



Properties relevant to atmospheric dispersal of the ice-nucleation active *Pseudomonas syringae* strain R10.79 isolated from rain water

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Abstract Transport of microbes in the atmosphere allows them to spread and to colonize new habitats. To survive the harsh environmental conditions encountered in the atmosphere, these microorganisms have to possess properties that allow them to resist atmospheric stress. We combined physiological experiments and genome analysis of *Pseudomonas syringae* strain R10.79, previously isolated from rain, to decipher the strain's ability to withstand these challenges and identify the mechanisms behind the

resistance. We used laboratory experiments to simulate different atmospheric stress factors and evaluated cell survival using fluorescent staining and flow cytometry. We found that while *P. syringae* R10.79 was resistant to oxidative, osmotic, UVB radiation-induced and freezing stress, it was sensitive to desiccation on surfaces. Strain R10.79 possesses a range of genes that would allow the strain to mitigate atmospheric stresses, such as cold shock and osmotic shock genes as well as genes encoding for properties that relate to UV irradiation resistance. Studying the substrate spectrum of strain R10.79, we uncovered that it can utilize carbon compounds abundant in the atmosphere including acetate, succinate and formate.

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In addition, strain R10.79 possesses metabolic pathways to utilize formaldehyde and methanol as sole carbon sources. These compounds could support the metabolic activity of strain R10.79 while suspended in the atmosphere during periods with sufficiently high relative humidity. Finally, we found that when growing on acetate, strain R10.79 produces several volatile organic compounds such as isoprene and methanol, for which we propose biochemical pathways and which are relevant for atmospheric chemistry and climate. The results reported in this study will support our general aim at establishing this strain as a model organism to study microbial responses to atmospheric conditions.

Keywords Atmospheric dispersal · Atmospheric stress · Atmospheric chemistry · Ice-nucleation activity · *Pseudomonas syringae* · Draft genome · Metabolism

1 Introduction

Atmospheric dispersal has been essential for microbial colonization of terrestrial and aquatic environments throughout Earth's history (Pearce et al. 2009). The ongoing importance of this process is indicated by the diversity of bacterial species and the significant number of cells found in air, cloud water, fog and precipitation (Šantl-Temkiv et al. 2020). In the atmosphere, bacterial cells are confronted with extreme conditions and consequently are challenged by several stress factors (Delort and Amato 2017). While the transition from a terrestrial or aquatic environment to the atmosphere is affected by species-independent factors, such as aerosolization triggered by wind, it has been proposed that survival in the atmosphere as well as subsequent dispersal and colonization is under strong selection (Tong and Lighthart 1997, 1998; Amato et al. 2015; Tesson and Šantl-Temkiv 2018). Therefore, it is likely that microorganisms with properties that allow them to better cope with atmospheric stress factors spread more successfully by atmospheric transport than microorganisms that lack these properties (Šantl-Temkiv et al. 2018; Amato et al. 2017, 2019; Ng et al. 2017, 2018).

Common atmospheric stress factors are UV radiation, low temperature, large temperature fluctuations and freeze–thaw, fluctuating osmotic conditions, desiccation, low pH and the presence of toxic compounds such as reactive oxygen species (Delort and Amato 2017). Joly et al. (2015) found that microbial strains isolated from the atmosphere could mitigate atmospheric stress factors, coping particularly well with oxidants and UV radiation and less well with freeze–thaw. Cloud droplets contain a high concentration of nutrients and organic acids that can be used by microorganisms (Sattler et al. 2001; Šantl-Temkiv et al. 2013a; Deguillaume et al. 2014) and may therefore support active metabolisms in airborne cells (Dimmick et al. 1975; Fuzzi and Mandrioli 1997; Sattler et al. 2001; Temkiv et al. 2012; Šantl-Temkiv et al. 2013b, 2018; Klein et al. 2016; Amato et al. 2017, 2019). Recently, a meta-transcriptomic study of cloud microbial communities confirmed that bacteria were transcriptionally active, and were expressing genes associated with central metabolic pathways as well as with major stress response mechanisms (Amato et al. 2019). It has been shown experimentally that aerosolization triggers expression of stress resistance genes and sorbitol metabolism genes in *E. coli* (Ng et al. 2018), that possession of selected stress-resistance and metabolic genes increases the survival rate in aerosolized *E. coli* (Ng et al. 2017, 2018), and that airborne *Sphingomonas aerolata* metabolically responds to gaseous substrates (Krumins et al. 2014). Systematically obtained and environmentally relevant experimental data on the role of gene expression in the survival of bacteria during atmospheric dispersal is still lacking.

