for inoculation. Several attempts were made to sterilize with an alcohol-dipped swab, but they were unsuccessful. Furthermore, to enhance reliability, the number of replicates per strain was increased from 5 to 6 and the inoculation was done in younger plants to avoid testing the fitness during senescence of the leaves. All these adjustments in the process were offset by consistent outcomes.

2.5 Criteria for selecting lichen samples in metabolomic studies

In this part of the project, our objective was to collect samples from various species belonging to three lichen genera, with one of them being *Peltigera*. The aim was to compare the untargeted metabolic profiles of the lichens, specifically searching for compounds that may be present, absent, or exhibit different abundances solely in *Peltigera* or the non-*Peltigera* genera. This exploration aimed to identify potential compounds influencing the presence of *P. syringae* (more information in Paper III).

To ensure a comprehensive and representative sampling of lichen genera, we opted for a strategic approach. Our criteria included the selection of three distinct genera, each comprising at least two species, to enhance the diversity within each genus.

Ideally, we would have analyzed the 10 lichen genera from the initial phase of the project. However, this approach was not economically or practically viable. The inclusion of *Peltigera* was imperative, providing a benchmark for comparing other lichens that exhibited the presence of *P. syringae*.

In choosing the additional two genera, we sought lichens from environments akin to those of *Peltigera*, minimizing the variability introduced by habitat differences. Our selection considered the potential preferences of humid environments by *P. syringae* excluding crustose lichens, known for their drier nature. The selected genera, *Cladonia* and *Stereocaulon* (Figure 7), in spite of growing in similar environments, belong to the order Lecanorales while *Peltigera* is from the order *Peltigera*les, difference that we had in mind during the metabolic data analyses. Furthermore, the selection process factored in the suitability of genera with ample existing research, such as *Cladonia* and *Stereocaulon* facilitating our research and enhancing the depth of our comparative analyses.

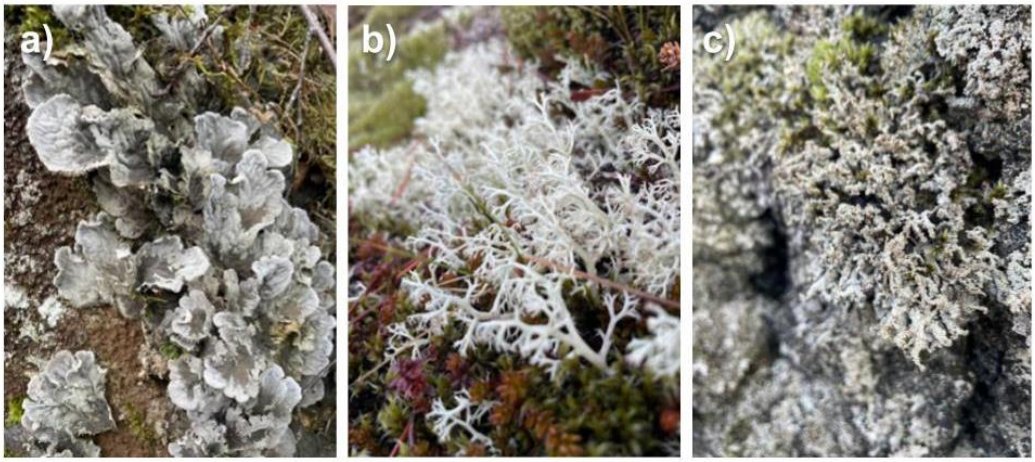


Figure 7. Pictures of some of the lichen species used in the metabolic studies of this thesis a) *P. membranacea*; b) *C. arbuscula*; c) *S. vesuvianum.*

3 Summary of main result

In this project, *P. syringae* was isolated from lichens for the first time. While previous metagenomic studies (Sigurbjörnsdóttir et al. 2015) had suggested the presence of *P. syringae* or closely related pseudomonads in *Peltigera membranacea*, this had not been verified by culturing until now. Indeed, the isolation of this potential plant pathogen was achieved from all *Peltigera* lichen species tested, but, interestingly, not from and of the other 9 lichen genera tested. Strains isolated from *Peltigera* lichen were categorized into four different PGs. Notably, the most prevalent are PG10 and PG13, constituting 60% and 22%, respectively, and are commonly associated with strains from non-natural environments. The remaining phylogroups, PG2 and PG1, account for 14% and 4%, respectively (Figure 8). These PGs are typically considered more aggressive and are commonly associated with epidemic strains.

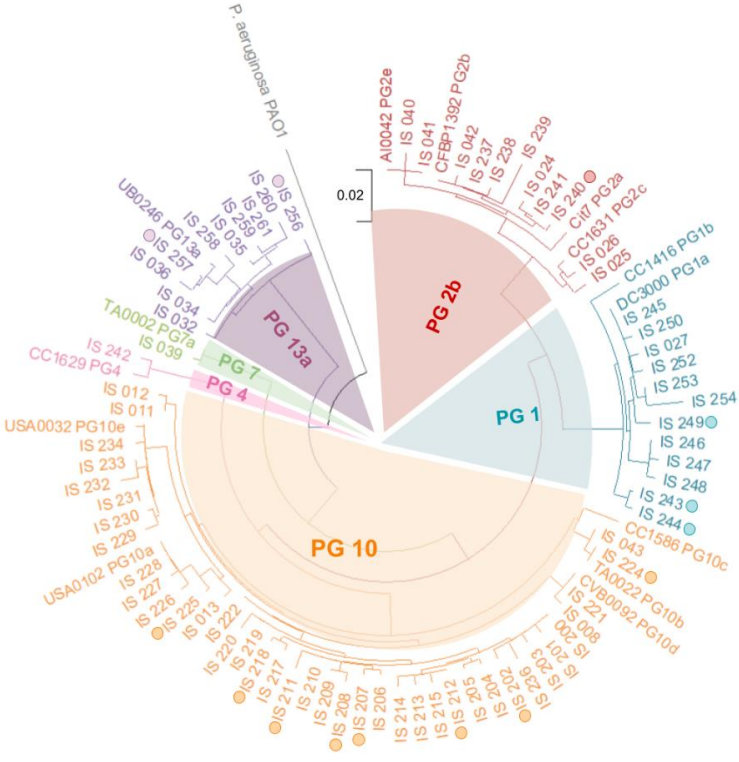


Figure 8. Neighbor-joining phylogenetic tree, based on 101 concatenated cts sequences of *P. syringae* from Iceland (IS). The tree comprises haplotypes from lichens, tracheophytes, and moss sampled in this study, along with tracheophytes from Morris et al. (2022) Sequences, 295 bp long with gaps, were used, resulting in 89 haplotypes (461 isolates). Identical sequences were condensed, leaving one representative per haplotype. PGs, classified following Berge et al. (2014), are denoted by different colors, and examples are referenced. Fourteen haplotypes from *Peltigera* lichens are marked with dots on the tree. See Paper I for more details.

To address questions on the biogeography and host specificity of the *P. syringae* population in lichens, we compared genetic distance within and among the geographically widely separated sampling sites. The findings revealed a greater resemblance in *P. syringae* populations within diverse hosts at the same sampling sites compared to those within the same *Peltigera* species at geographically distant sampling sites (Figure 9). This suggests a potential exchange of *P. syringae* strains between plants and *Peltigera* lichens, underscoring one of the leading hypotheses of the project, namely that *Peltigera* lichens can potentially serve as reservoirs for *P. syringae* in Icelandic habitats.

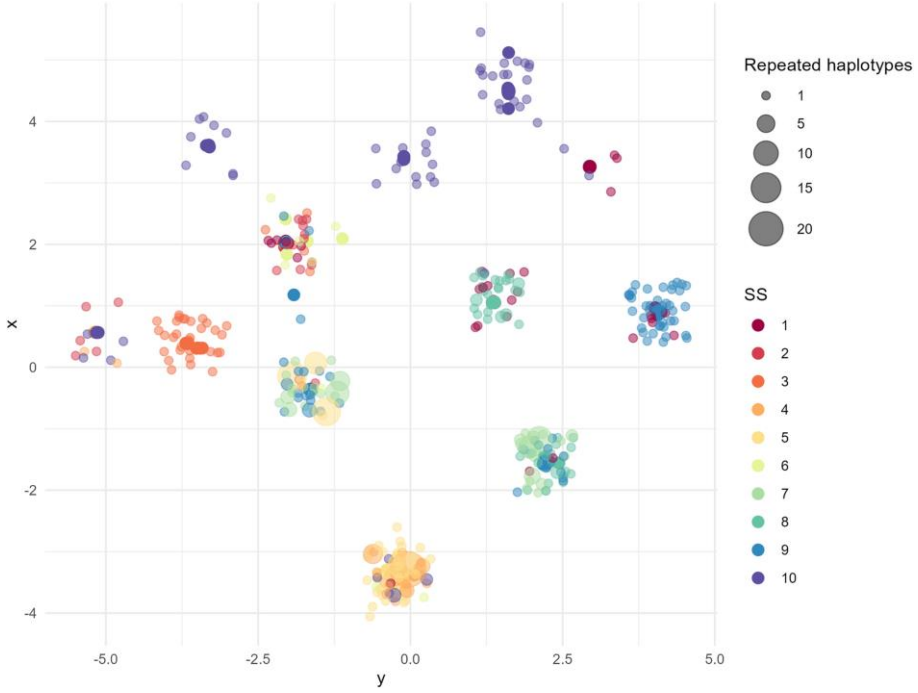
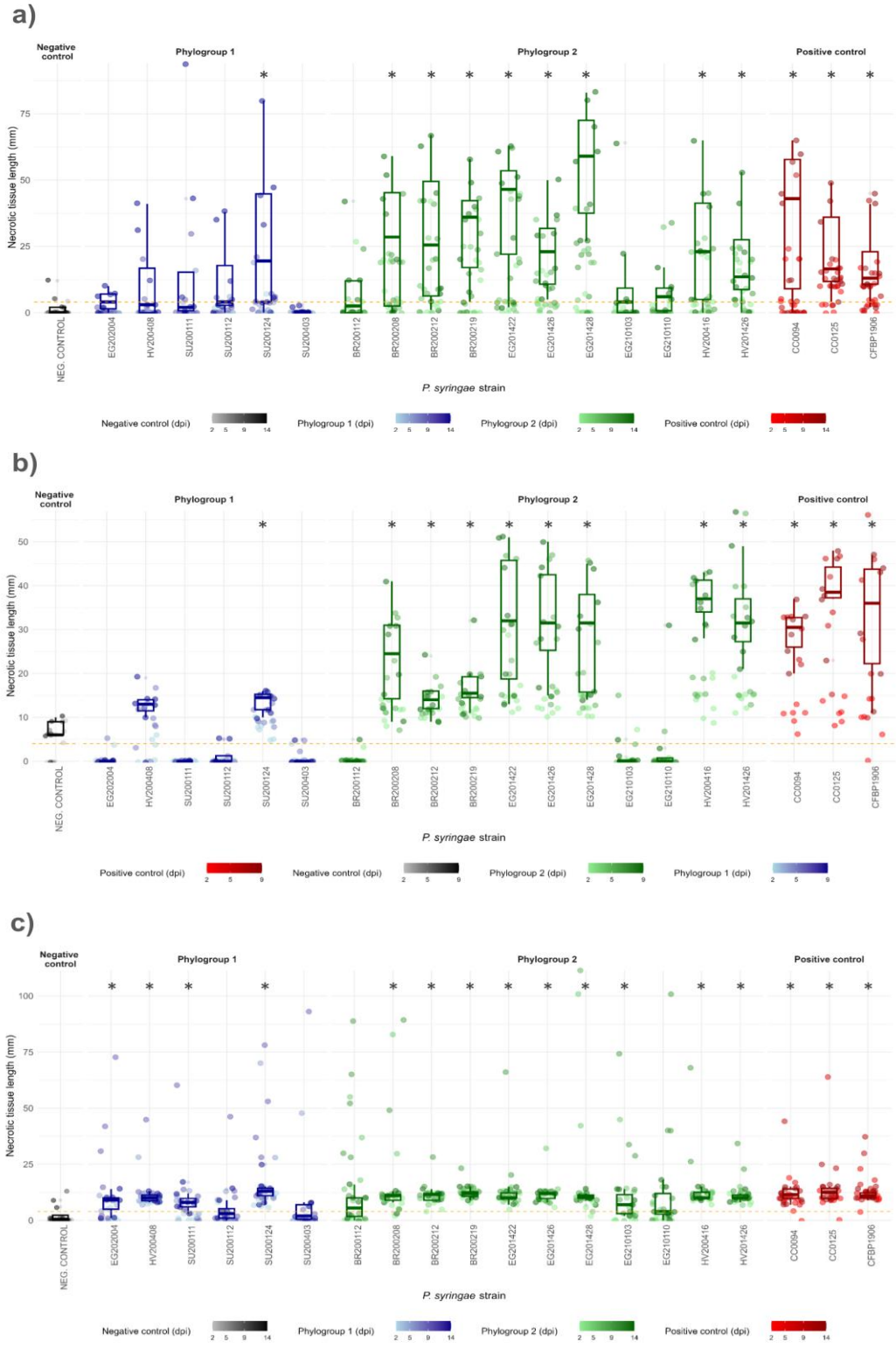


Figure 9. Genetic distance between *P. syringae* from 10 distinct sampling sites isolated from *Peltigera* lichen, tracheophyte and/or moss. t-Distributed Stochastic Neighbor Embedding

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 ten different sampling sites (see Paper I for more details).

The question of whether *P. syringae* strains from *Peltigera* lichens can potentially cause diseases in plants, strains assigned to phylogroups PG1 and PG2 were selected for pathogenicity and fitness analyses across various plant species, predominantly crops. The fitness evaluation involved four distinct strains, two from each PG, compared with two strains isolated from epidemic crops beyond Iceland (CC0094 and DC3000) across 10 different plan species. The fitness of *Peltigera* strains was generally comparable to epidemic strains, except in cucumber at 1-and 8 days post infection (dpi), and barley at 8dpi, where the epidemic strains exhibited higher fitness. Nevertheless, in tomato, *P. syringae* growth from *Peltigera* surpassed that of epidemic strains at 1dpi.

In the pathogenicity test, we chose 17 strains from *Peltigera* lichen and three strains (CC0125, CC0094, and CFBP1906) from other regions worldwide from the same PGs. Measurements of necrotic tissue length in the infiltrated leaves suggest that nine out of 17 strains from *Peltigera* lichen can be considered pathogenic in barley and cucumber, as they produced necrosis lengths significantly higher than the negative controls. The same strains, along with four additional ones, exhibited pathogenicity in the case of kale (Figure 10).


a)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
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0
25
50
75
NEG. CONTROL
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HV200408
SU200111
SU200112
SU200124
SU200403
BR200112
BR200208
BR200212
BR200219
EG201422
EG201426
EG201428
EG210103
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HV200416
HV201426
CC0094
CC0125
CFBP1906
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)
2 5 9 14
b)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
0
10
20
30
40
50
NEG. CONTROL
EG202004
HV200408
SU200111
SU200112
SU200124
SU200403
BR200112
BR200208
BR200212
BR200219
EG201422
EG201426
EG201428
EG210103
EG210110
HV200416
HV201426
CC0094
CC0125
CFBP1906
P. syringae strain
Positive control (dpi)
Negative control (dpi)
Phylogroup 2 (dpi)
Phylogroup 1 (dpi)
2 5 9
c)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
0
25
50
75
100
NEG. CONTROL
EG202004
HV200408
SU200111
SU200112
SU200124
SU200403
BR200112
BR200208
BR200212
BR200219
EG201422
EG201426
EG201428
EG210103
EG210110
HV200416
HV201426
CC0094
CC0125
CFBP1906
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)
2 5 9 14

Figure 10. Necrotic tissue length in mm on leaves infiltrated with *P. syringae*. Dots represent all measurements at different time points differentiate by the darkness of the color while the boxplot summarize the necrotic length on: a) Barley at 14 dpi; b) Cucumber on 9 dpi and; c) Kale on 14 dpi. All plants were incubated at 24 °C (14 h light, 10 h dark) and 75% humidity for up to 14 days. The *P. syringae* strains that exhibit statistical significance from the negative control (p value < 0.5) are marked with an asterisk on this graph.

The presence of *P. syringae* within lichens raises questions on the biochemical and physiological effects of the lichen thallus environment on *P. syringae*, and vice versa. To address these, an untargeted metabolomics analysis was conducted, which delved into the metabolic profiles of seven distinct species within the *Cladonia*, *Stereocaulon*, and *Peltigera* lichen genera. The complexity observed in all lichen metabolic profile is not surprising, given that it is composed of various organisms. It is also believed that a portion of the metabolites arises from the symbiotic interactions (Calcott et al., 2018). The richness analyses revealed greater diversity in non-*Peltigera* genera compared to *Peltigera* (Figure 11). On the other hand, the eveness as measured by a Simpson test shows significant higher levels in *Peltigera* samples compared with *Cladonia* and *Stereocaulon*. Indicating a more balanced abundance of the compounds isolated from *Peltigera* samples.

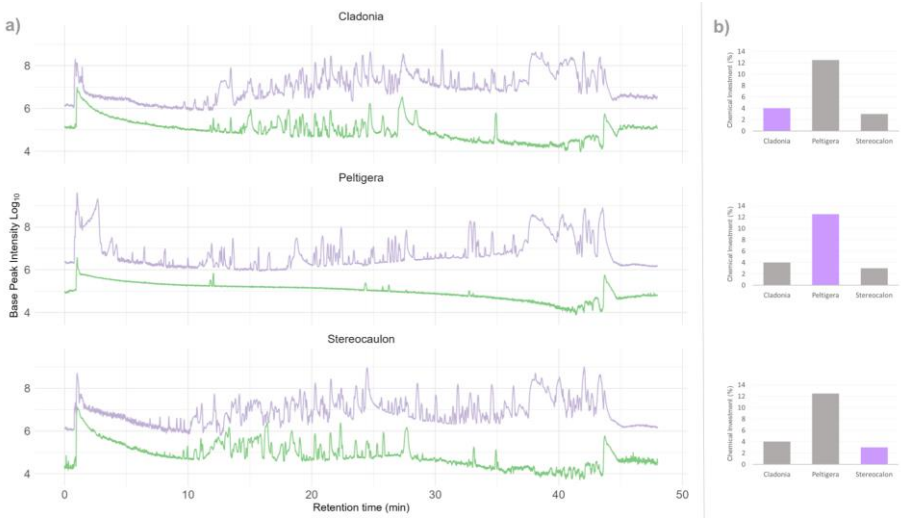

Figure 11. (A) Mass spectra (m/z) in purple (positive polarity) and green (negative polarity) for *Peltigera* sample 28, *Cladonia* sample 2, and *Stereocaulon* sample 42. (B) Represents the average chemical investment across the analyzed genera in this study. See Paper III for more details.

These results revealed genus and species-specific metabolic profiles (Figure 12.a), prompting further exploration into the differing metabolites. Furthermore, we could observe how *P. membranacea* is clearly separated from the other two lichen genera in the PCA (Figure 12.b). This distance is probably a result of the different photobionts that are part of *P. membranacea* where a cyanobacterium serves as the primary and exclusive photobiont, while the other two *Peltigera* species are tripartite, featuring both algae and *Nostoc* cyanobacteria (Paulsrud et al., 2001). This investigation aims to identify specific compounds that may play a role in the exclusive isolation of *P. syringae* from *Peltigera* lichen.

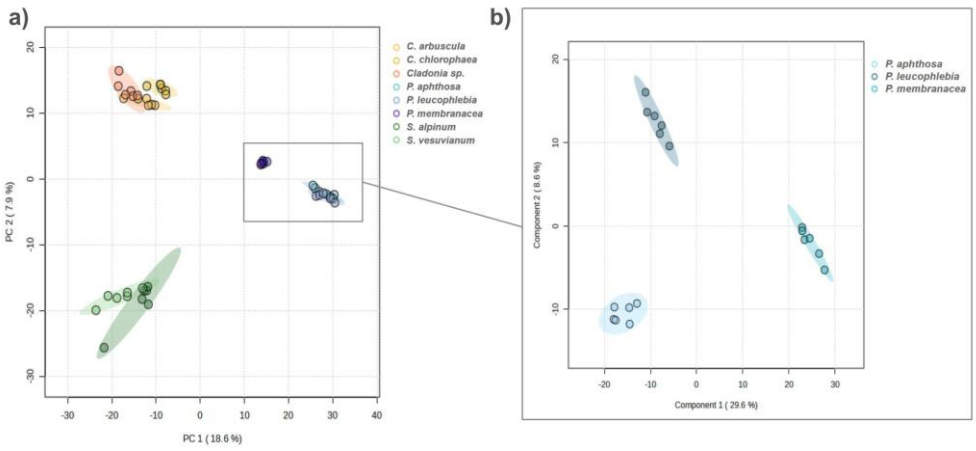

Figure 12. Principal Component Analysis (PCA) with each species differentiated by color. Ellipses indicate the 95% confidence area. a) All samples included; b) Only *Peltigera* samples included. Generated by Metaboanalyst.

Kinetic analyses reveal similar bacterial growth in lichen-supplemented media, except for *C. arbuscula*, which exhibited lower growth, and *P. aphthosa*, showing the closest bacterial growth and highest OD_{600} when compared to KB media. The inhibition disk test only highlighted inhibition from various *Cladonia* species and the two *Stereocaulon* against the antagonist of *P. syringae*, *B. velezensis*, while no inhibition was observed against *P. syringae*. The average lichen profile is characterized by a dominance of lipids in the positive polarity and mainly organic acids in the negative polarity.