The global leaf surface area is four times larger than the terrestrial ground surface area (Wéry et al. 2017). Thus, leaves comprise a very large microbial habitat supporting globally up to 10^{26} bacterial cells (Lindow and Brandl 2003), which are directly exposed to the atmosphere. Modelling and experimental studies show that ecosystem types, such as crops, forests, grasslands, and shrubs, are associated with some of the highest bacterial fluxes to the atmosphere (Lindemann et al. 1982; Burrows et al. 2013; Carotenuto et al. 2017). In addition, deposition of microorganisms onto the leaf surface through atmospheric dispersal, e.g. rainfall or dry deposition, is among key factors involved in the assembly of the epiphytic community (Schlechter et al. 2019). Thus, the atmospheric

transmission is particularly important for the spread of epiphytic bacteria. On plant leaves, there is a strong selection for cells with traits that would also favour survival in the atmosphere, such as coping with conditions of variable water availability, extensive radiation or large temperature variations (Morris et al. 2004). As epiphytic bacteria encounter similar stress factors in their primary habitat as they do in the atmosphere, it is not clear whether these survival traits were selected for as part of atmospheric dispersal or whether selection took place in their terrestrial habitats. Thus, we also cannot distinguish whether survival in the atmosphere is the primary or secondary benefit of these traits.

Among epiphytic bacteria, ice-nucleation active (INA) strains have attracted a lot of attention as they, when emitted into the atmosphere, have the ability to contribute to the structure and the lifetime of clouds, which in turn affects the global radiation balance (Morris et al. 2008, 2011; Phillips et al. 2009; Costa et al. 2014). The most efficient INA microorganisms, in terms of the temperature at which ice-formation is promoted, are found among members of genera *Pseudomonas*, *Pantoea*, *Erwinia*, and *Xanthomonas* (Morris et al. 2004). Possessing a specific protein, INA strains catalyse ice formation at temperatures up to $-2\text{ }^{\circ}\text{C}$, which is considerably higher than ice-nucleation activity of e.g. mineral dust that is rarely active above $-15\text{ }^{\circ}\text{C}$ (Kanji et al. 2017). Ice-nucleation activity also serves as a mechanisms that improves the chance of a successful colonization by reducing the residence time of cells in the atmosphere as the cells actively facilitate their deposition through formation of ice, leading to precipitation (Amato et al. 2015). Among INA bacterial species, *Pseudomonas syringae* is one of the best-studied species (Delort and Amato 2017) both with respect to the presence of *P. syringae* cells in the environment and their ability to survive conditions that prevail in the atmosphere. Thus, viable *P. syringae* cells have been found in the free atmosphere, in cloud droplets, and in precipitation including rain and snow (Joly et al. 2013; Monteil et al. 2014; Šantl-Temkiv et al. 2015; Stopelli et al. 2017). We recently demonstrated that cells of *P. syringae* strain R10.79, a strain isolated from rainwater, can survive aerosolization in small droplets followed by a short-term desiccation (Alsved et al. 2018). In addition, two strains of *P. syringae* were confirmed to withstand stress factors that cells would encounter during

prolonged periods of atmospheric dispersal (Joly et al. 2015). However, the mechanisms behind the ability of *P. syringae* cells to withstand atmospheric stress remain poorly understood.

In this study, we aim at identifying physiological, biochemical, and molecular mechanisms behind the properties relevant to atmospheric dispersal of *P. syringae* strain R10.79. Overall, we want to establish the strain as a model organism to study the response of microorganisms to conditions that prevail in the atmosphere. To achieve this, we firstly performed a series of experiments to determine the ability of the strain R10.79 to survive common atmospheric stress factors, to take up organic compounds commonly found in the atmosphere and to emit volatile organic compounds relevant for atmospheric processes. Secondly, we performed a genomic analysis in order to identify genes and propose mechanisms associated with improved survival in the atmosphere. Finally, we compared stress response genes in the *P. syringae* strain R10.79 to genes in a closely related epiphytic strain, *P. syringae* pv aptata to investigate whether similar stress-response mechanisms exist in strains obtained from their primary terrestrial habitat.

2 Material and methods

2.1 Bacterial strain

Pseudomonas syringae strain R10.79 was isolated from a rain sample that was collected in 2009 at 30 m above ground near the city of Roskilde, Denmark. (N $55^{\circ} 41' 29.51''$ /E $12^{\circ} 6' 17.52''$) as described in detail previously (Šantl-Temkiv et al. 2015). Briefly, a concentrated rain sample was plated in triplicates on R2A medium supplemented with 0.05 mg/ml of natamycin to inhibit fungal growth (Reasoner and Geldreich 1985). Cells were grown at $15\text{ }^{\circ}\text{C}$ for a week and colonies were picked and isolated on R2A medium. Isolates were tested for ice nucleation activity at $-7\text{ }^{\circ}\text{C}$ and identified based on their partial 16S rRNA genes sequences (Šantl-Temkiv et al. 2015).