A total of 6527 compounds were collapsed by Cytoscape with the positive polarity with a 4.5% annotated from which 297 were exclusively from *Peltigera* samples. Some candidates of the specific isolation of *P. syringae* from *Peltigera* are benzimidazoles that we only identified in *Peltigera* lichens aminoglycosides or guaiane, known to inhibit *B. subtilis* and other recognized antagonists of *P. syringae*. For instance, a compound exclusively absents in *Peltigera*, such as anthracene, serves as a carbon source utilized by *the antagonist of P. syringae, Bacillus subtilis* (Salamat et al., 2018). Furthermore, ubiquinones, recognized for their role in resistance against *P. syringae* in various plant species, were identified in the non-*Peltigera* lichens.

4 Discussion

The project concept emerged following the identification of *P. syringae* traces during metagenomic analyses of *P. membranacea* lichen conducted by Sigurbjörnsdóttir (2015). In this investigation, traces of other potential bacterial pathogens, including *Xanthomonas* and *Xylella sp.*, were also detected. This study focuses on *P. syringae*, a model organism for plant pathogens with a well-documented adaptation to cold environments, making it particularly relevant as a potential threat in Iceland. Furthermore, focusing on *P. syringae* was judged to complement ongoing work on this plant pathogen in different plant types in Iceland (Morris et al., 2019). The results not only showed a high abundance of the bacterium in the country but also pointed to the apparent isolation of the *P. syringae* Icelandic population from the mixing process of the rest of the globe for a long time (Morris et al., 2022).

4.1 Is *P. syringae* exclusively hosted by *Peltigera* lichen?

The work reported in Paper I indicated the presence of *P. syringae* in only one lichen genera *Peltigera*, commonly referred to as dog lichens or pelt lichens. It could, however, be argued that it ought to be isolatable from other lichen genera that, as *Peltigera*, offer a suitable intrathallic environment for growth of *P. syringae*. These characteristics, as briefly explained in Paper I, could include the amount of available water within the lichen, or, as was shown in Paper III, the presence of metabolites that could help *P. syringae* survival by, for example, inhibiting the growth of *B. velezensis*, a known competitor of *P. syringae,* or the lack of some metabolites that have antibiotic or antimicrobial effects against *P. syringae*.

4.2 *P. syringae* are perhaps more prevalent in lichens placed in cold climates

We are aware that attempts to isolate *P. syringae* from lichens in more temperate zones, including *Peltigera* species, have been unsuccessful. There could be reasons for *P. syringae* being more readily isolated from lichens in Iceland. These could include reduced competition in the habitat, as several bacteria found in temperate zones might struggle to survive or exhibit lower fitness in a cold climate. Indeed, *P. syringae* shows excellent adaptation to cold conditions, setting it apart from many other bacteria. Pseudomonads in Icelandic natural habitats tend to be cold adapted (Jóelsson et al., 2013; Markúsdóttir et al., 2013).

An alternative explanation might be associated with the observation that bacteria in cold habitats appear to have a lower biosynthetic potential than those in warmer environments (Pomeroy et al., 1991). Consequently, it is plausible that certain bacterial species with antimicrobial activity against *P. syringae*, found in *Peltigera* lichen within cold environments, may not produce certain metabolites, or produce them in lower quantities in this specific environment, potentially promoting the presence of this bacterium.

4.3 "Ideal conditions" for *P. syringae* outbreak are not common in Iceland

The conditions selected in the pathogenicity and fitness analyses aim to show if the strains are aggressive under the proper conditions for rapid growth. Even though the ideal conditions for infection might vary between strains, we selected 24°C and 80% of humidity for our samples which is an uncommon condition in Iceland.

This study highlights the potential of environmental bacteria, particularly those from lichens, to become pathogens. It underscores the need for increased awareness of potential outbreaks, especially as climate change pushes conditions

closer to the optimal for these bacteria, a situation that is becoming more likely in polar regions (Parry, 2019).

4.4 Metabolomics for analyses of host-microbe interaction

As lichens are immobile, they have evolved various mechanisms to engage with surrounding microorganisms. These mechanisms encompass defense strategies against pathogens and the synthesis of attractants for symbiotic organisms, such as sugars and sugar alcohols released by the primary lichen symbionts. These substances may also impact the composition of lichen-inhabiting bacteria. However, the chemical communication between the primary myco- and photobionts and microorganisms inhabiting lichens is not well-understood (Pichler et al., 2023)

Chen et al. (2021) demonstrated how metabolomics can be applied to functionally characterize specific metabolites influencing plant-microbe interactions. While this approach is intriguing for future studies in various organisms, it holds particular significance in lichens due to challenges associated with working on their thallus in the laboratory. These challenges include slow growth and difficulties in disinfection, especially when considering sampling for future inoculations.

4.5 Lichen profiles exhibit species and genera-specific metabolites

Our results showed a clear difference between the metabolic profile among different genera and species in both positive and negative run. A comparable result to ours is illustrated in Mittermeier et al. (2015), where they investigated various lichens isolated from Alpine and Ecuadorian environments. They observed that not only did samples from the same species or genera cluster

together in the PCA, but also that phylogenetically closer genera exhibited closer proximity in the plot.

Boustie et al. (2011) opened the question of the differentiation of lichen metabolic profile in response to environmental more than genetic factors. The lichens for this study were sampled in March 2023 in Reykjavik (Iceland) in days with -15 to -18 °C which we can consider rather extreme temperatures. Despite the anticipated changes in metabolic profiles due to environmental or other factors that we have not tested in this study, our findings suggest that different lichen species belonging to the same genera shared a big part of their metabolic profile. This might be because these genera share strategies or pathways among species within the same genus or maybe they retain certain fundamental metabolites specific to each species or genus. The outcomes of our study, involving samples from different genera in the same area and sampled at the same time, point to a clear differentiation likely stemming from genetic adaptation.

4.6 Untargeted metabolomic analyses reliability

Lichens and their metabolites are intricate, originating from diverse symbionts, resulting in a highly diverse metabolic spectrum. Untargeted metabolic analyses provide extensive information, albeit with reduced precision. Our experiment was meticulously designed to report only metabolites with results we are confident in, employing multiple replicates, pseudo-replicates, and strict statistical parameters. While we can trust the reported findings, caution is warranted regarding unreported data, as there is a possibility of loss due to the chosen metabolomic approach or the data filtering and cleaning processes.

We used the bacteria's growth on various lichen media and inhibition tests as references to analyze the differing metabolites among species showing significant differences in these analyses. Afterwards, we aimed to establish connections between the identified metabolites in various samples and their potential impact on the exclusive isolation of *P. syringae* from *Peltigera* lichen, drawing insights from information found in peer-reviewed journals. We

acknowledge the diverse roles that different compounds can play under various conditions. For instance, investigations into the antimicrobial effects of lichen extracts on the same or different microorganisms have occasionally produced disparate results. Typically, such differences are attributed to factors like extraction methods or the geographical locations of the lichen samples (Ranković et al., 2009). Our study aimed to speculate and analyze potential candidates, recognizing that this information may require validation through further research.

5 Suggestions for future work

Part of the work performed during the present project will be published after the submission of the thesis:

5.1 Exometabolomics of *P. syringae* in lichen-enriched growth media

During the metabolomics analyses, we gathered a substantial amount of information, which, upon compilation for a paper, proved to be excessively extensive. Consequently, we decided to divide the content into two articles. In one article, we concentrated on the lichen metabolites that could potentially influence the presence of *P. syringae* (Paper III). The other article encompasses the inhibition assay, kinetic analyses of selected *P. syringae* and a *B. velezensis* strain in lichen-supplemented media, along with the analysis of exo-metabolites produced by these bacteria during a 48-hour growth period in some of the lichen-supplemented media (Figure 13) (paper not included on the thesis).

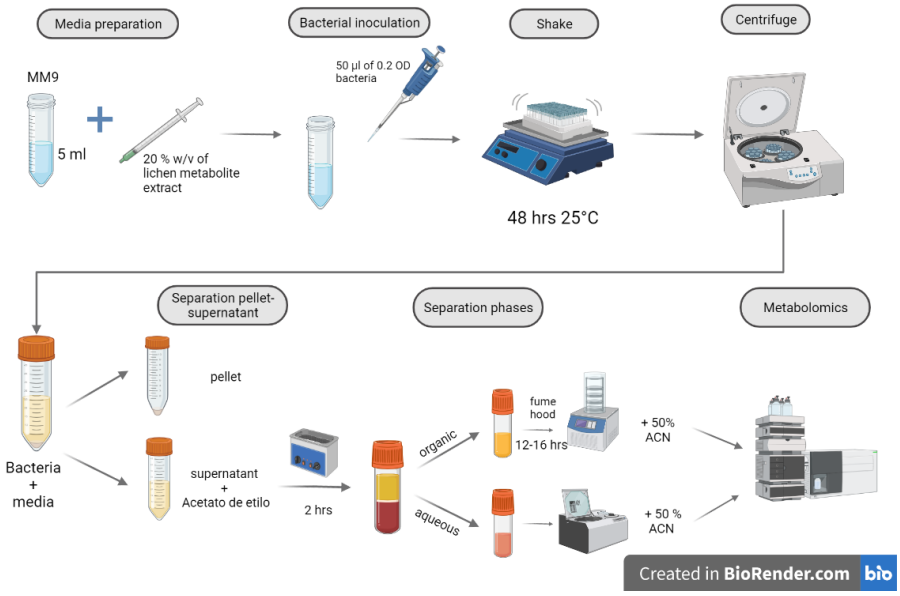

Figure 13. Schematic illustration of the exometabolite analyses of *P. syringae* and *B. velezensis* in diverse lichen media.

5.2 Bacterial selection for the experiments of *P. syringae* growing on lichen supplemented media: kinetics, inhibition and exo-metabolites analyses

The selection of the bacteria strains to be used in this part of the study was focused on the analyses of *P. syringae* belonging to different PGs. The PGs isolated from *Peltigera* lichen are PG10, PG13, PG2 and PG1 in order of abundance (Ramírez et al., 2023). As controls, we selected some strains from other parts of the world that belong to the same PGs (Table 1). Furthermore, we include *B. velezensis* to understand the capability of this known *P. syringae* competitor to grow on different lichens and therefore shaping *P. syringae* presence.

Strain name	Isolation country	Substrate	Isolation organism	Phylogroup	Pathogenicity	Fitness	Control	Source
EG201428	Iceland	lichen	*Peltigera canina*	2b	high	high		
SU200101	Iceland	lichen	*Peltigera leucophlebia*	10d	NA	NA		
HV200414	Iceland	lichen	*Peltigera membranacea*	13a	NA	NA		
CFBP1392	United Kingdom	angiosperm	*Syringa vulgaris*	2b	Oat, Pepper,Eggplant, Cantaloupe, Tomato, Sugar beet, Onio and Pea (Morris et al., 2019)		X	Morris et al., 2000
CC1557	France	snow	-	10d	Sorghum and Sugar beet (Morris et al., 2019)		X	Morris et al., 2008
UB0246	France	river water	-	13a	no (Monteil et al., 2013)	no (Monteil et al., 2013)	X	Diallo et al., 2012
Bacillus velezensis	United Kingdom	tree	Ash tree	-	-	-	X	Hinton et al, (not published)

Table 1. Overview of strains utilized in metabolomics, kinetics, and inhibition experiments, featuring details such as isolation country, substrate, organism, PG, pathogenicity, and fitness as outlined in Paper II. The inclusion of information indicating whether a strain served as a control and references to relevant literature sources is also provided.

5.3 Low intensity signal from *P. syringae* growing 48 hrs in lichen supplemented media

The first untargeted metabolomics run of exo-metabolites from *P. syringae* growing in minimum media supplemented with lichen metabolites showed low peaks impeding noise removal and confidence of the results. To increase the peak size all samples used in this essay were dried in a vacuum centrifuge concentrator and diluted this time to 10 mg/ml of the organic phase instead of 1 mg/ml of the aqueous phase to obtain higher peaks. The obtained peak intensities were adequate for the analyses and differentiation from noise.

5.4 Whole genome sequencing of 16 *P. syringae* strains isolated from *Peltigera* lichen, moss and tracheophytes

Moreover, we submitted 16 *P. syringae* strains obtained from both plants and *Peltigera* isolated in Iceland for whole-genome sequencing (Table 2). The strain selection aimed to cover a diverse range of *P. syringae* strains, including representatives from PG1, PG2, PG10, and PG13, isolated in Iceland from various *Peltigera* lichen, tracheophytes, and moss species, with a particular emphasis on the *Peltigera* lichen strains. Whole-genome sequencing was conducted using the Bacteria *de novo* sequencing platform with Pac Bio Sequel II for the reference

strain SU201201, while the remaining strains were analyzed through Bacterial whole-genome resequencing.

We intended to analyze these sequences to enhance our understanding of how Icelandic *P. syringae* strains differentiate from those found elsewhere globally as well as to observe if there are differences between strains from *Peltigera* lichen compared with plant strains and analyze the possible horizontal gene transfer between *Peltigera* lichen and the strains isolated from this genus. Finally, we would like to analyze the presence of Type III effectors, correlate them with fitness and epidemic performance of Paper II and compare them with the ones from epidemic strains.

Strain name	PG	Substrate organism	Species	Symptom in-planta	Fitness experiment done
SU201201	10d	Lichen	*Peltigera membranacea*		
HV200416	2b	Lichen	*Peltigera membranacea*		
BR200113	10d	Lichen	*Peltigera membranacea*		
HV200427	2b	Lichen	*Peltigera membranacea*		
EG201428	2b	Lichen	*Peltigera canina*	strong	Y
SU200111	1a	Lichen	*Peltigera leucophlebia*	weak	
SU200112	1a	Lichen	*Peltigera leucophlebia*	weak	
SU200124	1b	Lichen	*Peltigera leucophlebia*	weak	Y
HV200408	1a	Lichen	*Peltigera membranacea*	weak	Y
HV200426	2b	Lichen	*Peltigera membranacea*	medium	Y
BR200208	2b	Lichen	*Peltigera leucophlebia*	strong	
EG201416	13a	Lichen	*Peltigera canina*		
HV200510	13a	Tracheophyte	*Alchemilla alpina*		
EG201109	1a	Tracheophyte	*Rubus saxatilis*		
EG202203	2b	Moss	*Hylocomium splendens*		
EG201929	10a	Moss	*Rhytidiadelphus sp.*		

Table 2. Strains submitted for whole genome sequence and their isolation substrate, PG, fitness, or pathogenic test done and symptoms observed based on Paper II.

5.5 WGS contamination problems

The sequencing process for these strains turned out to be more challenging than initially expected. Due to the lack of whole-genome sequencing capabilities at the university, we opted to have the bacteria sequenced by an external company BGI Genomics. Initially, we sent the cell pellet of the bacteria, anticipating the company to handle the DNA extraction at their facilities. However, near the completion of the process, they informed us of sample contamination. Subsequently, we sent 12 out of the 16 strains for sequencing again, but this time, we conducted the DNA extraction ourselves and sent the extracted DNA to them. The samples were sequenced successfully in this second trial.

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Paper I

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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.

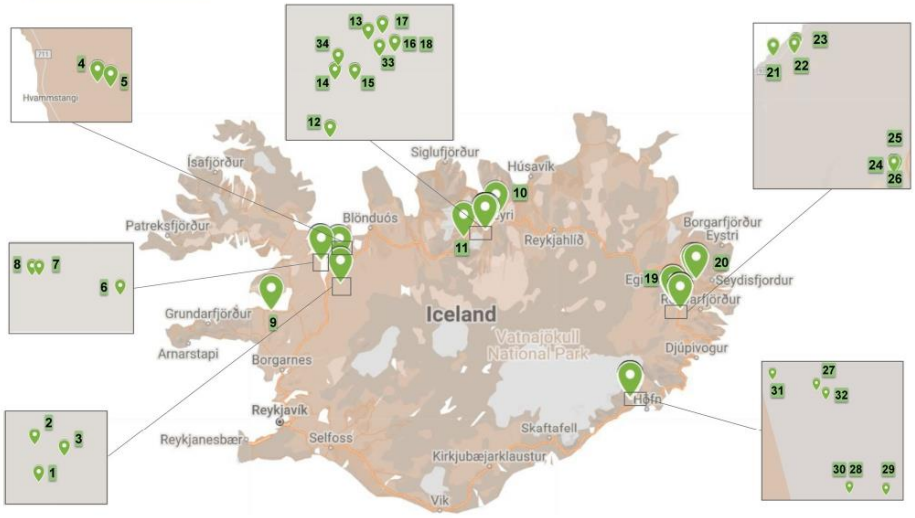

FIGURE 1 Sampling sites in Iceland. The 68 samples analysed in this work were collected in 34 locations and based on prevailing habitat types to represent a wide range of the sub-Arctic heathlands characteristic of northern and eastern Iceland.

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

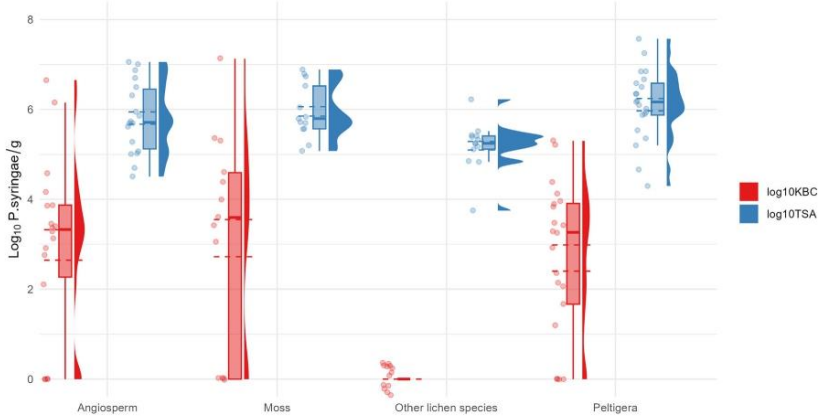

FIGURE 2 Total (TSA) and *Pseudomonas syringae*-like (KBC) populations on different plants and lichens. Bacterial numbers are shown as Log_{10} per g of fresh tissue. Dots indicate the individual samples for the summary statistics that are represented by the box and violin plots. Thick solid lines indicate 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

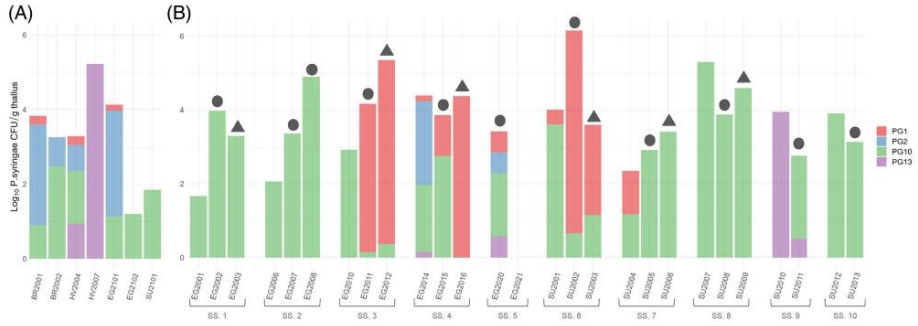

FIGURE 3 *Pseudomonas syringae* population size and proportion of the four dominant phylogroups. Different colours in the bars correspond to the proportions of *P. syringae* PG01, PG02, PG10, and PG13 whereas the height of the bar represents the population size. (A) Data from seven sites where only *Peltigera* 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.

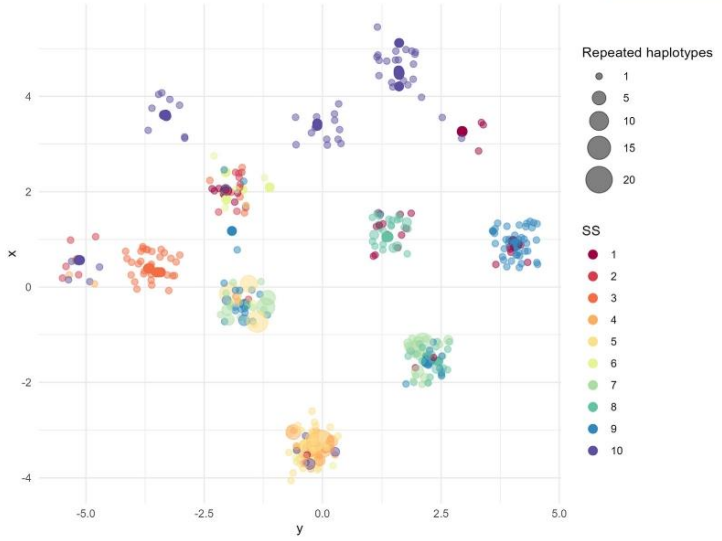

FIGURE 4 Genetic distance between *Pseudomonas syringae* from 10 distinct sampling sites isolated from *Peltigera* lichen, tracheophyte, and/or moss. *t*-Distributed Stochastic Neighbour Embedding graph (t-SNE) created to display 487 *P. syringae* strains that were collected from *Peltigera* lichen thalli, 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

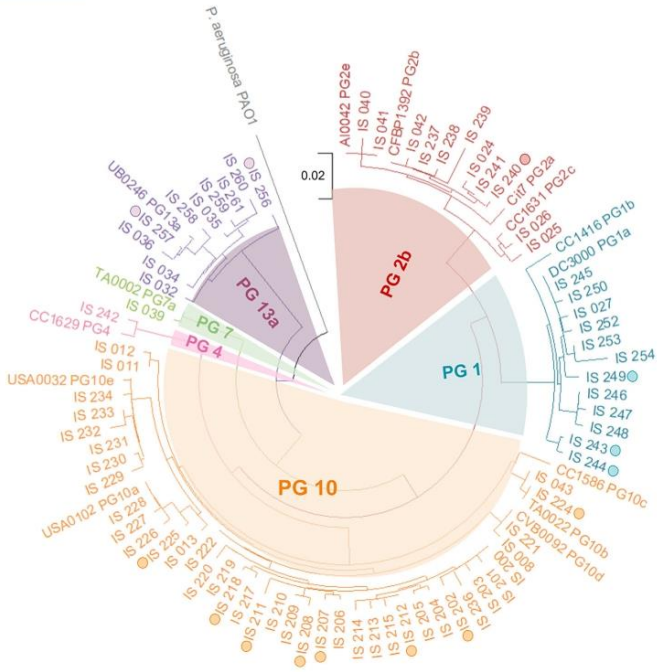

FIGURE 5 Neighbour-joining phylogenetic tree constructed using 101 concatenated cts sequences of *Pseudomonas syringae* isolated in Iceland (names starting with "IS"), with a bootstrap value of 1000. The tree includes haplotypes from lichens, tracheophytes, and moss that were sampled for this study, 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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SUPPORTING INFORMATION

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ORIGINAL ARTICLE

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From lichens to crops: Pathogenic potential of *Pseudomonas syringae* from *Peltigera* lichens is similar to worldwide epidemic strains

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Abstract

The presence of bacteria belonging to the *Pseudomonas syringae* complex in the natural vegetation of several Icelandic habitat types has been recently reported, raising questions about the risk to Icelandic crops, particularly given the expected increase in agricultural activity due to climate warming. This study takes advantage of Iceland's unique characteristics and the discovery of *P. syringae* in *Peltigera* lichens to gain a better understanding of the potential risk posed by this newly discovered ecological niche. The main objective was to evaluate the pathogenic potential and fitness in crops of *P. syringae* strains isolated from *Peltigera* lichen sampled in Iceland, focusing on strains that belong to phylogroups 1 and 2, which commonly contain epidemic strains. The results indicate that *P. syringae* strains isolated from Icelandic *Peltigera* lichen have a comparable fitness to epidemic strains in 8 out of 10 tested plant species (rice, tomato, thale cress, annual mugwort, spinach, garlic chives, tobacco and kale). Furthermore, pathogenicity assessment on three plant species highlighted that certain strains also caused similar symptoms and disease severity compared to epidemic strains. These findings provide valuable insights into the potential risks posed by *P. syringae* from Icelandic natural habitats and illustrate how strains from these habitats have a wide pathogenic potential to crops without having encountered these crops in the last several thousand years of their presence in Iceland.

KEYWORDS
epidemic strains, non-agricultural habitat, pathogenicity test, plant pathogen

1 | INTRODUCTION

Bacterial strains within the *Pseudomonas syringae* complex are present in connection with diverse biotic and non-living substrates worldwide. The ability of *P. syringae* to adapt to a wide range of habitats linked to the water cycle is thought to be a driver of its broad host range (Morris et al., 2013). This idea is reinforced by the observation that numerous strains from this group of bacteria isolated from non-agricultural environments are phylogenetically closely related to plant-associated strains and have also been shown to be pathogenic on plants such as kiwifruit and tomato (Morris et al., 2019). For this reason, recent research has continued to explore the ecology and pathogenicity of *P. syringae* outside the context of crops.

According to Ellis et al. (2010), agricultural land is becoming increasingly prevalent compared to other vegetated areas on Earth. However, Iceland stands out as an atypical region with a limited amount of cultivated land, thereby providing a unique opportunity to study the adaptation of *P. syringae* without a predominant

influence of local agriculture. Iceland's flora is characterized by a relatively small number of native species of vascular plants, comprising around 530 species (Wąsowicz, 2020). Additionally, the country is home to a diverse range of lichens (755 species; Kristinsson & Heiðmarsson, 2009) and mosses (around 600 species; Jóhannsson, 2003). However, the vegetation of Iceland evolved in the absence of large herbivores and subsequently is vulnerable to grazing and human activities (Runólfsson, 1987). Furthermore, Iceland's position on the border between Arctic and Atlantic waters and air masses, known as the polar front, makes for an interface (Jónsdóttir et al., 2005) that creates favourable climate conditions for *P. syringae* with average temperatures between 2 and 14°C throughout the year (Ogilvie & Jónsson, 2001).

A lichen is a composite organism resulting from a symbiotic association between a fungus, known as the mycobiont, and one or more photosynthetic partners, termed photobionts. The photosynthetic partners are typically green algae or cyanobacteria. This mutualistic relationship forms a unique structure known as a thallus, which is the visible body of the lichen. However, they also harbour internal bacterial communities (Boustie & Grube, 2005; Cardinale et al., 2006; Feuerer & Hawksworth, 2007; Leiva et al., 2021), as well as fungi, sometimes pathogenic for the lichen (Bates et al., 2012; Spribille et al., 2016), archaea (Bjelland et al., 2011; Garg et al., 2016) and viruses (Eymann et al., 2017). While lichens heavily depend on the atmosphere for water intake, their remarkable resilience in challenging environments is partly attributed to the microbiome associated with lichens, playing a crucial role in their survival (Bates et al., 2011; Bjelland et al., 2011; Cardinale et al., 2006, 2008, 2012; Grube & Berg, 2009; Hodkinson & Lutzoni, 2010; Leiva et al., 2021; Mushegian et al., 2011; Pisani et al., 2011; Selbmann et al., 2010; Sigurbjörnsdóttir et al., 2015).

Previous research has shown that *P. syringae* is prevalent in Iceland on wild vascular plants and moss (Morris et al., 2022), confirmed by the observations of *P. syringae* genes in the lichen metagenome of *Peltigera membranaceae* (Sigurbjörnsdóttir, 2016; Sigurbjörnsdóttir et al., 2015). This prompted researchers to investigate its ubiquity across several types of plants and lichens in Iceland, hypothesizing that lichens may serve as nonhost reservoirs for *P. syringae* (Vilhelmsson et al., 2016). Morris et al. (2022) unveiled how the genetic lines of *P. syringae* in the Icelandic region are monophyletic, indicating that they may have evolved separately from the *P. syringae* populations elsewhere in the world during the relatively short geological history of Iceland. However, these monophyletic haplotypes represent different phylogroups (PGs) (Morris et al., 2022). This illustrates the extraordinary adaptive properties throughout the *P. syringae* complex.

P. syringae was found in lichens in Iceland, specifically in species of the genus *Peltigera* (Ramírez et al., 2023). To delve deeper into this discovery, a phylogenetic analysis of *P. syringae* strains collected from various sources, including lichens, tracheophytes and moss, was conducted. The analyses revealed significant differences among strains between geographical locations, showing a greater similarity of *P. syringae* within a site across all vegetation types rather than within vegetation types across sites. Moreover, *Peltigera* thalli harboured a consistent population density of *P. syringae*, although it was lower than that on moss and tracheophyte samples (Ramírez et al., 2023). This finding underscores the adaptability of *P. syringae* to inhabit a diverse range of vegetation beyond higher plants, offering novel insights into its evolutionary dynamics.

Assigning *P. syringae* strains to phylogroups based on their citrate synthase gene sequences (Berge et al., 2014) is a useful tool for understanding their phenotypic variations. Many of the strains isolated from *Peltigera* lichens can be assigned to phylogroups PG01 and PG02. These phylogroups contain a wide range of epidemic strains, as documented by Berge et al. (2014). Roughly 50% of the lichen thalli were found to host PG01 and/or PG02 strains. Among the *P. syringae* isolates, PG02 included approximately 14% of strains and PG01 accounted for a mere 4% of the overall *P. syringae* population derived from *Peltigera*, while the remaining 82% were assigned to the environmental habitat-associated phylogroups PG10 and PG13 (Ramírez et al., 2023).

In light of the ubiquity of bacteria in the *P. syringae* complex on vegetation in Iceland and, in particular, the presence of the PG01 and PG02 phylogroups showing high frequencies of strains displaying a functional type III secretion system (Berge et al., 2014), our goal was to assess the fitness and pathogenic potential—mainly on crops—of strains from PG01 and PG02 from Icelandic *Peltigera* compared to strains in the same phylogroup isolated from epidemics on crops elsewhere in the world.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

P. syringae strains belonging to PG01 and PG02 were randomly selected from a collection of strains isolated from *Peltigera* lichen that previously tested positive in a hypersensitive response (HR) test, conducted according to Morris et al. (2007), to represent the range of diversity of these phylogroups associated with this group of lichens (Figure 1). As positive controls, strains belonging to PG01 and PG02 from epidemic occurrences were chosen due to their well-established pathogenic potential and consistent behaviour, as revealed by previous work (Morris et al., 2019). For fitness assessment, the reference strains included CC0094 (PG02d) and *P. syringae* pv. *tomato* DC3000 (PG01a). For the evaluation of pathogenicity, CC0125 and CFBP1906 (PG02b) and CC0094 (PG02d) were employed as reference controls, aligning with the findings outlined previously (Morris et al., 2000).

2.2 | Inoculum preparation

Bacterial inoculum was prepared from 24 to 72 h growth on King's B (KB) medium (King et al., 1954). A loopful of growth was resuspended

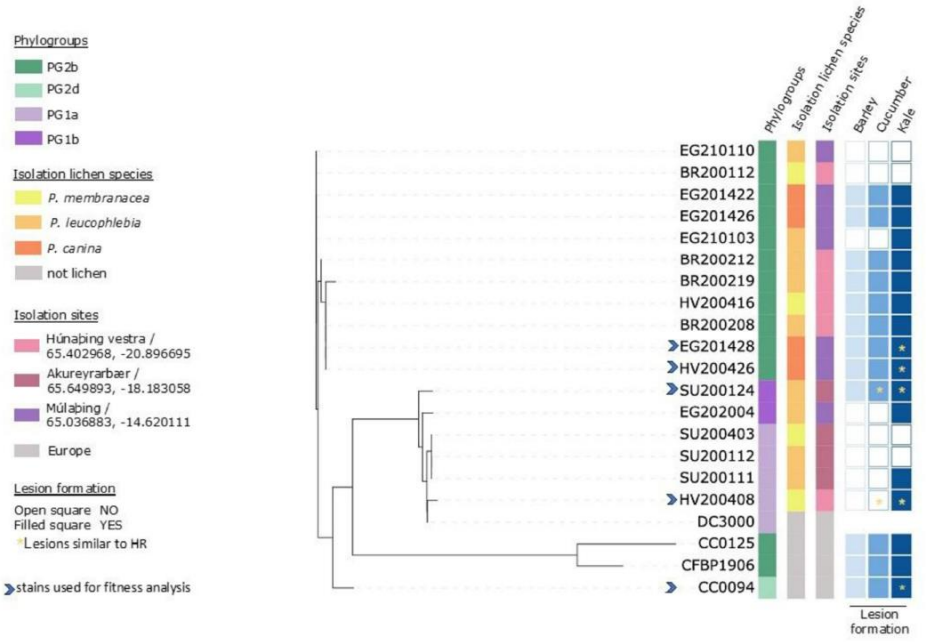


FIGURE 1 Phylogenetic tree illustrating the strains included in the study, along with details on phylogroup classification, lichen of isolation, site of isolation, and lesion formation in barley, cucumber and kale. The colour code represents different categories, and an arrow indicates the strains selected for fitness analyses.

in phosphate buffer (8.75 g/L K_2HPO_4, 6.75 g/L KH_2PO_4, pH 6.9) and adjusted with a spectrophotometer to 10^8 cfu/mL ($OD_{600\ nm}$ = 0.1). The inoculum was further diluted in phosphate buffer, resulting in a density of 10^7 cfu/mL for the pathogenicity tests and 10^6 cfu/mL for the in planta fitness tests. Inoculum concentration was verified by dilution plating.

2.3 | Plant material

For fitness tests, plant species belonging to 10 families were used: rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), thale cress (*Arabidopsis thaliana*), annual mugwort (*Artemisia annua*), spinach (*Spinacia oleracea*), garlic chives (*Allium tuberosum*), tobacco (*Nicotiana tabacum*), kale (*Brassica oleracea*), cucumber (*Cucumis sativus*) and barley (*Hordeum vulgare*). Pathogenic potential was evaluated on the last three plant species, which are cultivated in Iceland. Cultivar details, growing conditions and cultivation dates can be found in Table S1. The duration of cultivation in the greenhouse was tailored to the specific plant species. In the greenhouse environment of Montfavet, France, the plants were cultivated during a period spanning from September 2022 to January 2023. Adequate watering was provided in accordance with the plants' needs. Plants were grown in TS3 substrate mixture (Klasmann-Deilmann).

2.4 | Evaluation of fitness in planta

Fitness of strains in plants was determined in terms of population growth after inoculation using a modified protocol based on methodologies outlined by Clarke et al. (2010), Donati et al. (2020) and Kim et al. (2022). Leaf tissue was wounded and strains were inoculated into plants by placing a 5 μL-drop of inoculum (10^6 cfu/mL), letting the inoculum be absorbed by the plant. An emery board/emery paper was used to gently create a fresh wound on the surface of the leaf. Each plant received a single inoculation, and there were 10 replicate plants per strain.

Three to five plants were inoculated with phosphate buffer as negative control for each plant species. For a given plant species, all strains were inoculated simultaneously to facilitate between-strain comparisons. Via a randomized complete block design, the plants were set up in a growth chamber and incubated at 24°C (14 h light,

10 h dark), under a light intensity of 151–165 μmol/s/cm^2 and 75% humidity.

At 1 and 8 days post-inoculation (dpi), five leaves were collected from each plant species for each strain, except in the case of *A. thaliana*, where six leaves were collected. Prior to maceration, leaves bearing soil residues were gently cleaned using dry paper. Each leaf was gently macerated for 2–3 min in sterile phosphate buffer (10 μL of 0.1 M buffer per mg of fresh plant weight amounting to 1–30 mL depending on the leaf size) in sterile stomacher bags. Aliquots of serial dilutions of the macerate were plated on KB medium. The plates were placed in a dark environment and incubated at room temperature for a period of 48–72 h. *P. syringae* colonies were identified based on their shape, size, colour, texture, elevation and margin (Morris et al., 2022). The abundance of *P. syringae* in the inoculated leaves was expressed per leaf.

2.5 | Inoculation, incubation and disease assessment for pathogenicity assays

We employed a modified protocol inspired by Bartoli et al. (2015) and Cazorla et al. (1998). One leaf per plant was infiltrated with a needleless syringe with 5–10 μL of inoculum (10^7 cfu/mL). Six to eight replicate plants were used per plant species per strain. The negative control consisted of phosphate buffer. Plants were arranged in a growth chamber in a randomized complete block design and incubated at 24°C (14 h light, 10 h dark), under a light intensity of 151–165 μmol/s/cm^2 and 75% humidity for 14 days.

Symptoms were scored at 2, 5, 9 and 14 dpi in terms of the maximum length of the necrosis that developed at the inoculated site, except for cucumber that was observed only until 9 dpi. Symptoms were also photographed and described at each scoring date. Strains were classified as pathogenic if the necrotic length was significantly different than the negative control.

To verify that symptoms were caused by *P. syringae*, isolations were made from at least three infected tissue replicates per strain. Sections of tissue (at the interface of necrotic and the green surrounding tissue) were aseptically collected and placed on KB plates and incubated at 24°C in darkness. After 2–3 days, colony morphology, colour and fluorescence under 366 nm were assessed.

2.6 | Statistical analysis

Statistical analyses were conducted using Microsoft Excel and R Studio. This included *t* tests, graph plotting with the ggplot2 package and one-way analysis of variance (ANOVA). The *t* test was applied to determine the differences between strains inoculated into the same plant species at various time points. Moreover, one-way ANOVA was employed, followed by post hoc Tukey–Kramer analysis to unravel strain statistical differences in necrotic tissue length.

3 | RESULTS

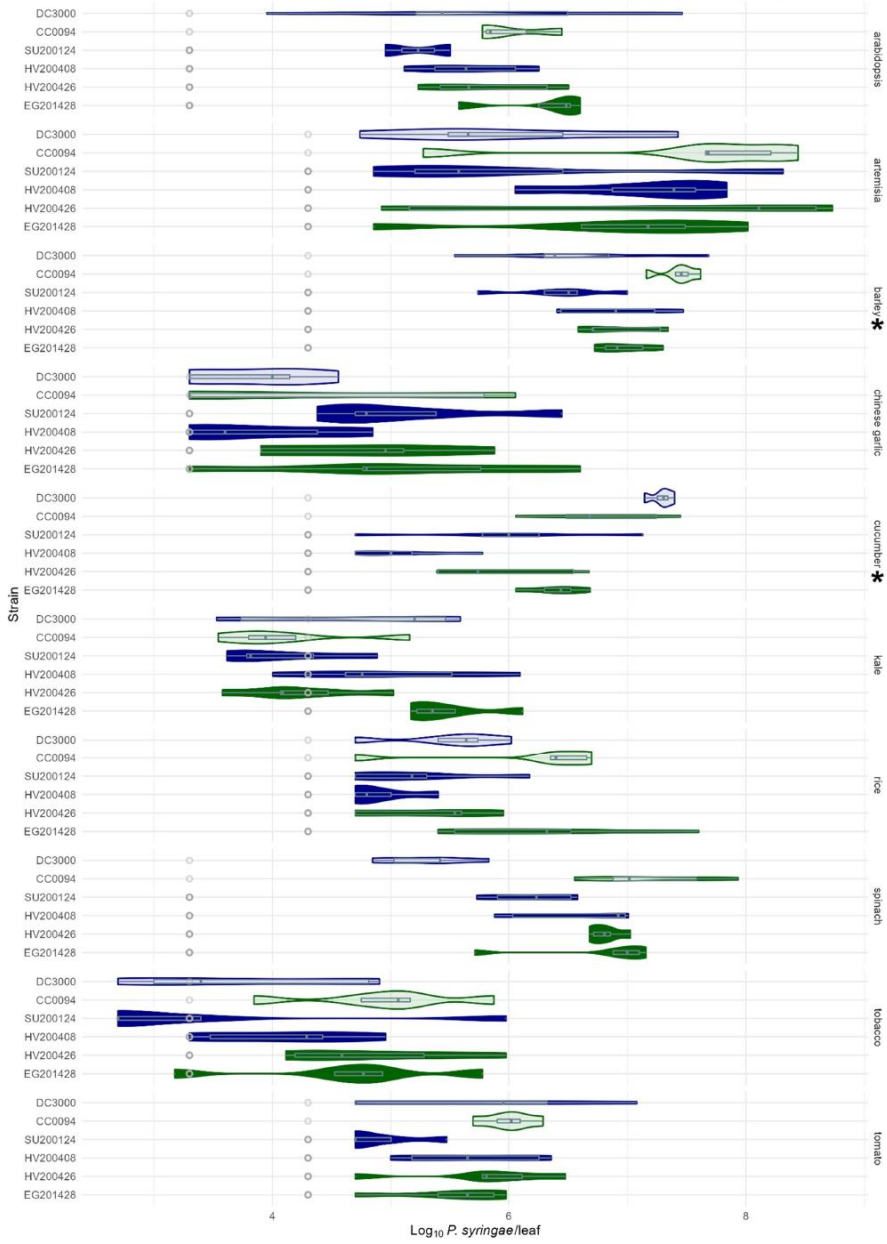
3.1 | Fitness of *P. syringae* isolated from Icelandic *Peltigera* lichens compared to epidemic strains

The fitness analysis revealed that *P. syringae* strains SU200124, HV200408, EG201426 and EG201428 isolated from Icelandic *Peltigera* lichen (hereafter referred as lichen strains) exhibited overall population growth levels similar to the epidemic strains CC0094 and DC3000 at both 1 and 8 dpi under the specified culture conditions across the 10 plant species tested, except for cucumber, barley and tomato. Indeed, differences emerged in tomato at 1 dpi, with the population size of *Peltigera* lichen *P. syringae* strains reaching higher densities compared to epidemic ones (Figure S1), although no difference was further observed at 8 dpi. Conversely, despite a similar population density at 1 dpi, the *Peltigera* strains showed a lower population level at 8 dpi compared with epidemic strains in cucumber and barley (Figure 2). However, for the latter, considering both time points, the comparison of the total population of lichen strains was significantly higher than the population density of epidemic strains ($p < 0.05$). This indicates that *Peltigera* strains display, as a whole, the same in planta fitness as epidemic strains, with some variability among plant species.

Interestingly, each lichen strain behaved like the epidemic one belonging to the same phylogroup. Indeed, the main differences were observed more at phylogroup level, with PG02 (including CC0094, HV201426 and EG201428) showing higher bacterial densities compared to PG01 (DC3000, SU200124 and HV200408) at 1 dpi only, in 7 out of the 10 analysed plant species. Such discrepancy, which was not noticeable anymore at 8 dpi, suggests a better capacity of PG02 strains to adapt and start growing in plants independently of their origin.

On the other hand, when examining strains individually, at 1 dpi, CC0094 consistently showed a greater population size compared with DC3000 in all plant species but *Arabidopsis*, and higher than all strains but HV200426 in tobacco (Figure 2). Conversely, at 8 dpi, distinctions were observed only in cucumber, with DC3000 having greater population sizes than all lichen strains, and in barley, where CC0094 had a significantly higher population size than SU200124 ($p < 0.05$; Figure 2). Finally, looking at the dynamic of population growth, we observed that, in general, all strains (epidemic and lichen)

FIGURE 2 *Pseudomonas syringae* population in inoculated leaves of 10 plant species at 8 days post-inoculation. Violin and boxplot graphs represent the values of *P. syringae* per leaf for five or six replicates per strain inoculated in each plant. The Icelandic strains isolated from lichens are represented by a solid colour while the epidemic strains used as control are plotted as transparent. PG1 strains are represented in blue, while PG2 strains are depicted in green. Grey dots indicate the minimum detection level. Asterisk denotes a plant species where statistically significant differences in *P. syringae* population sizes between epidemic and *Peltigera* strains were observed.


DC3000
CC0094
SU200124
HV200408
HV200426
EG201428
arabidopsis
artemisia
barley
chinese garlic
cucumber
kale
rice
spinach
tobacco
tomato
Strain
4
6
8
Log10 P. syringae/leaf

displayed a population increase between 1 and 8 dpi in all plant species, except for HV200408 and SU200124, which decreased over time in cucumber (Figure S2), while no strain but EG210103 grew in kale between 1 and 8 dpi (Figure S3).

3.2 | Pathogenic potential of *P. syringae* from *Peltigera* compared to strains from crop epidemics

For this analysis, the set of lichen strains was enlarged to a total of 17, including the four strains used for in planta fitness investigation, together with four and nine additional strains belonging to PG01 and PG02, respectively. To account for nonspecific reactions of the plants due to wounding, the size of the necroses on plants inoculated with selected strains was compared to those observed on control plants. Nine out of the 17 strains of *P. syringae* isolated from lichen produced necrotic symptoms that were significantly longer than any of the necroses observed on the negative controls in barley (Figure 3a) and cucumber (Figure 3b), and comparable to the lesions caused by epidemic strains. These same nine *Peltigera* strains and four additional strains, for a total of 13 out of 17, also caused lesions that were significantly longer on kale when compared to damage on control plants, and in the same size range as those observed with epidemic strains (Figure 3c).

According to lesion size, symptoms were most severe on barley and cucumber compared to kale, although we cannot rule out the possibility that such differences could be attributable to technical aspects (e.g., plant growth conditions, plant age). Moreover, not only the number of PG02 strains (seven in barley and cucumber, nine in kale) causing significant lesions was higher than the number of PG01 strains (one in barley and cucumber, four in kale) but, considering only necrosis-inducing strains, symptoms caused by PG02 strains were overall more severe than those caused by PG01 strains. Indeed, symptom severity (lesion length) in most cases was within the same range as those caused by the strains from crop epidemics (Figure 3).

In barley, in addition to necrosis, other symptoms such as chlorosis, leaf collapse, wilting, water-soaked areas or a slightly pale appearance were also recorded, mainly following infection with necrosis-inducing strains (Figure 4.1). Similarly, the infiltration of the *Peltigera* strains in kale caused symptoms such as chlorosis, water-soaked areas sometimes surrounded by a yellow halo and pale appearance, in addition to necrotic lesions (Figure 4.2). The observed symptoms in cucumber included necrosis, chlorosis and lesions with a yellow halo.

However, in some cases, necroses resembled hypersensitive-like cell death, that is, occurring shortly after infiltration and apparently localized to the site of infection. In particular, such a phenotype was observed in cucumber inoculated with the *Peltigera* strains belonging to PG01, namely HV200408 and SU200124, which induced smaller lesions, reaching a maximum length of around 15 mm, compared to the lesions induced by PG02 strains with a lesion size between 30 and 40 mm (Figure S2). Interestingly, the two above-mentioned strains showed a decrease in population size in cucumber 8 dpi compared to 1 dpi, probably related to the induction of HR leading to the restriction of bacterial growth.

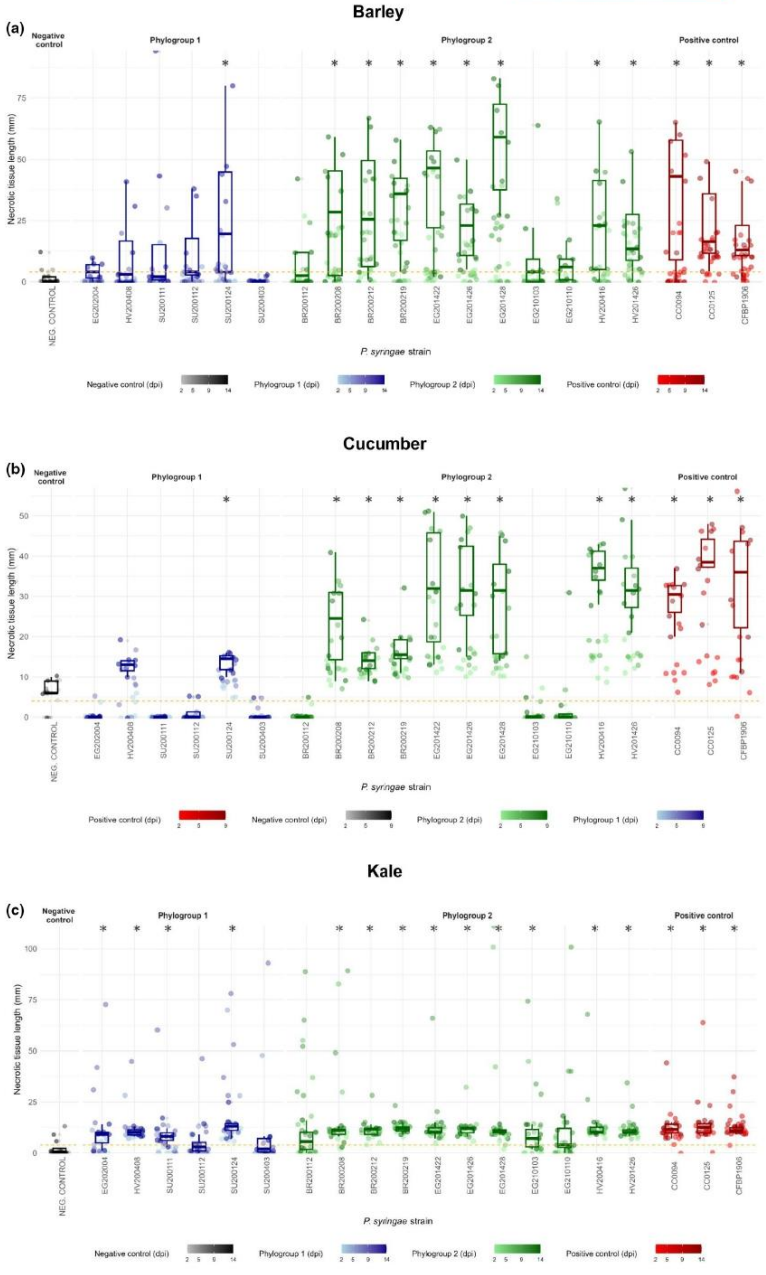
The induction of HR-like cell death was also common to numerous strains tested in kale, including the lichen strains HV200408 and SU200124 from PG01, and HV201426 and EG201408 belonging to PG02, together with the epidemic strain CC0094 (Figure S3). Interestingly, the lesions induced by these strains were already clearly visible after only 2 dpi, but did not increase further up to 14 dpi. Considering the absence of bacterial population increase between 1 and 8 dpi in fitness experiments (Figure 2), these results indicate the capacity of these *Peltigera* strains to induce a rapid HR in kale that in turn stops bacterial growth. Conversely, the PG02 lichen strain EG210103 induced significant lesions in kale only after 5 dpi and these necroses were characterized by the presence of chlorosis (Figure S3b). Moreover, this strain was the only one capable of growing between 1 and 8 dpi in fitness experiments, thus supporting the absence of HR in this case.

4 | DISCUSSION

Here, we have revealed that *P. syringae* isolated from *Peltigera* lichen in Iceland has fitness and pathogenicity levels comparable to strains isolated from epidemic crops worldwide. As a component of pathogenicity, some *Peltigera* strains were capable of inducing HR. Hence, even though we do not know what the host range would be in a cropping system, the strains from lichens possess a functional system to deliver effectors and to be recognized by a plant as something more than a saprophyte.

Numerous studies have demonstrated that *P. syringae* retains its pathogenic capability even in the absence of significant agricultural pressures (Morris et al., 2008, 2013; Morris & Moury, 2019), especially in those strains that have a wider range of habitat. The hypothesis suggests that the pathogenicity of *P. syringae* could be more pronounced and evident in agricultural contexts due to the lack of genetic diversity of the host and some practices for cultivation that might favour a rapid growth of *P. syringae*, even though it can also grow in non-agricultural plants such as *A. thaliana*, grass and ornamental plants (Jones et al., 1986; Katagiri et al., 2002; Sato et al., 2001). However, the unique context of the Icelandic

FIGURE 3 Necrotic tissue length in mm on leaves infiltrated with *Pseudomonas syringae*. Dots represent all measurements at different time points distinguished by the darkness of the colour; green corresponds to PG1 strains and blue to PG2 strains, while the boxplots summarize the necrotic length on (a) barley at 14 days post-inoculation (dpi), (b) cucumber at 9 dpi and (c) kale at 14 dpi. All plants were incubated at 24°C (14 h light, 10 h dark) and 75% humidity for up to 14 days. The *P. syringae* strains that exhibit statistical significance from the negative control ($p < 0.05$) are marked with an asterisk.


Barley
(a)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
NEG. CONTROL
EC202004
HV200408
SU200111
SU200112
SU200124
SU200403
BR200112
BR200208
BR200212
BR200219
EC201422
EC201426
EC201428
EC210103
EG210110
HV200416
HV201426
CC0094
CC0125
CFBP1906
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)
Cucumber
(b)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
P. syringae strain
Positive control (dpi)
Negative control (dpi)
Phylogroup 2 (dpi)
Phylogroup 1 (dpi)
Kale
(c)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)

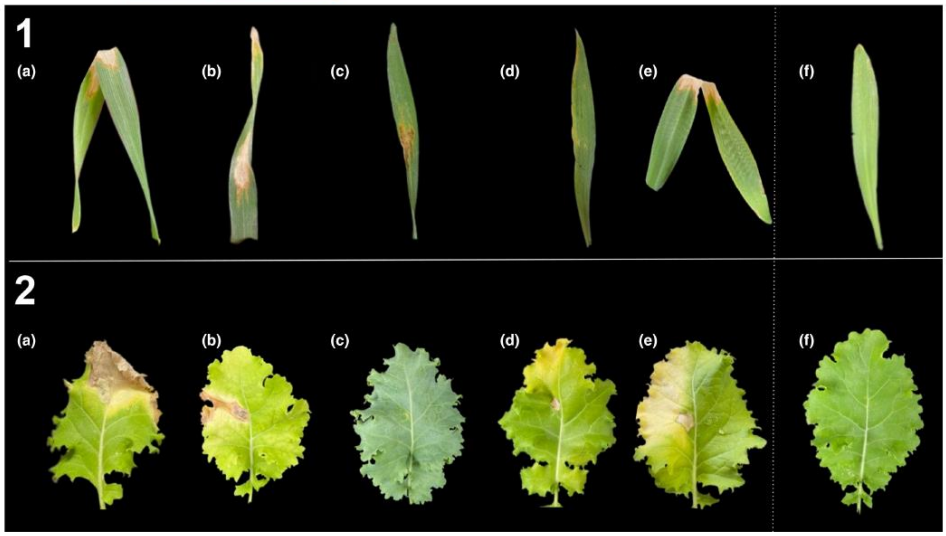


FIGURE 4 Leaf symptoms at 14 days post-inoculation. The leaves represent some of the most common symptoms observed on each plant species. (1) Barley infected with strains (a) HV201426), (b) EG201422, (c) EG201428, (d) HV201426, (e) positive control CFBP1906 and (f) negative control. (2) Kale infected with strains (a) HV201426, (b) EG210110, (c) SU200403, (d) EG201426, (e) positive control CFBP1906 and (f) negative control.

P. syringae strains that have evolved in Iceland for thousands of years makes them an interesting case study (Morris et al., 2022) as it illustrates the ancestral nature of the traits that confer pathogenicity and their maintenance in populations in natural habitats (Xin et al., 2018).

Although the pathogenicity observed in controlled conditions do not precisely reflect the pathogenicity of bacterial strains in the field, our results clearly illustrate the pathogenic potential of Icelandic strains, under favourable conditions. Thus, while this suggests the potential threat posed by *P. syringae* in Iceland, it is crucial to keep in mind that *P. syringae* is ubiquitous in the environment. In this light, it is notable that there have been no reported instances of *P. syringae* causing diseases in local crops in Iceland nor to the native vegetation. This highlights the role that environmental factors, such as cold temperatures that reduce the cultivation season or average temperatures under the optimal for *P. syringae* to develop aggressive traits (Bender et al., 1999), probably play in constraining bacterial proliferation and safeguarding plant health. The interplay of environmental conditions significantly contributes to the assessment of disease potential (Morris et al., 2023).

P. syringae in Iceland has evolved over the past 10,000 years, at least, separate from contact with plants that are common to agricultural landscapes such as rice and tomato (Morris et al., 2022). Nevertheless, Icelandic strains display some level of fitness in these plants, which could be then considered as novel potential hosts for such strains. This raises intriguing questions about the establishment of host specificity. However, the aggressiveness detected for Icelandic *P. syringae* might be related to the adaptation to environmental habitats, which have been linked to the evolution of their pathogenicity (Morris et al., 2010).

As previously demonstrated for *P. syringae* overall (Morris et al., 2019), it has not been possible to make any inference of pathogenic capacity/host range based on the substrate of isolation (habitat or lichen species) or according to the phylogroup of the Icelandic strains. This is also consistent with the observation of the mixing of populations of *P. syringae* between crop and environmental habitats, whereby they are not genetically distinguishable as different populations (Monteil et al., 2016; Morris et al., 2010). Moreover, the lichen strains resemble more the strains of *P. syringae* at a same site than other strains from lichens at distal sites (Ramírez et al., 2023). Thus, overall, these observations demonstrate that the habitat from which a strain is isolated is not necessarily the habitat on which it has spent the most time and via which selection pressures might have dominated.

The finding of *P. syringae* raised concerns about a possible threat to Icelandic crops in case the strains isolated showed pathogenic properties. The Icelandic cool climate can play an important role in pathogenicity of *P. syringae*, due to tissue damage that is intensified by frost episodes and humid environments, as it has been shown that the presence of a layer of free water is essential for infection (Lamichhane et al., 2015). Furthermore, certain studies have

indicated that lower temperatures enhance the functionality of type III and type VI secretion systems, which are typically associated with increased aggressiveness (Puttilli et al., 2022; Tribelli & López, 2022). Despite the limited agricultural land in Iceland (Denk et al., 2011), certain crops are cultivated on an industrial scale in greenhouses, with yields exceeding 1000 tonnes annually. These include tomatoes and cucumbers, while outdoor cultivation involves crops like barley and potatoes. Nevertheless, the anticipation is that the range of plant species grown outdoors and their yields in Iceland will rise over the coming decades due to the predicted rise in temperatures (Parry & Ruttan, 1991). The expansion of cultivable land, coupled with the anticipated milder, yet still relatively cold temperatures in Iceland, may pose a potential threat to Icelandic crops.

The results of this study are one more example of *P. syringae* strains isolated from environmental habitats, where they are not causing any obvious damage, or where they might even be beneficial (Morris et al., 2013). However, these strains can affect some plant species under specific conditions—conditions that might be increasingly probable with the changing climate and land use in Iceland.

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CONFLICT OF INTEREST STATEMENT

The authors affirm that the research was conducted without any commercial or financial affiliations that could be construed as potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Paper III

Exploring the Exclusive Isolation of *Pseudomonas syringae* in *Peltigera* Lichens with Metabolomic Profiles and Kinetic Analysis

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Keywords: *Pseudomonas syringae*, untargeted metabolomics, LC/MS, *Peltigera* lichen, kinetics, inhibition, metabolite

Abstract

The exclusive isolation of potentially plant-pathogenic *Pseudomonas syringae* from *Peltigera* lichens raises questions about the factors influencing the bacterium's apparent host specificity among lichens. To identify potential factors involved, an untargeted metabolomics approach was applied to seven lichen species belonging to three genera (*Cladonia*, *Peltigera*, and *Stereocaulon*). Additionally, we assessed, the growth of *P. syringae* strains in media prepared from extracts from each lichen species. The overall profile exhibited reduced metabolite richness in *Peltigera* compared to other genera. However, chemical investment analysis indicates a higher allocation of resources by *Peltigera*, suggesting a focused production of specific compounds. Growth kinetics analysis indicated comparable *P. syringae* growth across lichen-supplemented medium, with the notable exceptions of *C. arbuscula* versus *Cladonia sp*., with the first showing lower growth rates. An inhibition test using disks soaked in lichen extracts showed no inhibition against *P. syringae*. The average lichen metabolome profile is characterised by a dominance of lipids and organic acids. Furthermore, specific compounds like aminoglycosides positively influence the presence of *P.*

syringae in *Peltigera*, known to inhibit *B. subtilis* and other recognised antagonists. Additionally, compounds absent in *Peltigera*, such as anthracene, may serve as a carbon source for other *P. syringae* inhibitors like *B. velezensis*.

Introduction

Lichens are a widespread and diverse group of cryptogam species formed by a symbiosis between fungi and photosynthetic symbionts (Boustie and Grube, 2005; Feuerer and Hawksworth, 2007). The lichen thallus comprises intertwined fungal hyphae and photosynthetic cells, consisting of either algae, cyanobacteria, or a combination of both. Beyond these constituents, lichens also host internal communities of bacteria (Cardinale et al., 2006; Leiva et al., 2021), fungi (Bates et al., 2012; Spribille et al., 2016), archaea (Bjelland et al., 2011; Garg et al., 2016), and viruses (Eymann et al., 2017). While lichens heavily depend on the atmosphere for water intake, their remarkable resilience in challenging environments is partly attributed to their microbiome, which plays a crucial role in their survival. (Leiva et al., 2021; Pisani et al., 2011)

Given their widespread distribution, lichens can be found in environments with variable climates, including subpolar oceanic zones such as Iceland. Terricolous lichens are a significant constituent of Iceland's vegetation, accounting for approximately 3.64% of vegetation coverage (Ramírez et al., 2023). Some of the most common Icelandic lichens include members of the genera *Peltigera*, *Stereocaulon*, and *Cladonia*. *Stereocaulon* is a cosmopolitan lichen genus comprising approximately 125 fruticose species (Coppins and Smith, 2009), including the three-part symbiotic species *S. alpinum* and *S. vesuvianum* (Torres et al., 2023). *Cladonia* is a genus characterised by a primary crustose or squamulose thallus followed by a secondary fruticose thallus. This genus encloses approximately 475 species (Škaloud et al., 2015), from which *C. arbuscula* and *C. chlorophaea* adhere to a bipartite arrangement involving an algal partner (DePriest, 2004; Pisani et al., 2011). The lichen genus *Peltigera* are typically foliose and exhibit broad lobes. This diverse genus encompasses around 100 lichen species (Stenroos et al., 1994), with examples like *P. leucophlebia* and *P. apthosa* containing a combination of both algae and cyanobacteria as photobionts (Cordeiro et al., 2012), and species such as *P. membranacea* that exclusively harbor cyanobacteria (Miao et al., 1997).

Due to their complex nature, lichens also possess a broad metabolism, drawing considerable interest due to their distinct chemistry and biotechnological prospects. The metabolome of lichens can be divided into primary metabolites such as proteins, lipids, and carbohydrates, which are essential for lichen

metabolism and structure (Huneck et al., 1996), and secondary metabolites. Lichen's secondary metabolites are intricate yet non-essential small molecules that can comprise 0.1% to 30% of the lichen thallus' dry weight (Molnár and Farkas, 2010). Throughout history, the compounds derived from lichens have been thought to be synthesised by the dominant fungal partner. However, recent studies indicate that photobionts and bacteria associated with lichens also contribute to a variety of potentially valuable molecules, and although some of those metabolites may not directly benefit the microorganisms, they are advantageous to the overall lichen (Calcott et al., 2018).

Over 1050 recognised lichen metabolites are catalogued in the Lichen DataBase (LDB). These metabolites have multifaceted functions within the lichen symbiosis and their ecological niche. Some of them serve as allelopathic agents, influencing the growth and survival of neighbouring organisms, thereby giving lichens a competitive advantage (Paukov et al., 2019). Others affect the palatability of lichens to herbivores, acting as a form of defence. Furthermore, certain secondary metabolites enhance the permeability of the cell membranes of phycobionts, the algal partners within lichens, potentially aiding in nutrient exchange and survival in challenging conditions. Additionally, these metabolites serve as shields against excessive ultraviolet (UV)-B radiation, helping to protect the photosynthetic components of lichens from harmful UV rays (Paukov et al., 2019). Moreover, it is noteworthy that a substantial portion of studied lichen species synthesises substances with varying degrees of antimicrobial activity (Romagni and Dayan, 2002), which could have implications for their interactions with other microorganisms in their environment. Overall, these protective compounds collectively contribute to the unique and enduring conditions for lichen existence, resulting in remarkable longevity, with certain lichens living for several millennia (Denton and Karlén, 1973).

Earlier investigations in Icelandic lichens of the genera *Peltigera* indicated a particular prevalence of *P. syringae* as part of their microbiome (Morris et al., 2022; Ramírez et al., 2023). Interestingly, *P. syringae* was isolated consistently from only this lichen genus out of ten evaluated. Furthermore, these bacteria were found in 4 different *Peltigera* species collected. The genetic lines of *P. syringae* discovered in the Icelandic region are remarkably monophyletic, suggesting independent evolutionary trajectories from *P. syringae* populations in other global regions. These monophyletic haplotypes are observed across various phylogroups (PGs) (Morris et al., 2022). Notably, certain *P. syringae* strains associated with PG01 and PG02 underwent pathogenicity and fitness assessments across different plant species, revealing pathogenicity levels comparable to those of epidemic *P. syringae* strains such as DC3000 or CC0094 (Ramírez et al., 2023).

Following the exclusive isolation of *P. syringae* from lichens of the *Peltigera* genus, we became intrigued by the potential distinctions between *Peltigera* and other lichen genera. We initially hypothesised that the lack of *P. syringae* isolated in other lichen species could be due to the higher amount of water in *Peltigera*, which correlates with the observation of a higher culturable bacteria amount (Ramírez et al., 2023). A second hypothesis was posited based on the apparent coincidence between the defence mechanism of the suborder *Peltigerineae* lichen that focused on the production of high quantities of superoxide instead of diversifying their defence in the production of several secondary metabolites as the main part of the lichens (Beckett et al., 2003). Notably, *P. syringae* is not affected by superoxide (Minardi and Mazzucchi, 1988), potentially assisting its colonisation in the *Peltigera* microbiome. A third hypothesis was postulated, taking into account the lichens' chemistry. We hypothesized that a metabolite(s) within *Peltigera* lichens could be enhancing *P. syringae*'s survival or reducing the growth of known *P. syringae* antagonistic bacterial strains such as other *Pseudomonas velezensis* (Rabbee et al., 2019). Conversely, non-*Peltigera* lichen genera may contain metabolites inhibiting *P. syringae* growth or enhancing the population of antagonistic microbes on their thallus, which could reduce *P. syringae* population on the thallus.

In this context, this research aimed to elucidate if the reasons behind the exclusive isolation of *P. syringae* from the lichen genus *Peltigera* were related to the lichens' chemical profile. For this purpose, three different approaches were taken. First, we analysed, the metabolic profiles of lichens of different species from the *Peltigera*, *Cladonia* and *Stereocaulon* genera were obtained by LC/MSMS to evaluate if there were distinctions between the different genera. Afterwards, the growth profile of the different bacterial strains, including *Bacillus velezensis*, in medium supplemented with lichen extract to determine if the growth of the strain was enhanced by the presence of the lichen metabolites. Finally, the antibacterial activity of the lichen extracts was tested against selected *P. syringae* strains isolated from the Icelandic lichens and a control, the *P. syringae* antagonistic bacteria *B. velezensis*.

Experimental Procedures

Sampling lichens

In March 2023, sample collection occurred in the southwestern region, specifically in the snow-free areas of Iceland in that season, including Heiðmörk forest, Öskjuhlíð hill, and the shores of Elliðaá in Árbæjarstífla. Following collection, all samples were cleaned of other organisms attached, such as moss or debris. Promptly after, all lichen thalli were flash-freezed in liquid nitrogen *in situ* and stored at -80°C. A total of 31 samples were collected, representing three distinct genera: *Cladonia, Peltigera,* and *Stereocaulon*, and seven specific species: *C. arbuscula, C. chlorophaea, P. aphthosa, P. leucophlebia, P. membranacea, S. alpinum,* and *S. vesuvianum.* Additional details regarding the sampling process, dates and location are available in Table S1 All specimens underwent morphological analysis, and corresponding vouchers have been deposited at the Icelandic Institute of Natural History.

Tissue preparation

After lyophilisation during 48-72 hours at -52°C in LyoDry Benchtop Freeze Dryer (MechaTech Systems), samples were mashed until reaching a dust-like consistency with the help of a pestle. Samples of the same lichen species were pooled together in equal amounts and stored at -80°C for metabolite extraction as detailed below.

For the metabolite extraction, 80 milligrams of each of the lichen grounds were placed into an Eppendorf tube, and 500 µL of 100% methanol (LC/MS grade, Fisher Scientific UK Limited) were added. The samples were thoroughly mixed using a vortex mixer for one minute and sonicated for 20 minutes at room temperature, with an ultrasound frequency of 37 kHz and a power of 80 W (Fisherbrand, FB15051). After ultrasonication, the samples were centrifuged at room temperature for 10 minutes at 13,000 rpm. And the resulting supernatant was collected. This process was repeated thrice, with fresh solvent added to each round. The obtained crude extract was dried using a vacuum concentrator (Eppendorf® Concentrator Plus) at 45°C. Afterwards, the total chemical investment (hereafter referred to as chemical investment) was calculated as reported by Salazar et al. (2018) measured as the average thallus dry-mass percentage of secondary compounds.

HPLC-MS/MS analysis

Before analysis, the samples were reconstituted to a final concentration of 10 mg ml^{-1} in 50% acetonitrile (ACN, LC/MS grade, Fisher Scientific UK Limited) and filtered through a PTFE 0.22 µm filter (Fisherbrand™). A pooled sample (QC) was created by combining 10 µl from each extract to monitor the analysis performance and correct batch effects (if observed). Similarly, 50% ACN was employed as a blank control and included in the batch alongside the QCs samples, to be analysed once every ten (randomised) lichen samples.

For HPLC-MS/MS analysis, 10 µl of each extract was injected into a Dionex UltiMate 3000 chromatographic system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). This analysis utilised a C18 column (Hypersil GOLD, 100X2.1 mm, particle size: 1.9 µm, Phenomenex, Torrance, USA). The mobile phase consisted of 95% H_2O (LC/MS grade, Fisher Chemical™) with 0.1% formic acid (FA, Op tima™ LC/MS Grade, Fisher Chemical™) as solvent A, and 95% ACN with 0.1% FA as solvent B. The flow rate was set at 250 µl min^{-1}.

The chromatographic program was configured as follows: 1% B from 0-1 min, a gradient increase from 1 to 99% B between 1-40 min, an isocratic phase at 99% B for one minute, followed by a one-minute return to 1% B and a 10 min re-equilibration phase at 1 % B. Data-dependent acquisition of MS/MS spectra was conducted in Electrospray Ionization (ESI) positive and negative modes. ESI parameters included a 40 arbitrary units (AU) sheath gas flow, 10 AU auxiliary gas flow, and 3 AU sweep gas flow. The auxiliary gas temperature was set to 100°C, the spray voltage was set to 3.9 kV in positive mode, and to 3 kV in negative mode. The inlet capillary was heated to 275°C, and the S-lens level was adjusted to 50V.

The MS scan range was set from 100 to 1800 Daltons, with a resolution of 140,000 and one micro-scan. For MS acquisition, the maximum ion injection time was 100ms with an automatic gain control (AGC) target of 1×10^6. Up to five MS/MS spectra per duty cycle were acquired at m/z 200 of 17,500. The maximum ion injection time for MS/MS scans was 50ms with an AGC target of 1×10^5 and a minimum AGC target of 8×10^3.

The MS/MS precursor isolation window was set to m/z 4.0, and the normalised collision energy was stepped from 25% to 35% to 45% with z=1 as the default charge state. Dynamic precursor exclusion was conFigured for 10s. For additional

details regarding the parameters, programs, and analyses, please refer to Table S1.

Data pre-processing and *in silico* compounds annotation.

After the HPLC-MS/MS analysis, raw files underwent conversion into centroid mode (.mzML) using Msconvert (Chambers et al., 2012). Subsequently, Mzmine 3.2.8 was employed for feature finding, chromatogram deconvolution, alignment, gap filling, feature filtering, and ion identity (IIN), as detailed in Table S2 (Schmid et al., 2023). The results were exported in the form of feature abundance tables (.csv), MS/MS spectra files (.mgf), and ion identity networking results (.csv).

To enhance data quality, solvent-related features were removed using the data acquired from the blank samples, and features with a relative coefficient variation exceeding 30% in the QC samples were filtered out. In addition, adducts and in-source fragments identified by the IIN algorithm were eliminated to reduce the presence of overrepresented features, retaining only representative ions for subsequent analysis.

An extra spectral file (.mgf) was exported from MzMine for utilisation in SIRIUS 4.0, facilitating in silico compound classification through CANOPUS (CSI: FingerID; MSI, level 3) (Djoumbou Feunang et al., 2016; Dührkop et al., 2019; Kim et al., 2021). Feature class assignments were annotated using the ClassyFire chemical ontology output and the NPC library for specific cases where the annotation was more accurate and corresponded in chemical composition with the one from ClassyFire. Assignments with a probability exceeding 0.8 were considered valid, while those with a lower probability were categorised as “Unknown”.

Chemical diversity assessment

To analyse the impact of the tested factors, genera and species, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted using the adonis2 function in the ‘vegan’ package (R 4.1.0). This involved assessing Bray-Curtis dissimilarity matrices created for each dataset. NMDS plots were generated using ggplot2 to visualise sample dispersion. Post-hoc pairwise PERMANOVA for each lichen genera was performed, adjusting for multiple comparisons with the pairwise.adonis function and Bonferroni correction.

For alpha diversity analysis of extracts, feature richness and the Simpson diversity index were calculated. Feature richness represented the number of detected features from each genera/species. The inverse Simpson index reflected

the abundance-weighted diversity of metabolites in each sample. Alpha diversity indices differences were evaluated using a one-way ANOVA followed by a Tukey-Kramer HSD post hoc test ($p<0.05$). Spearman correlations between factors and response variables were computed using 'corrplot' with Bonferroni-corrected p-values.

Beta diversity was evaluated through principal component analysis (PCA), Hierarchical Clustering Analysis (HCA), and orthogonal partial least-squares discriminate analyses (OPLS-DA) in Metaboanalyst 6.0. OPLS-DA models were constructed to compare genera and species. Variable Importance in Projection (VIP) features with scores higher than 1.5 were selected as discriminant variables. The relative abundance of discriminant features was evaluated with unpaired t-tests and controlled for false discovery rates at 1%. Two-way ANOVA and Tukey's multiple comparison tests were used to test differences between genera and/or species. PCA and OPLS-DA scores (R^2X, R^2Y, Q^2Y) were employed to assess variance coverage and predictability, while permutation tests (n=999) were run and analysed to ensure the significance, stability, and non-randomness of OPLS-DA models.

Antibacterial activity assay of lichen extracts

A range of bacterial strains were used for antibacterial assays. Three *P. syringae* isolates (EG201428, SU200101, HV200414) were obtained from Icelandic lichens in a previous study (Ramírez et al., 2023), whilst the remaining strains (CC0094, CFBP1392, UB0246 and CC1557) correspond to *P. syringae* type strains from PG2, PG10 and PG13. An antagonist of *P. syringae*, *B. velezensis* (KHWW02) isolated from an ash tree in the UK was used as a non-*Pseudomonas* control. The inhibitory effects of metabolites from 31 lichen samples, grouped by species, against various strain of *P. syringae* and *B. velezensis*, was assessed and interpreted using the Kirby-Bauer disk diffusion assay (Matuschek et al., 2014). In brief, 10 ml of King's B broth (KB; (King et al., 1954)) was inoculated with 100 µl the bacterial strains and incubated for 16 hours at 27 °C and 120 rpm. After incubation, the cell density was adjusted to 0.2 Optical density (OD_{600}, equivalent to 10^8 CFU ml^{-1}), then further diluted 1:100 in 20 ml of KB (King et al., 1954)) agar (0.75% w/v agar) and poured into 90 mm Petri dishes.

The lichen metabolites extracted were dissolved in 50% ACN and 25 µl of each lichen extract was added to a sterile 6 mm paper disk and allowed to dry. Once

the solvent evaporated, three paper disks per extract were placed in each Petri dish and incubated for 24 hours at 27 °C. The diameter of the observed inhibition zones was recorded, and differences in antibacterial activity among treatments were assessed through one-way ANOVA, followed by a Tukey-Kramer HSD test ($p < 0.05$).

Bacterial growth assays

For the bacterial growth assay, lichen metabolites were extracted in 100% methanol, dried and dissolved in 20% methanol. Minimal medium (M9, without carbon source (Sambrook et al., 1989)) was supplemented with metabolites of each lichen with a ratio 20:1 (weight/volume) after being filtered with PTFE 0.22 µm filter. The bacterial strains were cultured overnight in KB medium at 27 °C with shaking. The bacterial suspensions were centrifuged and washed with phosphate-buffered saline three times to remove all KB traces. After the washing, all strains were diluted in M9 without glucose until 0.4 OD_{600}. This concentration was diluted to half when it was mixed with the medium containing each lichen extract in a 96-well plate. The growth assay was conducted in a TECAN SPARK® Multimode Microplate Reader (Tecan Trading AG, Switzerland) at 27 °C, with shaking (500 rpm) for 47 hrs; three replicates of each treatment were done and two per control (M9 and KB). The distribution of the plates was done by the combinations: *P. membranacea- P. apthosa*; *S. vesuvianum- S. alpinum*; *P. leucophlebia- C. chlorophaea* and *C. arbuscula*.

Results and discussion

Peltigera shows higher chemical investment focused on fewer compounds

Peltigera species produces a permissive growth environment to *P. syringae* due to; 1. A more diverse range of metabolites are produced by *Peltigera* supporting *P. syringae* growth; 2. *Peltigera* produces *P. syringae* specific metabolites, which presumably may be absent from *Cladonia* and *Stereocaulon;* 3. *Cladonia* and *Stereocaulon* produce inhibiting chemical(s) that prevents *P. syringae* growth.

To test whether *Peltigera* produces a more diverse range of metabolites untargeted metabolomics was employed to obtain chemical profiles from eight lichen species (three *Peltigera*, three *Cladonia* and two *Stereocaulon*), generating a dataset with 6527 features in positive mode and 1504 in negative mode (Table S5). Multivariate data analysis was applied to statistically assess these features, revealing differences among the studied species. Additionally, metabolite annotation using GNPS and SIRIUS platforms provided insights into specific compound presence. Out of the dataset, only 4.5% and 1.7% (corresponding to 297 and 25 features) were fully annotated in the positive and negative modes, respectively.

The *Peltigera* lichens demonstrated a higher chemical investment (extract weight/lichen dry weight ratio), ranging from 12% to 13%, in contrast to the considerably lower investment observed in the *Cladonia* and *Stereocaulon* lichens (2-4%). However, non-*Peltigera* lichens exhibited higher richness (number of detected metabolites) than *Peltigera* in both polarities (positive – average richness in *Peltigera, Cladonia,* and *Stereocaulon*: 717, 1084.78, 1086.17, respectively; negative – 45.44, 81.72, and 96.75) (Figure 1). A Shannon diversity index test indicated that there was no significant difference between genera in the positive mode, while in the negative mode dataset, there were differences among all genera ($p < 0.05$). In terms of metabolite evenness based on the Simpson index, *Peltigera* samples showed significantly higher levels compared with *Cladonia* and *Stereocaulon* (*Cladonia-Peltigera* p= 0.0002; *Stereocaulon-Peltigera*, p= 2.044×10^{-5}), while *Cladonia-Stereocaulon* comparison indicated a more balance abundance of the compounds isolated from *Peltigera* samples (p= 0.4563). Conversely, in the negative polarity, *Stereocaulon* exhibited higher evenness compared to the other two genera, which did not differ (*Peltigera-Stereocaulon,* p= 1.14×10^{-11}; *Cladonia-Stereocaulon*, p= 2.53×10^{-14}) while there was no significant difference between *Peltigera-Cladonia* (p= 0.4665). This suggests that *Peltigera*'s chemical composition is characterised by a substantial

concentration of a few metabolites, while other lichen species display a more even distribution of a wider variety of metabolites in relatively similar quantities. The substantial allocation of energy and carbon resources towards the production of these lichen secondary metabolites suggests they may have significant physiological and ecological roles (Białońska and Dayan, 2005). Overall, these results suggest that metabolite diversity is not the key factor that allows *P. syringae* to grow differentially in *Peltigera*.

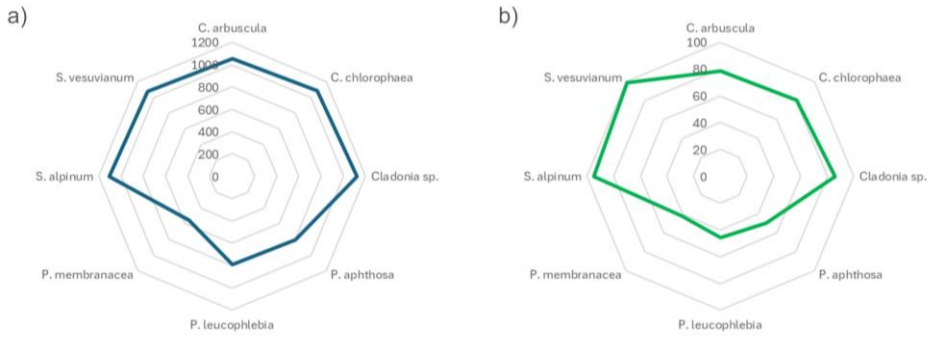

Figure 1. Average richness of metabolites within lichens differs across species. A) Positive Electrospray Ionization (ESI) metabolites and b) Negative ESI metabolites. The blue and green lines show the number of features detected by R.

Since *P. syringae* strains were isolated from all *Peltigera* species sampled, we analysed the *Peltigera* chemical profiles for three species of *Peltigera* to determine their common metabolites that support *P. syringae* colonisation within the *Peltigera* microbiome. *P. membranacea* showed less diverse metabolic profile exhibiting 2060 compounds, while *P. leucophlebia* and *P. aphthosa* displayed 2939 and 3005 compounds, respectively (Figure 1) (Richness difference *P. membranacea- P. leucophlebia*: 5.38×10^{-9}; *P. membranacea- P. aphthosa*: 1.70×10^{-8}, and *P. leucophlebia- P. aphthosa*: 6.30×10^{-1}). Despite this, there is a slightly higher chemical investment in *P. membranacea*, suggesting that this species may concentrate its metabolic production in a smaller number of compounds as described before. No VIP values greater than 1.5 were identified in the negative run. Based on these results, we could determine that in the metabolites present in *P. membranacea* are included the essential those that enhance the presence of *P. syringae* or that all *Peltigera* species analysed in this study lack some metabolite that inhibit the presence of *P. syringae.*

The metabolic profiles allow discrimination between lichen species and genera

Based on the previous observation, we tested whether *Peltigera* produces *P. syringae* specific metabolites that were absent in *Cladonia* and *Stereocaulon*. The chemical profile of the *Peltigera*, *Cladonia* and *Stereocaulon* lichens (Figure 2) varies both across genera (positive mode: PERMANOVA F: 4.0841; p= 1.00 x 10^{-4}, negative mode: PERMANOVA F: 88.1250; p= 1.00 x 10^{-4}) and species (positive mode: PERMANOVA F: 3.1864; p= 1.00 x10^{-4}, negative mode: PERMANOVA F: 335.8548; p= 1.00 x10^{-4}). Clear clustering was observed for both species and genus level. This observation aligns with previous findings that pointed to secondary metabolites as valuable tools in lichen taxonomy and systematics (Phi et al., 2022; Schmid et al., 2023; Schmitt and Lumbsch, 2004). Nevertheless, caution is warranted when attributing differences in metabolites solely to genetic relatedness, environmental factors can lead to the emergence of chemosyndromes in species (Paukov et al., 2019a) or phenomena like homoplasy have been studied in lichens (Nelsen and Gargas, 2008). To determine the main differences between genera, the features detected on each mode (positive or negative) were classified at the superclass and subclass levels using SIRIUS 4 (Figure 3).

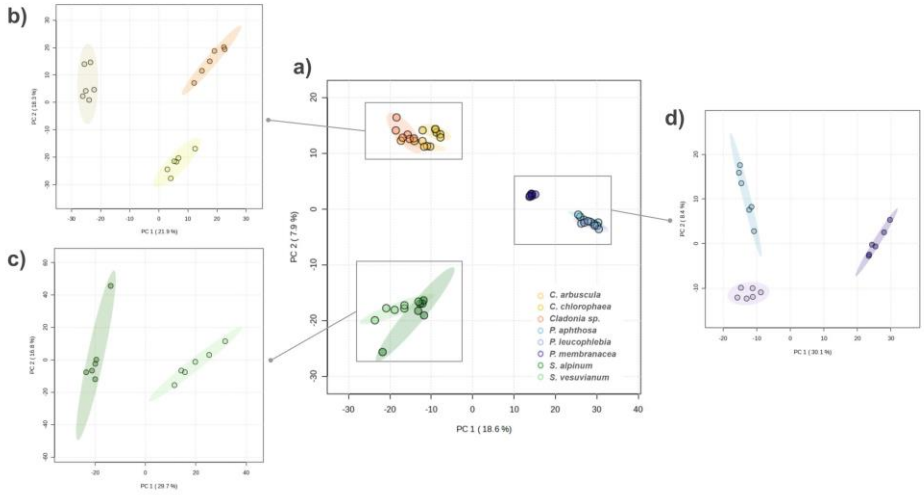

Figure 2. Lichens produce metabolites that cluster by species and by genus. Principal component analysis of the metabolites from ESI positive metabolites isolated from samples utilized in this study a) All samples b) PCA comparing only *Cladonia* samples, c) PCA with a database of only *Stereocaulon* samples, d) PCA comparing only *Peltigera* samples. The image was created by Metaboanalyst.

In the positive mode dataset, lipids (and related compounds) were the more abundant superclass in all the genera aligning with previous studies (Dembitsky, 1992), followed by organoheterocyclic compounds (Figure 3b). *Peltigera* exhibited lower relative abundances of lipids in negative polarity compared to other genera. The role of lipids in lichen has been related to the response and adaptation to environmental factors such as temperature, elevation, light, or high levels of sulphur or radiation (Bychek and Bychek, 1996; Bychek-Guschina et al., 1999; Piervittori et al., 1995; Shapiro et al., 1998). Notably, the lipids subclass diversity was variable among genera. For example, *Peltigera* lichens had a higher abundance of triterpenoids while it produces lower amounts of fatty acids, fatty alcohols, and bile acids (Figure S3.a). Organoheterocyclic compounds form a diverse group, encompassing some highly effective fungicides like captan, iprodione, and vinclozolin (Agrios, 2005), as well as antiviral and antimicrobial agents such as benzimidazole and pyridines. They also exhibit antioxidant and antiherbicidal properties (Kabir and Uzzaman, 2022). Additionally, other common superclass among our samples, phenylpropanoids, have been associated with plant defense against pathogens including *P. syringae* (Dixon et al., 2002; López-Gresa et al., 2011), were less abundant in *Peltigera* and some *Cladonia*.

Conversely, organic acids and benzenoids were the major superclasses in the negative mode dataset (Figure 3b). In the negative mode, we observed small variations among replicates compared with the positive mode (Figure 3). Organic acids showed variations among genera, being higher in *Peltigera* and *Cladonia* (excluding *C. arbuscula*). Among the most abundant subclasses of organic acids in the negative mode dataset, amino acids were much lower in *Peltigera* than in the other two genera while alpha keto acids were not detected in *Peltigera*. Analysis of the benzenoids superclass in the negative mode showed a high percentage in the *Stereocaulon* and *C. arbuscula* metabolic profiles, while they seem to be minority in *Peltigera* especially *P. leucophlebia* (Figure 3b). Previous experiments reveal that plants (and microorganisms) generate and release benzenoids in response to stress, leading to think to scientist that their function may be associated with chemical communication and stress protection (Keen and Taylor, 1975; Misztal et al., 2015). The subclass benzoic acid and the anisoles showed lower relative abundance in *Peltigera* (Figure S3.b).

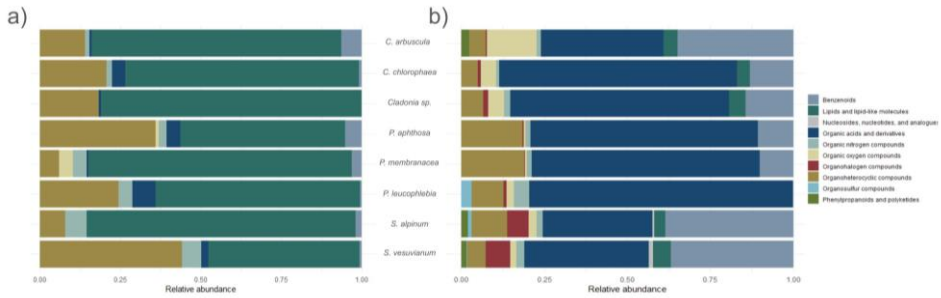

Figure 3. Metabolites grouped by superclass show lipids and organic acids are the most abundant metabolites in the lichens. Identification of compound superclasses from lichens determined by ClassyFier with values expressed in relative abundance. a) Positive polarity b) Negative polarity.

Unique metabolites comparison between to *Peltigera* and non-*Peltigera* genera

To test whether *Stereocaulon* and *Cladonia* produce unique metabolites that might be inhibitors we examined whether there were unique or more abundant metabolites that were present in non-*Peltigera* genera. To do this, we examined the 333 metabolites identified as VIP>1.5 that distinguish *Peltigera* and non-*Peltigera* lichen genera (*Cladonia* and *Stereocaulon*), 28 were found in both *Peltigera* and non-*Peltigera*, 115 were exclusive to *Peltigera*, and the remaining 190 were exclusive to non-*Peltigera* genera. Among the VIPs identified, 77% were lipids, the most prevalent chemical family, followed by organic acids and their derivatives (Table S4).

Several metabolites with known antimicrobial properties have been identified only in *Peltigera*. Aminoglycosides are a diverse group of antibiotics which some of them affect a wide range of bacteria including *P. syringae* and some of their antagonist as *B. subtilis, P. aeruginosa*, *Enterobacter,* and *Serratia sp* (Cameron and Sarojini, 2014; Krause et al., 2016). Among the predominant compounds in *Peltigera* we had pyridinones which has antimicrobial effect against some gram-negative bacteria as *P. aeruginosa* (Duh et al., 1990; El-Sayed et al., 2017). On the other hand, some metabolites with antimicrobial properties were only identified in non-*Peltigera* such as cyclic depsipeptides or azolines which inhibit *P. aeruginosa, B. subtilis, Staphylococcus aureus* among others. In the negative mode the potent antimicrobial usnic acid (Molnár and Farkas, 2010) was almost absent on the *Peltigera* analysed samples. Usnic acid strongly inhibiting some of

the *P. syringae* competitors as *B. subtilis* and *S. aureus* (Maciąg-Dorszyńska et al., 2014). However, usnic acid is not active against gram negative bacteria (Açıkgöz et al., 2013).

On the other hand, some metabolites predominantly present in *Cladonia* and *Stereocaulon* might inhibit the presence of *P. syringae* in their thallus by other methods. This is the case of ubiquinones identified as regulators of basal resistance in plants against *P. syringae* and stress mediated by reactive oxygen species (Dutta et al., 2015). Also, salicylic acid only isolated from non-*Peltigera* genera, has been observed in different plant species after *P. syringae* infection (Brooks et al., 2005; Huang et al., 2003; Rasmussen et al., 1991) suggesting to increase plant resistance (Ribeiro et al., 2022). Choline levels are notably higher in the non-*Peltigera* samples and has been reported on plants enhancing growth and stress protection from plant pathogens including *P. syringae* and *B. subtilis* (Chen et al., 2013). Contrary, we identified compounds only in *Peltigera* as benzimidazoles which are a set of compounds that play a role in *P. syringae*-plant interactions, not inhibiting *P. syringae* growth, but providing protection to the plant (Smaili et al., 2019). Also, triterpenoids mainly found in *Peltigera* are involve in protection of plants against pathogens (Alcerito et al., 2002), which might also be the role in lichens. Prostaglandins absent in *Peltigera* are related with defence mechanisms in microalgae (Di Dato et al., 2017). *Peltigera* exhibited an absence of xanthines in both polarities but was found in the other two genera. The enzyme xanthine oxidoreductase exhibits superoxide-producing activity, effective against several pathogens but not with *P. syringae* (Minardi and Mazzucchi, 1988).

Additionally, the non-detection of anthracene in *Peltigera* (Table S5), used by species like *B. subtilis, B. velezensis*, and *P. aeruginosa* for nutrition, implies a substrate competition that could inhibit *P. syringae's* presence (Ilori and Amund, 2000; Salamat et al., 2018; Sultana et al., 2021; Vargas et al., 2011). Based on the observations of these metabolic analyses, the data suggest that could be specific metabolites supporting *Peltigera* growth of inhibiting in *Stereocaulon* and *Cladonia*.

Testing whether *Peltigera* extract differentially support *P. syringae* growth or whether *Stereocaulon* and *Cladonia* extracts inhibit *P. syringae* growth

One potential reason that *P. syringae* is isolated only from *Peltigera* is that this lichen produced something that other lichen species do not. To test this, growth assays using lichen extracts were conducted on a range of *P. syringae* isolates and compared against growth in rich medium (KB); *B. velezensis* was used as an outgroup control. Given the number of strains and growth substrates being used,

four growth trials were carried out where two lichen species were used in each trial. Growth of strains in each trial were compared as pairs. Overall, growth in KB was generally 3-4 times higher (reaching OD_{600} values of >1.28) than growth in the M9 (reaching OD_{600} values of 0.4111 in M9 + lichen extract; no strains were able to grow in M9 without lichen extract). All *P. syringae* isolates (except CFBP1392 in *Stereocaulon*) were able to grow in M9 supplemented with lichen extracts (Figure 4). Variations in growth were observed between *Cladonia* sp. and *C. arbuscula* extracts, with all strains growing best in the medium supplemented with *Cladonia* sp. (*Cladonia* sp. maximum OD_{600} :0.12-0.2; *C. arbuscula* maximum OD_{600}: 0.005-0.06). Also, bacterial growth comparison among *P. syringae* phylogroups calculated with all samples pooled together showed a statistically higher growth in PG13 compared to PG2 in lichens, an effect not observed in enriched control media (KB), which supports both PG2 and PG13 equally and to a lesser extent, PG10 (Figure S6).

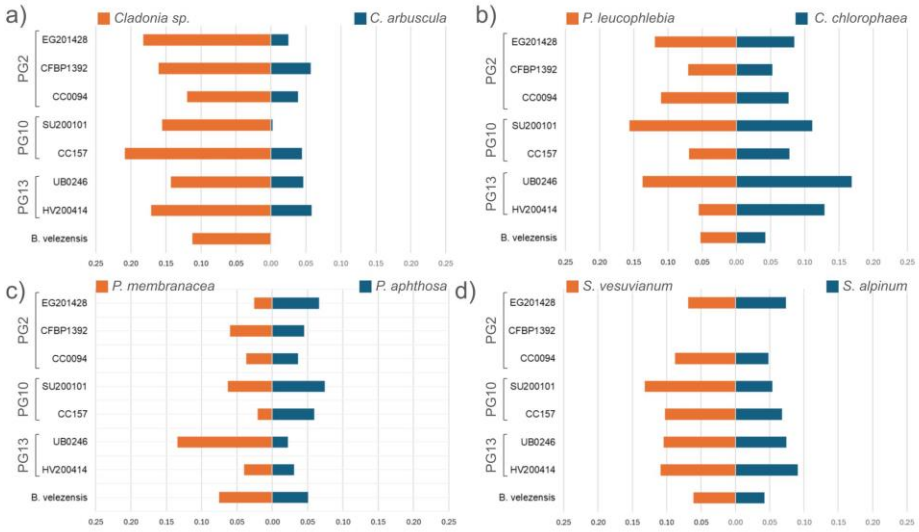

Figure 4. *P. syringae* can utilise lichen extracts for growth. The bacterial culture density (OD_{600}) was observed after 47 hrs incubation in M9 minimal medium containing different lichen extracts. Values are presented by pairs tested in the same trials and the values represent the average of three replicates. a) *C. arbuscula* and *Cladonia sp.*; b) *C. chlorophaea* and *P. leucophlebia*; c) *P. apthosa* and *P. membranacea*; d) *S. alpinum* and *S. vesuvianum.*

One possible reason for why *P. syringae* is able to colonise *Peltigera* is that it can tolerate the lichen environment compared to other lichens. To test this, the antimicrobial properties of lichen extracts from each studied species was used

against different *P. syringae* bacteria isolated from *Peltigera* lichen and other strains isolated from plants of snow from three different countries as well as *B. velezenzis*. Notably, clear halos indicating bacterial growth inhibition were observed only in *B. velezensis* and not *P. syringae* strains (Figure 5). This effect was strongest when using lichen extracts from *Cladonia* and *Stereocaulon* species (Figure 5a), but no inhibitory effect was observed when using the *Peltigera* extract. This indicates the host-specificty effect is unlikely to be due to toxic products being produced by *Cladonia* and *Stereocaulon*. Interestingly, all lichens caused the appearance of a hazy halo for some of the *P. syringae* strains, appearing 'wetter' than the rest of the plate (Figure 5). This possibly indicates the production of a surfactant by the bacterium. This phenomenon previously reported as a result of surfactin production by *P. syringae* (Burch et al., 2010), appears to vary between *P. syringae* strains, with a wider halo observed in PG13, followed by PG10, and finally PG02 (Fig 5b).

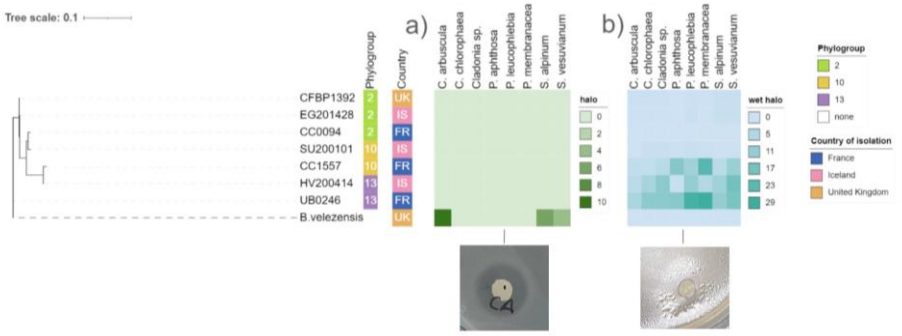

Figure 5. *Cladonia* and *Stereocaulon* inhibit *B. velezensis* but trigger some *P. syringae* to produce a surfactant-like 'wet' halo. Selected *P. syringae* strains from different phylogroups are shown as well as *B. velezensis*. The diameter of halos (in millimetres) is colour-coded a) inhibition halo, b) "wet" halo.

Conclusion

In this study, we explored the metabolic profiles of various lichens belonging to the genera *Cladonia, Stereocaulon,* and *Peltigera*. Our aim was to identify distinctive or commonly occurring metabolites within these lichens, with the intention of understanding how specific metabolites or their absence might contribute to the exclusive isolation of *P. syringae* from *Peltigera*. We highlight the complexity of the lichen metabolic profile being significantly higher in *Cladonia* and *Stereocaulon* than in *Peltigera*. The extensive diversity of

compounds presents a remarkable chemical complexity for organisms interacting with lichens. This could explain why a less diverse compound profile provided by *Peltigera* might be more favorable to *P. syringae.* We should be cautious when interpreting the metabolites identified in different lichens in this study, as the lichens were cleaned but not sterilised, it's possible that metabolites from other organisms present on the lichen surface, rather than within the lichen thallus itself, were also observed. This and other factors such as sampling place, lichen age or different health status of the lichens might explain why two of the samples was considered as *Cladonia sp.* instead of *C. chlorophaea* due to the difference not only in the metabolic profile but also in the kinetic analyses.

Our kinetic and inhibition analysis do not show clear tendencies of *Peltigera* supporting *P. syringae* growth as it would be expected from the isolation described in Ramírez et al. (2023) where no *P. syringae* was isolated from non-*Peltigera* samples. The only exception would be *C. arbuscula* where it was clear the difference of this lichen as nutrient source for the *P. syringae* compare with the rest of the lichen analysed in the study.

While our findings did not pinpoint a specific metabolite as the cause of *P. syringae's* selective behaviour, we identified certain metabolites that could potentially play a role. We hypothesise that this difference might be produced by a combination of metabolites or the loss of some metabolite that might be important during the extraction or other part of the process. Additionally, it raises the possibility that *Peltigera* actively supports the presence of *P. syringae* while the lichen is alive, adding complexity to the study due to the challenge of maintaining lichens in laboratory conditions. Another factor might be the structural differences in the cell wall among lichens that might facilitate *P. syringae* entrance in the thallus. Finally, another reasonable and strong reason might be the higher amount of water in *Peltigera* lichens compared with the other two genera analysed in this study, which might sound very reasonable due to the strong relation of this bacterium with water, being found in different surface water masses.

Acknowledgments

This work was funded by the Rannis Icelandic Research Fund under project number 1908-0151.

Supplementary material

Table S1. Description of the samples analyse with the species that belong, place of isolation, water content, sampling data and symbionts. The missing information is marked with an "X".

Table S2. Parameters used in feature finding, chromatogram deconvolution, alignment, gap filling, feature filtering, and ion identity by MzMine in both ESI

Run	Noise level MS1	Noise level MS2	Retention e (min)	Minimun highest ensity	Minimun intensity of secutive scans
Lichen positive	2.50×10^4	2.50×10^3	0.2	7.00×10^5	1.50×10^5
Lichen negative	5.50×10^3	7.00×10^2	0.15	1.10×10^5	5.70×10^3

Figure S3. Abundance of subclasses within the predominant superclass in each polarity. The abundance of the subclasses was converted to Log10 to enhance the clarity of representation for all compounds.a) "lipid-like molecules" from positive polarity b) organic acid and derivates from negative polarity

Table S4. List of VIP>1.5 *Peltigera* vs non-*Peltigera* comparison. Abundance is expressed in arbitrary units (AU).

Table S5. List of features from all samples with annotations at 60% confidence level or higher from both ESI modes

Figure S6. Bacterial growth of *P. syringae* strains and *B. velezensis* color by PG and with line type by isolation place ("IS"- Iceland or "O" – other). Separate graphs represent different media types, with numbers indicating the trial number.

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Paper IV

pathogens

MDPI

Article

Pseudomonas syringae on Plants in Iceland Has Likely Evolved for Several Million Years Outside the Reach of Processes That Mix This Bacterial Complex across Earth's Temperate Zones

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Abstract: Here we report, for the first time, the occurrence of the bacteria from the species complex *Pseudomonas syringae* in Iceland. We isolated this bacterium from 35 of the 38 samples of angiosperms, moss, ferns and leaf litter collected across the island from five habitat categories (boreal heath, forest, subalpine and glacial scrub, grazed pasture, lava field). The culturable populations of *P. syringae* on these plants varied in size across 6 orders of magnitude, were as dense as 10^7 cfu g^{-1} and were composed of strains in phylogroups 1, 2, 4, 6, 7, 10 and 13. *P. syringae* densities were significantly greatest on monocots compared to those on dicots and mosses and were about two orders of magnitude greater in grazed pastures compared to all other habitats. The phylogenetic diversity of 609 strains of *P. syringae* from Iceland was compared to that of 933 reference strains of *P. syringae* from crops and environmental reservoirs collected from 27 other countries based on a 343 bp sequence of the citrate synthase (*cts*) housekeeping gene. Whereas there were examples of identical *cts* sequences across multiple countries and continents among the reference strains indicating mixing among these countries and continents, the Icelandic strains grouped into monophyletic lineages that were unique compared to all of the reference strains. Based on estimates of the time of divergence of the Icelandic genetic lineages of *P. syringae*, the geological, botanical and land use history of Iceland, and atmospheric circulation patterns, we propose scenarios whereby it would be feasible for *P. syringae* to have evolved outside the reach of processes that tend to mix this bacterial complex across the planet elsewhere.

Keywords: time tree; phyllosphere; microbial ecology; evolutionary history

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1. Introduction

Strains of bacteria in the *Pseudomonas syringae* complex are found in association with a wide range of biological and inert substrates all over the world [1]. A remarkable feature of the *P. syringae* metapopulation is the worldwide ubiquity of certain genetic lines [2], and in particular, quasi-clones at the whole genome level isolated from locations as distant as France and New Zealand [3]. There is considerable evidence to support that this global mixing of *P. syringae* occurs mostly via the water cycle whereby cells of this group of bacteria are swept up into the atmosphere, travel long distances in the free troposphere, and fall back to Earth's surface with rain and snow [1,2,4]. Nevertheless, the prevalence of agriculture in or near the regions from which all known strains of *P. syringae* have been isolated creates doubt about the relative importance of spill-over—from agriculture to natural habitats—by strains that could have been disseminated with exchanges of crop

plant material. For strains in *P. syringae* phylogroups (PG) that have rarely been reported to be associated with cultivated plants, such as those in PG10 and PG13 for example [5], their world-wide prevalence is probably not due to exchanges of plant material but rather due to natural dissemination processes. However, for strains in PG01 and PG02, for example, that are associated with diseases of many cultivated plants [5], exchanges of plant material could contribute to worldwide movement, but to an unknown extent relative to that due to natural dissemination.

Iceland is ideal for exploring the natural biogeography of *P. syringae* given the small fraction of land dedicated to crop production. Only 1% of the total 10^5 km^2 of land surface of Iceland consists of arable land that is farmed whereas vegetation covers about 25% of the total land surface [6]. Average pesticide use in Iceland is about 20 g/ha of cropland due to the low level of insect pests in particular, leading to this country's ranking as one of the lowest consumers of pesticides among 160 countries (https://www.worldometers.info/food-agriculture/pesticides-by-country/; accessed on 23 December 2021). Therefore, the natural vegetation is exposed to little pesticide spillover. The spring and summer seasons are relatively cool and wet with maximum temperatures rarely exceeding about 25 °C. These conditions would be particularly conducive for growth of *P. syringae*.

P. syringae has not yet been reported from Iceland. The natural history of Iceland's biodiversity leaves room for speculation about its presence. Modern natural vegetation of Iceland consists of nearly 500 vascular plant species, most of European origin. The monocots are the most diverse among the vascular plant groups (145 of the 275 species of angiosperms). There are also about 600 species of bryophytes [6]. Although the oldest fossil records of plants in Iceland date back to about 15 million years ago [7], it is believed that nearly all of the modern flora arose from rather recent immigration of plants. Iceland was formed from rifting and the spreading of the sea floor as the East Greenland continental margin of the North American tectonic plate began to move away from the Scandinavian and the British Isles margin of the Eurasian plate about 55 million years ago (YA) [6,8]. In spite of the vegetation that colonized ancient Iceland, it is currently accepted that little of the original flora survived the Last Glacial Maximum [6] that occurred 21,000 YA covering Iceland, Greenland, Canada, the British Isles, and Scandinavia in the northern hemisphere [9]. Much of the modern indigenous plant flora of Iceland is considered to have immigrated to the island at the time of the late Weichselian deglaciation (at about 10,000 YA) [7] after the Last Glacial Maximum. Interestingly, 97% of the vascular plants native to Iceland have also been recorded in Norway and about 86% in the British Isles; 64% are also found in Greenland [6]. Precursors of this vegetation are likely to have been dispersed with drift ice, water currents and birds from European and Eurasian origins [6]. The contribution of coastal flora from Norwegian fjords is likely to have occurred around 10,000 YA during the glacial melt that was rapid with a massive input of freshwater and sediments into the ocean. This is believed to have created a gyre of water with reduced salinity that fostered spread of biota from southwestern Norway to northern Atlantic islands [10]. It seems likely that microbes would have accompanied these migrations. In contrast to the potential for ocean currents to disseminate plants carrying *P. syringae* and other microorganisms, aerial dissemination to the island might be more difficult. Over the past 8000 years, at least, Iceland has often been beyond the main flow of the jet stream [11]. The jet stream can move northward to 65°N latitude crossing the southern half of the island depending on the behavior of the North Atlantic Oscillation, whereas the northern half is mostly crossed by Arctic winds [11]. This suggests that much of Iceland could be rather isolated from the main processes of natural long distance migration of terrestrial organisms.

The objective of this work was to determine if strains in the *P. syringae* complex are indeed present on vegetation in Iceland and if so, to assess their abundance, their affinity for different plant types and habitats, and their diversity relative to that of *P. syringae* worldwide.

2. Results

2.1. P. syringae Is Ubiquitous and Abundant on Vegetation across Iceland

Strains in the *P. syringae* complex were detected on 35 of the 38 samples collected at densities that ranged over six orders of magnitude from about 50 to 10^7 cfu g^{-1}. None of the plants showed any apparent disease symptoms. The analytical methods used could not distinguish if the isolated bacteria were on or in plant tissues. The total culturable mesophilic bacterial populations on these same plants ranged over ca. 3.5 orders of magnitude from 10^5 to more than 10^8 cfu g^{-1} (Table 1). Among the different plant types, *P. syringae* densities were significantly greatest on monocots compared to those on dicots and mosses ($p < 0.05$ for all significant comparisons) but there were no significant differences between these latter two plant types (Figure 1A). Densities of total mesophilic bacteria showed the same significant trends as those of *P. syringae* among the habitat types and plant types. Ferns, leaf litter and lava fields were each represented by only one sample therefore precluding statistical comparisons. There were significant effects of type of habitat from which samples were collected or whether the plant tissue was from monocots, dicots or moss on population densities of *P. syringae* and total culturable mesophilic bacteria (ANOVA, $p < 0.05$). According to Tukey's HSD test, population densities of *P. syringae* were significantly greater in grazed pastures compared to all other habitats ($p < 0.012$ for all significant differences) whereas densities in the other habitats were not significantly different from each other (Figure 1B).

2.2. P. syringae in Iceland Is Genetically Diverse but Distinct from P. syringae Elsewhere

Assessment of the genetic diversity of strains was based on 343 bp of the citrate synthase (*cts*) gene. This segment of the *cts* gene aligned for a set of 609 *P. syringae* strains from Iceland and 933 strains of *P. syringae* from crops and environmental reservoirs collected from 27 countries on 8 continents or distinct geographical regions in North, South and Central America, Europe, UK, the Indian subcontinent, Africa, Asia, Australia and New Zealand. The *cts* sequences for the reference strains was obtained from our previous work [5] or from online sources (including https://www.ncbi.nlm.nih.gov/; accessed on 1 January 2020). This resulted in 259 haplotypes (i.e., unique *cts* sequences for the 343 bp) for the reference strains and 70 haplotypes for the Icelandic strains as listed in Supplementary Tables S1 and S2.

The haplotypes of *P. syringae* from Iceland were interspersed phylogenetically among the previously described diversity of this group of bacteria and represented PGs 1, 2, 4, 6, 7, 10, and 13 (Figure 2). Among the 70 haplotypes of strains from Iceland, none of them contained reference strains from outside of Iceland, illustrating an extreme extent of geographic isolation compared to the reference strains. In contrast, for reference strains there were 88 haplotypes containing more than one strain for which we assessed their geographic distribution. Of these haplotypes, 37 had strains from two or more continents representing 64% of the strains for these 88 haplotypes. The most widely dispersed case was one haplotype that contained strains from six continents or distinctly separated regions—Africa, Asia, the UK, North and Central America, continental Europe and Australia and New Zealand (Supplementary Figure S2A).

The distinction of the lineages did not seem to be related to their specialization to a type of plant or habitat. Many of the haplotypes were grouped into monophyletic lineages, of which seven lineages contained from 3 to 16 haplotypes (Figure 2). The largest of these lineages illustrated that diversification of these genetic groups of *P. syringae* were not specific to any plant type or habitat. For example, strains in lineage L02.1, that contained 12 haplotypes, were isolated from monocots, dicots and moss in forests and grazed pastures. Likewise, strains in lineage L10.8, that contained 16 haplotypes, were isolated from monocots, dicots, moss and fern in all of the four habitat categories (Supplementary Figure S2B). Furthermore, strains from nine haplotypes across all of the lineages were isolated from at least two plant types.

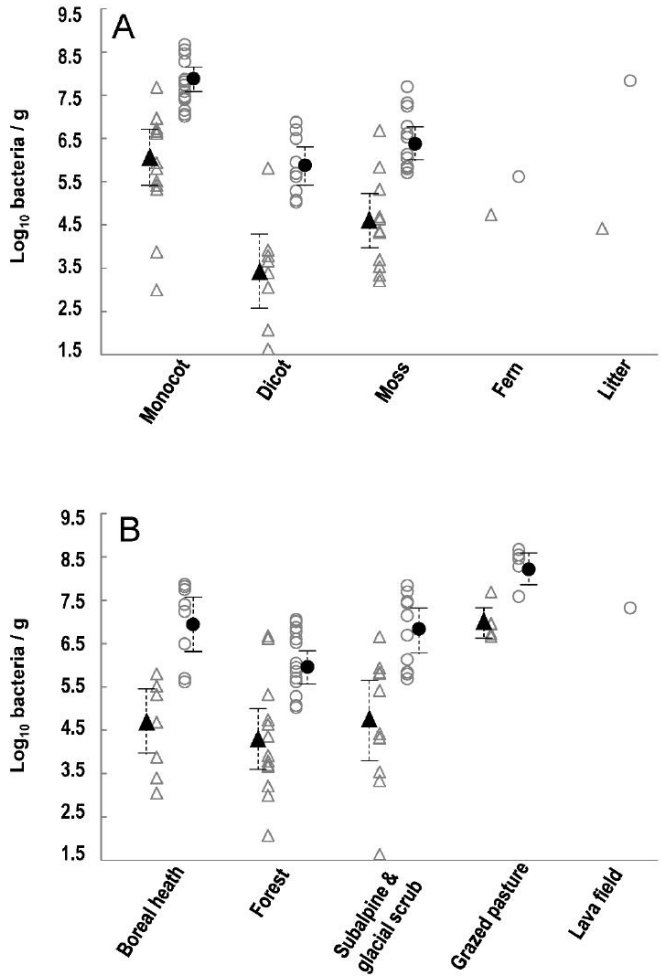

Figure 1. Population densities of *Pseudomonas syringae* (triangles) and of total mesophilic bacteria (circles) on plants collected across Iceland. Population sizes are presented according to (**A**) the type of plant and (**B**) the general habitat classification of the sampling site. Open symbols represent each of the 35 individual samples where population densities were greater than the detection level (ca. 40 cfu g^{-1}). Closed symbols indicate the mean population densities and the dotted lines indicate the 95% confidence intervals.

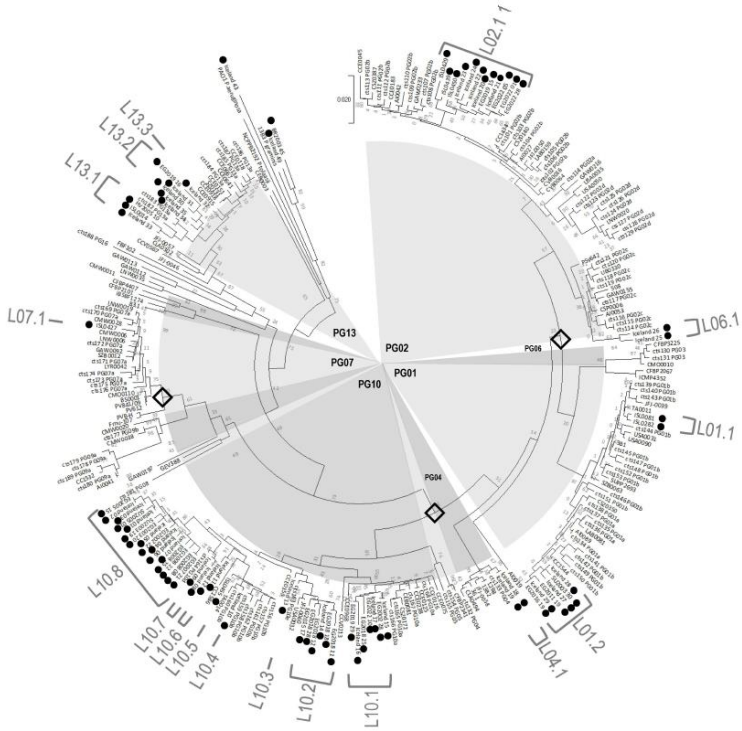

Figure 2. Phylogenetic situation of 329 haplotypes of *Pseudomonas syringae* representing 609 strains from Icelandic vegetation and 933 reference strains from 27 countries and a dozen types of substrates (crop plants, wild plants and various environmental substrates). The Neighbor-joining tree was constructed from a 343 bp sequence of the citrate synthase gene for one representative of each of the 329 haplotypes in the database based on 1000 bootstrap replications. The scale bar represents the p-distance. Haplotypes from Icelandic strains are indicated by black dots. Three additional Icelandic haplotypes and two reference haplotypes were included as outliers to root the tree. None of the Icelandic haplotypes included reference strains. Lineages (L) of Icelandic strains are denoted by grey bars and labels according to their phylogroup. Black diamonds on the tree branches indicate where time constraints were set to estimate the time of divergence of the Icelandic lineages (the divergence of the canonical group of *P. syringae* (PGs 1, 2, 3, 4 and 6) from the other phylogroups; the divergence of PG01 from PG02; and the divergence of PG07).

We also evaluated if the genetic isolation of *P. syringae* in Iceland was due in part to limited mixing and dissemination of populations of this bacterium across the island. To accomplish this, we fitted a linear regression between mean genetic diversity and geographic distance for 531 pairwise comparisons among the 34 sites where more than one strain was isolated (Figure 3). This resulted in an R^2 value for the regression between these two parameters of 0.014, indicating that only 1.4% of the variability in the difference in genetic diversity between sites is explained by geographic distance thus corroborating the hypothesis of mixing across the island. On the other hand, of the 70 haplotypes of *P. syringae* on Icelandic vegetation, 51 were found on only a single sample. Nevertheless, the most ubiquitous haplotype was in PG02 and was found on nine samples collected

at diametrically opposed locations on the east and west coasts. Likewise, a haplotype of PG13 was detected on eight samples with a geographic distribution similar to that of the ubiquitous haplotype of PG02.

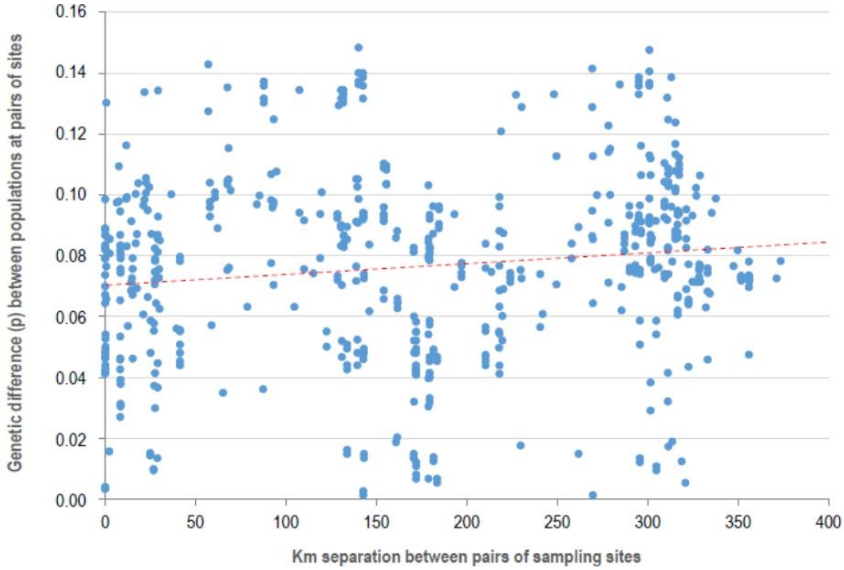

Figure 3. Effect of the geographic distance between sampling sites in Iceland on the genetic difference of the populations of *Pseudomonas syringae* they harbored. The genetic difference between sites was expressed as the percent dissimilarity of the 343 bp sequence of the citrate synthase gene used for phylogenetic analyses. The dotted line represents the regression curve: Genetic distance = 0.07134 + 0.00003123 × km.

2.3. The Size, Composition and Co-Occurrence of Certain Phylogroups of P. syringae Populations on Vegetation Depend on Habitat and Plant Type

For the samples in which *P. syringae* was detected, PGs 1, 2, 10 and 13 were detected most frequently: on plant types that were sampled repeatedly (33 samples of angiosperms and moss), strains in PG10 were the most frequently detected (on 82% of the samples) followed by PG13 (36%) and PG01 and PG02 (each at 27%). PGs 4, 6 and 7 were detected in fewer than 6% of these samples. There was co-occurrence of different phylogroups in the same sample for about 60% of the samples, but, interestingly, PG01 and PG02 were the only phylogroups that did not occur together in any of the samples (Figure 4).

Vegetation type (monocots, dicots or moss) had an effect on the abundance of certain phylogroups of *P. syringae*. Whereas monocots tended to harbor larger populations than dicots and mosses for most phylogroups (Figure 5A), populations of PG01 were virtually absent on monocots (detected on only one of 14 samples). Furthermore, PG10 had the largest populations of all phylogroups consistently for monocot, dicots and ferns (Figure 5A). On the one sample of fern collected (not shown in Figure 5), PG10 constituted 100% of the 5.4×10^4 cells of *P. syringae* detected. However, the differences in population size of the phylogroups among plant types was statistically significant only for PG13 (ANOVA, $p = 0.017$).

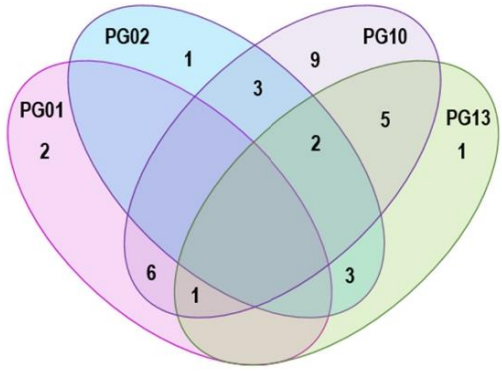

Figure 4. Venn diagram of the co-occurrence of strains of *Pseudomonas syringae* in phylogroups (PG) 1, 2, 10 and 13 in 33 samples of monocots, dicots and moss from Iceland. Integers represent the number of samples with co-occurrence of these phylogroups.

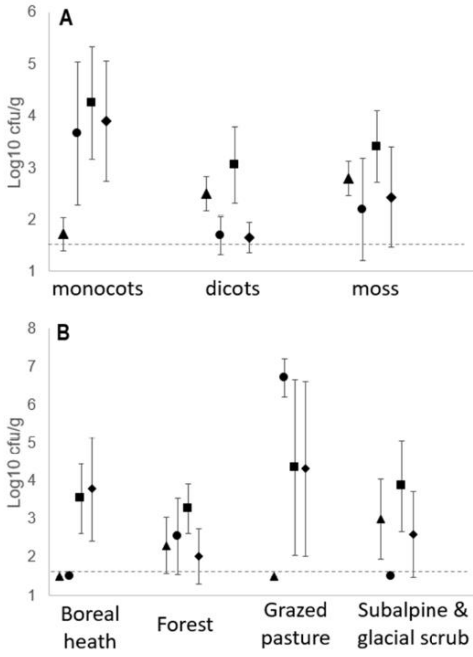

Figure 5. Population densities of the four phylogroups of of *Pseudomonas syringae* most frequently detected on vegetation in Iceland for different types of plants (**A**) and in different habitats (**B**). Mean population densities of PG01 (triangle), PG02 (circles), PG10 (squares) and PG13 (diamonds) were calculated based on results for all samples in each category. Values for samples with population sizes under the detection level were set to just below the detection limit ($10^{1.5}$) rather than excluding the sample from the calculation. Error bars represent the 95% confidence interval. The dotted line represents the limit of detection of *P. syringae* in these samples.

Across the different habitats sampled, only PG10 and PG13 were consistently present in all habitats. Neither PG01 nor PG02 were detected in any of the seven Boreal heath samples. In addition, PG01 was not detected in the five grazed pasture samples, and PG02 was not detected in any of the ten samples from subalpine and glacial scrub. Among all phylogroups, PG02 was the only one to be significantly influenced by habitat, having the significantly greatest abundance in samples from grazed pastures.

2.4. Haplotypes of P. syringae on Icelandic Vegetation Diverged near the Time of the First Fossil Evidence of Plants on Iceland as Well as Very Recently

The estimated time of divergence of the 70 *cts* haplotypes of *P. syringae* from Icelandic vegetation ranged from 16 million years ago to too recent to calculate (Figure 6). The time of divergence of all haplotypes in PGs 1, 2, 4 and 7 was estimated to have occurred at the time of, or after, the Last Glacial Maximum. This estimate is compatible with a process of colonization and evolution driven by the modern flora after the period of deglaciation. In contrast, the estimated time of divergence of all haplotypes of PGs 6 and 13 and most of PG10 are markedly older than the last glacial maximum.

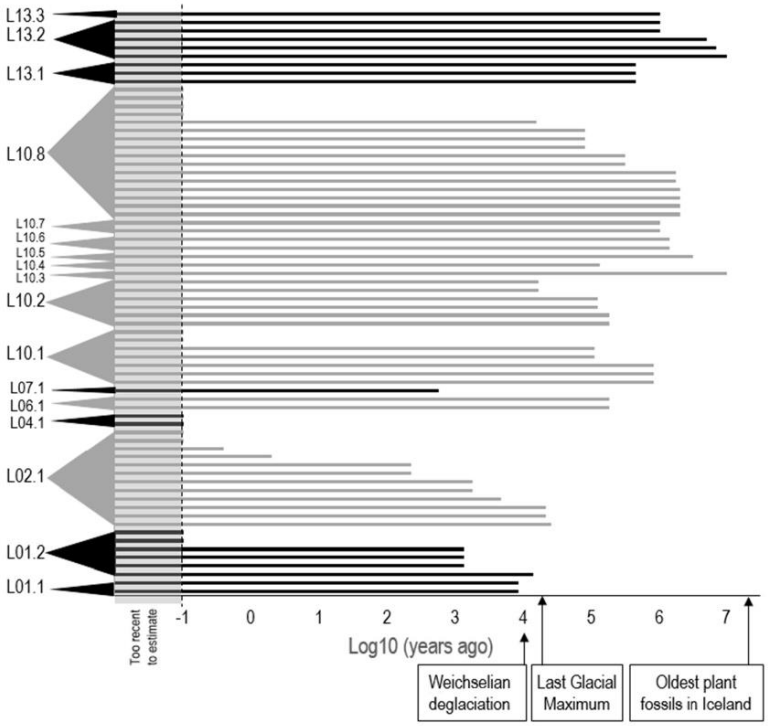

Figure 6. Estimated time of divergence of the 70 haplotypes of *Pseudomonas syringae* isolated from plants in Iceland. Each bar represents a haplotype. Haplotypes are grouped according to their lineages (L) in phylogroups 1 to 13 as indicated in Figure 2. Times of divergence, presented on a log scale, ranged from 16 million years ago to too recent to estimate (less than the value indicated by the dotted line). Each successive phylogroup is presented by either black or grey bars to help the reader differentiate among adjacent phylogroups in the figure.

3. Discussion

Understanding how plant pathogens evolve is essential for anticipating disease emergence and the durability of resistant plant varieties. Overwhelmingly, knowledge of the evolution of plant pathogens has been founded mostly on studies of the specific crop-pathogen interactions of interest in a context of modern agricultural practices and settings. Such studies provide only partial information, ignoring the consequences of stresses and selective pressures that plant pathogens could endure in reservoirs and natural habitats. This biases our understanding of evolutionary processes that microorganisms with pathogenic capacities endure. The lack of insight on stresses and selective pressures other than in agricultural contexts inevitably biases the importance accorded to agriculture in the evolution of plant pathogens. Over the past several decades there have been efforts to disentangle anthropogenic forces from non-anthropogenic forces in the evolution and emergence of zoonotic pathogens and parasites [12–14] leading to the establishment of comprehensive surveillance systems under the concept of One Health [15]. However, for plant pathogens it is legitimate to wonder whether it is possible to find contexts in which to study the influence of non-agricultural habitats on pathogen evolution. The preponderance of crops in the planetary landscape and the influence of long distance dissemination that can move microorganisms between natural and agricultural contexts make it difficult to find such contexts. In a previous study we determined that more than half of the haplotypes of *P. syringae* in headwaters of rivers were endemic to the site where they were isolated (in either France, the US or New Zealand) [2]. However, the possible influence of agriculture and aerial dissemination on those headwater sites make it difficult to assess if these haplotypes diverged mostly under the influence of their aquatic habitat or if they were refugees from agriculture.

Our results illustrate that the geographical, geological and ecological contexts of Iceland create a remarkable situation where we can assess the influence of natural landscapes on the diversification of a group of bacteria that contain plant pathogens. We have shown that monocots, dicots, moss and ferns across four habitat types (boreal heath, forests, grazed pastures, subalpine and glacial scrub) all harbor *P. syringae*, but that grazed pastures in particular have the greatest influence on the fitness of *P. syringae*. Some of the pastures were sown monocultures but other were natural mixed stands. Strains in PG01 and PG02 occurred only in two of the four habitats, their frequencies of occurrence have opposing trends on monocots and dicots, and they seemed to exclude each other in the samples we analyzed. Interestingly, these two groups are represented by important plant pathogens in the overall metapopulation of *P. syringae* [5]. In contrast, strains in PG10 and PG13—phylogroups for which there are no reports of them causing plant disease outbreaks-occurred with roughly equal frequency and abundance across all the habitat types and plant types we examined. This suggests a greater adaptability of phylogroups 10 and 13 compared to PG01 and PG02. Nevertheless, as we noted for populations of *P. syringae* in headwaters in France, the US and New Zealand [2], we observed in Iceland that PG02 has the capacity to generate haplotypes that are more ubiquitous than haplotypes of other phylogroups. In other words, although representatives of PG02 are not necessarily the most frequently encountered in the environment compared to PG10 and PG13, when the occurrence of individual haplotypes are noted, those of PG02 are the more ubiquitous than those from other phylogroups. This suggests that the pangenomic traits that assure adaptability tend to be packed into individual haplotypes of PG02 more so than in the other phylogroups where these traits might tend to be distributed across individual haplotypes. This hypothesis could be explored by searching for particular genomic features of ubiquitous PG02 haplotypes compared to haplotypes that are endemic to various geographic locations.

We believe that it is possible to attribute the trends described above mostly to processes that do not directly involve crop cultivation. This is because cultivation of pastures and other crops is very recent on the evolutionary scale of *P. syringae* and a very small fraction of land is used for cultivation in Iceland. Furthermore, the composition of *P. syringae* populations in Iceland is apparently separate from the influence of immigrants from

regions of the world where crop cultivation is much more predominant. By comparing Icelandic strains with a comprehensive data set of *P. syringae* strains from 27 countries and a dozen types of habitats, we revealed the uniqueness of Icelandic strains and the unlikelihood that some of them had emigrated recently from other cultivated regions of the globe. Indeed, this result is based on the data set of reference strains that we established. This data set is very comprehensive in terms of geographic and environmental origins of strains (Supplementary Table S1, Supplementary Figure S3A). Both data sets, for Icelandic and for reference strains, illustrate that for many of the haplotypes and genetic lineages of *P. syringae*, their corresponding strains are found in several habitats or geographic locations and in association with several types of substrates. This observation argues against the notion that Icelandic strains are specialized to a given habitat or plant type and it gives additional credence to our conclusion that there is an impediment to the long distance dissemination of *P. syringae* toward Iceland. Nevertheless, future samples that can expand on this diversity might lead to nuances in the interpretation of the results. In particular, our data set of reference strains does not contain any samples from latitudes greater than 64° whereas all of the Icelandic strains are from sites situated farther north than 64° which seems to be the limit of the reach of the jet stream in the Northern Hemisphere [11]. The geographic range of many of the plant species we sampled here, including *Alchemilla alpina*, *Empetrum nigrum*, *Vaccinium uliginosum*, and the mosses *Hylocomium splendens* and *Rhytidiadelphus loreus*, for example, includes expanses of North American, northern Europe and Greenland north of 64°. Assessing the diversity of *P. syringae* from these plants at different locations could contribute to validating the uniqueness of the Icelandic strains. That Iceland is an island separated by at least 300 km of ocean from the nearest neighboring land mass might exaggerate phenomena that could be occurring elsewhere at high latitudes. Experiments to capture *P. syringae* in rain from offshore winds along the eastern coast of Iceland, from where most rain events arrive, could further corroborate the limited access of Iceland via the atmosphere. Notwithstanding, the apparent seclusion of Icelandic populations of *P. syringae* is coherent with knowledge of the range of atmospheric circulation [11] that would have limited dissemination to the island from other continents where *P. syringae* is abundant. In this light we propose that the diversity of *P. syringae* from vegetation in Iceland is, in large part, a reflection of evolutionary processes occurring in habitats with very little or no crop cultivation. However, grazing—that involves regular wounding to plants (mostly grasses) and input of nitrogen-laden fecal matter by herbivores—enhances the fitness of most phylogroups of *P. syringae* with the notable exception of PG01. Grazing of pastures is a practice that started in Iceland in about the 900′s with the introduction of sheep, before which there were no herbivorous mammals on the island. A unique aspect of cold environments is that mosses, in addition to grasses, can also be grazed—by geese, sheep and reindeer [16]. Iceland presents the unique opportunity to determine if grazing of mosses has the same impact on populations of *P. syringae* as does grazing of grasses, and to evaluate the dynamics of this impact under the grass-moss-herbivore feedback that is forecast in a warming climate [17].

By estimating the time of divergence of the unique Icelandic lineages of *P. syringae*, we can propose a hypothetical scenario of the origin and evolution of *P. syringae* in Iceland in the context of the geological and botanical history of the island. The precision of our estimates of age could be improved if longer sequences (such as concatenated housekeeping genes or whole core genomes) were available. Nevertheless, the estimates we have made here allow us to propose a scenario that has not been told previously for a plant pathogen. The ages of the Icelandic lineages of PG01, PG02 and PG07 are compatible with the hypothesis that progenitors of these lineages arrived with plants that invaded and colonized Iceland just after the Last Glacial Maximum leading to today's modern flora of the island mostly from Norway and partially from the northern reaches of present-day Scotland [6]. These immigrant lineages apparently diversified, thereby giving rise to the very young endemic lineages we detected for PG01 and PG02 (Figure 6). We suspect that a similar scenario of immigration after the Last Glacial Maximum, and then subsequent diversification of

lineages, occurred for PG04 but that the older lineages were too rare to detect in our sampling or have died out.

In contrast to PGs 1, 2 and 7, the ages of the lineages of PG10 and PG13 suggest that they were present before, and survived, the Last Glacial Maximum. This is indeed what is believed to have occurred for certain microscopic freshwater crustaceans that have evolved unique genetic lines endemic to Iceland; they probably survived glaciation under the ice sheet [18]. There is evidence that bacteria could have survived glaciation in the ice sheet and/or in subglacial lakes. Concerning subglacial lakes, their temperature and chemical conditions can be very similar to mountain lakes and headwaters that harbor *P. syringae* [2]. The pH, temperature and conductivity of the subglacial lake in the Grímsvötn volcanic caldera in Iceland, for example, are within the range of those in freshwaters where *P. syringae* has been frequently detected [19]. The Grímsvötn volcanic caldera lake and its sediments harbor culturable, aerobic, psychrophilic bacteria at concentrations of ca. 6×10^6 L^{-1} and 8×10^6 g^{-1}, respectively [19]. Bacterial populations in the water and sediments of this subglacial lake consist mostly of beta- and gamma-Proteobacteria with *Pseudomonas* spp. detected in the sediments [19]. To have survived within ice during the Last Glacial Maximum, *P. syringae* would have needed to endure, at most, the period that started about 33,000 YA when the ice sheet covering the North Atlantic started growing [20] to about 10,000 YA when rapid deglaciation occurred [7], i.e., a period of roughly 20,000 years. Although there is no direct evidence that *P. syringae* could survive this duration, diverse viable bacteria have been isolated from cores of 20,000 year-old ice [21]. Furthermore, metabolic activity has been demonstrated for bacteria in ice from Lake Vostoc that is 110,000 to 240,000 years old [22]. These observations illustrate the capacity of bacteria to survive for tens to hundreds of thousands of years in ice and they are the basis for concern about the diversity of microorganisms that are being liberated from melting permafrost and glaciers [23]. For *P. syringae*, its capacity to survive under annual snow pack near the snow-ground interface and its subsequent percolation into karsts and rivers upon melting of the snow in the spring have been demonstrated [24,25], but direct observations for longer periods have not been reported. Whatever the processes by which PG10 and PG13 survived the Last Glacial Maximum, the age of their lineages in Iceland suggest that they colonized the island via the same processes that brought the original flora to Iceland. For PG10, we observed that these original lineages diversified to give rise to the younger lineages we observed. For PG13, our samples did not lead us to observe subsequent diversification if it has occurred.

If *P. syringae* has had the opportunity to evolve in relative seclusion from the rest of the metapopulation of this bacterial complex elsewhere on Earth, it is tempting to wonder if other plant-associated microorganisms typically disseminated via the atmosphere have also evolved in similar seclusion. Comparative ecology between *P. syringae* and other broad-host-range, aerially disseminated pathogens, such as *Botrytis cinerea*, has led to elucidation of general ecological concepts applicable to numerous organisms and to the particularities of each organism [26,27]. Icelandic landscapes provide a unique opportunity to pursue comparative ecological studies on the relative impacts of agricultural and nonagricultural contexts on the diversification of plant pathogens.

4. Materials and Methods

4.1. Samples and Sampling Sites

In the summer months of 2018, 2019 and 2020, 38 samples of plants (angiosperms, moss and ferns) (Table 1) were collected from 32 sites in the Western Northwestern, Northeastern and Eastern Regions of Iceland (i.e., all regions except the Westfjords, Reykjanes and the Southern Region) (Figure 7). The sampling sites were classed into habitat categories (boreal heath, forest, subalpine and glacial scrub, grazed pasture, lava field) based on classifications of the Icelandic Institute of Natural History (https://vistgerdakort.ni.is/; accessed on 29 June 2021), the European Nature Information System classifications for

Iceland (https://en.ni.is/flora-funga/habitat-types/terrestrial-habitat-types; accessed on 29 June 2021) and on ground-based observations at each of the sampling sites.

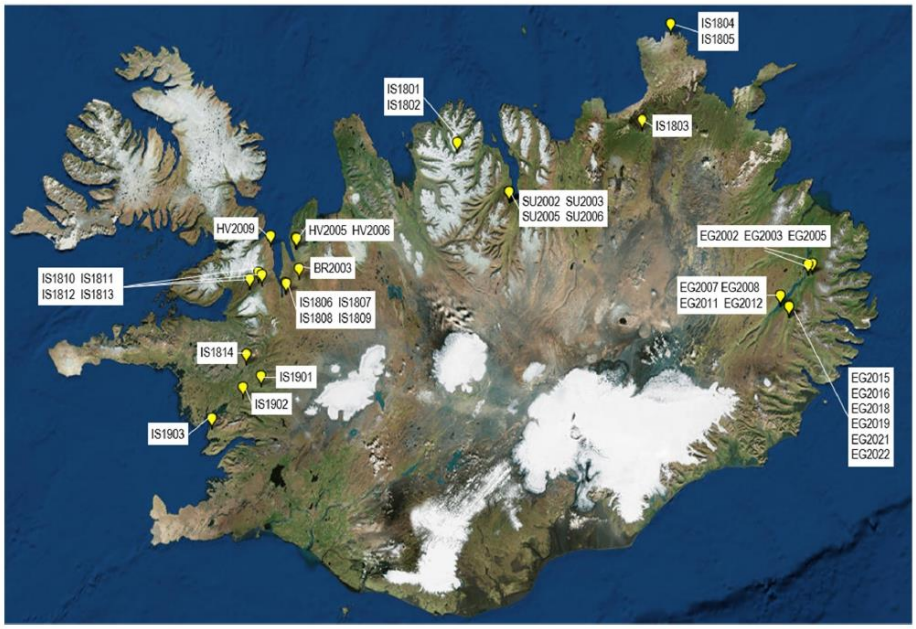

Figure 7. Location of the 38 sites where vegetation was sampled in 2018, 2019 and 2020 across Iceland. Codes for each site are the sample names indicated in Table 1. The map was made with tools at GPSVisualizer.com, accessed on 29 June 2021.

Plant tissue was collected aseptically into clean, single-use plastic bags. Most plants were from natural stands of mixed species. For each sample, a quadrat of about 10 × 10 cm was delimited and all plants of a given species or type (dicot, monocot or moss) in the quadrat were collected separately. Single species were sampled separately where they could be identified, as was the case for dicots. Otherwise, plants in the same quadrat were bulked by recognizable groups such as monocots, dicots or moss. Samples consisted of a bulk of the plants sampled from three, randomly placed quadrats per each geographic site. They were stored in a cooler while transported to the laboratory.

Table 1. Population densities of *Pseudomonas syringae* and total culturable mesophilic bacteria on plant samples collected across Iceland. The locations where samples were collected are indicated in Figure 7 according to the sample names.

Sample Name	Date	Latitude °N	Longitude °W	Habitat Type	Category of Plant Sample	Identity of Sampled Material	$\log_{10}$ cfu *P. syringae*/g Tissue	$\log_{10}$ cfu Total Bacteria/g Tissue
BR2003	Augustust 2020	65.239038	−20.851623	Forest	monocot	mixed grasses	6.62	7.01
EG2002	September 2020	65.262654	−14.378452	Forest	moss	*Hylocomium splendens*	3.70	6.05
EG2003	August 2020	65.262654	−14.378453	Forest	monocot	*Festuca* sp.	2.99	7.06
EG2005	September 2020	65.267478	−14.331496	Forest	dicot	*Empetrum nigrum*	2.07	5.07
EG2007	September 2020	65.096122	−14.732982	Forest	moss	*Hylocomium splendens*	3.21	5.86
EG2008	September 2020	65.096122	−14.732983	Forest	fern	*Equisetum pratense*	4.74	5.62
EG2011	September 2020	65.094289	−14.734456	Forest	dicot	*Rubus saxatilis*	3.79	5.28
EG2012	September 2020	65.094289	−14.734457	Forest	moss	*Rhytidiadelphus loreus*	5.32	6.61
EG2015	August 2020	65.036883	−14.620112	Forest	dicot	*Salix arctica*	3.67	5.95
EG2016	September 2020	65.036883	−14.620113	Forest	moss	*Rhytidiadelphus squarrosus*	4.36	6.78
EG2018	September 2020	65.036937	−14.620176	Forest	dicot	*Vaccinium uliginosum*	3.92	5.03
EG2019	September 2020	65.036937	−14.620177	Forest	moss	*Rhytidiadelphus loreus*	4.63	6.52
EG2021	September 2020	65.036937	−14.620176	Forest	dicot	*Alchemilla alpina*	nd[1]	6.87
EG2022	September 2020	65.036937	−14.620177	Forest	moss	*Hylocomium splendens*	6.68	5.70
HV2005	August 2020	65.402968	−20.896696	Boreal heath	dicot	*Alchemilla alpina*	3.06	6.50
HV2006	August 2020	65.400877	−20.884433	Boreal heath	dicot	*Alchemilla alpina*	nd	5.62
HV2009	August 2020	65.411194	−21.215754	Boreal heath	dicot	*Salix herbacea*	3.40	5.69
IS1801	June 2018	65.904903	−18.838611	Subalpine & glacial scrub	monocot	sparse mixed grass	5.94	7.43
IS1802	June 2018	65.925386	−18.823611	Subalpine & glacial scrub	monocot	sparse mixed grass	6.66	7.47
IS1803	June 2018	66.024216	−16.493893	Subalpine & glacial scrub	litter	litter on forest floor	4.42	7.84
IS1804	June 2018	66.5145298	−16.132639	Subalpine & glacial scrub	monocot	mixed grasses and mosses	5.42	7.15
IS1805	June 2018	66.514528	−16.132639	Subalpine & glacial scrub	moss	mixed grasses and mosses	4.33	6.13
IS1806	June 2018	65.163587	−21.032756	Boreal heath	monocot	mixed grasses and mosses	5.52	7.81
IS1807	June 2018	65.163587	−21.032756	Boreal heath	moss	mixed grasses and mosses	3.88	7.40
IS1808	June 2018	65.162114	−21.019500	Boreal heath	monocot	mixed grasses and mosses	5.33	7.86
IS1809	June 2018	65.162114	−21.019500	Subalpine & glacial scrub	moss	mixed grasses and mosses	5.84	7.69
IS1810	June 2018	65.205417	−21.326132	Boreal heath	monocot	mixed grasses and mosses	5.80	7.74
IS1811	June 2018	65.205417	−21.326132	Boreal heath	moss	mixed grasses and mosses	4.68	7.24
IS1812	June 2018	65.218333	−21.370000	Grazed pasture	monocot	farm grass	6.97	8.54
IS1813	June 2018	65.184495	−21.480230	Grazed pasture	monocot	farm grass	6.74	8.28
IS1814	June 2018	64.777373	−21.520000	Lava field	moss	moss on lava rock	nd	7.32
IS1901	September 2019	64.660454	−21.338979	Grazed pasture	monocot	pasture grass and clover	7.69	8.67
IS1902	September 2019	64.601361	−21.563346	Grazed pasture	monocot	pasture grass	6.95	8.46
IS1903	September 2019	64.428018	−21.959702	Grazed pasture	monocot	pasture grass	6.67	7.58
SU2002	August 2020	65.649893	−18.183058	Subalpine & glacial scrub	dicot	Empetrum nigrum	5.82	6.70
SU2003	August 2020	65.649893	−18.183058	Subalpine & glacial scrub	moss	Hylocomium splendens	3.54	5.84
SU2005	August 2020	65.650320	−18.182976	Subalpine & glacial scrub	dicot	Empetrum nigrum	1.63	5.69
SU2006	August 2020	65.650320	−18.182977	Subalpine & glacial scrub	moss	Hylocomium splendens	3.34	5.80

4.2. Isolation, Characterization and Quantification of Bacteria

Samples were processed within 4 days of collection. A subsample (about 10 g) was weighed and stomached for 2–3 min in 0.1 M sterile phosphate buffer (8.75 g K_2HPO_4 and 6.75 g KH_2PO_4 per liter, pH 6.8) (5–10 mL buffer/g of tissue) in sterile stomacher bags. Macerations were dilution-plated on KBC medium [28] and 10% tryptic soy agar (TSA) as described previously [29] and incubated at room temperature (ca. 22–25 °C) in the dark. Colonies were counted after 2 and 3 days of incubation: total colonies on TSA and *P. syringae*-like colonies on KBC. Various colony-types were targeted as putative *P. syringae* to account for the morphological diversity of colonies across the *P. syringae* complex. The ultimate identity was then confirmed based on phylogenetic relationships with reference strains. *P. syringae*-like colonies are round with lace-like or irregular edges with various distinguishing features for some of the phylogroups (Supplementary Figure S1). For strains in all phylogroups except PG07 and PG08, colonies are about 0.5 mm in diameter with a surface that is smooth and translucent and not rough nor opaque. The colonies are not pigmented. Colonies of strains in PG07 and PG08 are up to 1 mm in diameter and slimy with a yellow/orange pigment. Many strains of *P. syringae* display phase variation where a round, smooth colony gives rise to a juxtaposed slimy colony. Most, but not all, strains of *P. syringae* produce fluorescent pigments on medium with low iron content; however, this is difficult to observe on KBC medium. Up to 30 *P. syringae*-like colonies were randomly chosen per sample and purified. Their identity as part of the *P. syringae* complex was verified based on the phylogenetic relationship of each strain with reference strains, established from partial sequences of the citrate synthase (*cts*) gene, according to the phylogenetic context, primers for *cts* amplification and conditions for PCR described by Berge and colleagues [5]. Amplified DNA was sent to GenoScreen (Lille, France) or Macrogen Europe B.V. (Amsterdam, The Netherlands) for sequencing. Values for population densities of *P. syringae* on plant tissues were calculated based on the percent of *P. syringae*-like colonies sampled from KBC that were verified to be part of the *P. syringae* complex among the total number of *P. syringae*-like colonies observed. A total of 736 strains from *P. syringae*-like colonies were characterized for which 609 were confirmed to be in the *P. syringae* complex. A list of all strains collected in Iceland, their origin and their associated cts sequence are presented in Supplementary Table S2.

Sizes of total and *P. syringae* populations were calculated as log10 colony-forming units (cfu) g^{-1} for each individual sample. Calculation of descriptive statistics (mean, standard error, confidence intervals) and comparative statistics (*p*-values from ANOVA) were calculated with Statistica 10 (StatSoft www.statsoft.fr; accessed on 27 August 2019).

4.3. Evaluation of Genetic Diversity and Divergence

Genetic diversity was assessed among Icelandic strains and in comparison to a data set of *cts* sequences from 933 reference strains that included those from our previous work [5] and from open access sources representing a dozen habitat types (wild and cultivated plants, surface freshwaters, irrigation water, ground water, lithic biofilms, leaf litter, cloud water, rain and snowfall, snowpack and soil) and 27 countries of isolation in the Northern and Southern hemispheres. The names, origins and associated cts sequences for these 933 reference strains are in Supplementary Table S1. Sequences were aligned and cut and then Neighbor-joining trees were constructed with MEGA 10.2.6 (https://www.megasoftware.net/; accessed on 26 June 2021) based on 1000 bootstrap replications as described previously [5]. Time of divergence of haplotypes from Iceland were approximated with the Timetree interface in MEGA 10.2.6 [30] using the maximum likelihood estimator with uniform distribution of age. Age constraints for calculation of divergence times were set according to previous estimates of the age of the most recent common ancestor of various phylogroups. The time of divergence of the canonical *P. syringae* phylogroups (PGs 1, 2, 3, 4, and 6) from the other phylogroups was set at 150–183 million years ago according to O'Brien and colleagues [31]. These same authors estimated the divergence of PG01 and PG02 at 3–10 million years ago. The age of divergence of PG07 was set at 0.3 million years

ago according to estimates of Karasov and colleagues [32]. The specific placements of these time constraints on the phylogenetic tree are indicated in Figure 2.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11030357/s1, Figure S1: Morphology of Pseudomonas syringae colonies; Figure S2: Graphical representation of source categories of haplotypes of strains of Pseudomonas syringae from Iceland and from reference collections used in this study; Table S1: Data set of reference strains used in this study; Table S2: Data set for strains of Pseudomonas syringae isolated from vegetation in Iceland for this study [5,33–36].

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