2.2 Investigating the effect of freeze–thaw, oxidative and osmotic stress on cell survival

Assays to investigate the resistance to freeze–thaw cycles, oxidative stress and osmotic stress were designed according to Joly et al. (2015) with the modification that cell survival in the present study was not determined by plate counts but mostly by live–dead staining as described in detail below.

Three biological replicates of strain R10.79 cells were grown to stationary phase in R2 medium (Reasoner and Geldreich 1985) at room temperature (RT). When the cultures reached the stationary phase, the cell concentration was adjusted to $\sim 10^7$ cells/mL in fresh R2 medium. Freeze–thaw experiments were carried out by freezing the cells at $-20\text{ }^{\circ}\text{C}$ for 2 h and thawing them at $4\text{ }^{\circ}\text{C}$ for 3 h before determining the number of viable cells. The resistance to oxidative stress was investigated by suspending cells in R2 medium containing 0, 0.1, 0.25, 0.5 or 1 mM of hydrogen peroxide (final concentration), respectively. The cells were incubated at RT in the dark and cell survival was evaluated after 90 min of exposure. The resistance to hyper-osmotic stress was investigated by suspending cells in RA medium containing NaCl at final concentrations of 0.1 M, 0.5 M, 1 M or 2.5 M NaCl, respectively. The experiment was carried out at RT and the cells were subjected to the respective NaCl concentrations for 90 min. After exposure to hydrogen peroxide or NaCl, the cells were pelleted by centrifugation at $13,000\times g$ for 5 min at RT. The supernatant was removed and the pellet was re-suspended in 0.1% NaCl.

Cell survival was investigated by staining treated and control cells using the Live/Dead BacLight Bacterial Viability Kit (Invitrogen) as described previously (Alsved et al. 2018). The kit contains two dyes: propidium iodide (PI) that only stains cells with defective cytoplasmic membranes, i.e. cells assumed to be dead, and SYTO9 that stains all cells. The cells were stained with $5\text{ }\mu\text{M}$ of SYTO9 and $30\text{ }\mu\text{M}$ PI, and were incubated for 15 min in the dark before the fraction of live and dead cells in the samples was determined by flow cytometry (NovoCyte ACEA, Biosciences Inc). Data were analysed using the FlowJo software (FlowJo LLC) (Alsved et al. 2018).

The minimum growth inhibitory concentration (MIC) of NaCl was determined in triplicate as

described in Joly et al. (2015). The assay was performed in microtiter plates that were inoculated with $\sim 10^5$ cells per well and incubated at RT for one week; thereafter, the optical density at 600 nm (OD) was determined with a plate reader. A standard curve was constructed using dilutions of a stationary-phase culture, for which the cell numbers were determined by flow cytometry (data not shown).

2.3 Examining the effect of UVB radiation on the survival of strain R10.79

The effect of UVB radiation on the survival of cells of strain R10.79 was studied using a stationary-phase culture grown in R2 medium. Cells were pelleted by centrifugation at 7000 rpm for 3 min at $4\text{ }^{\circ}\text{C}$, and thereafter washed in PBS buffer three times. Ten mL of the washed cell suspensions were transferred to sterile Petri dishes, placed on ice in a Stratalinker® UV Crosslinker and irradiated with UVB light for 30 min. After 1, 5, 15 and 30 min, $100\text{ }\mu\text{L}$ of the cell suspension was removed from the Petri dish, and the number of colony-forming units (CFU) was quantified on R2A plates using the drop-plating technique. R2A agar plates were divided in four sectors and five $10\text{ }\mu\text{L}$ droplets of each dilution were placed in a separate sector of the plate. The UV dose received by the cells (expressed as $[\mu\text{J}/\text{cm}^2]$) was calculated using the following equation: $\mu\text{J}/\text{cm}^2 = \mu\text{W}/\text{cm}^2 \text{ s}$.

2.4 Utilization of carbon sources

Sole carbon source utilization of strain R10.79 was assessed using an assay based on Biolog PM1 plates (Biolog, Hayward, CA). This assay consists of a defined medium with a unique carbon compound in each well of the 96-well PM1 microtiter plate. Tests were carried out according to the manufacturer's instructions, and growth was evaluated by OD measurements as described in Temkiv et al. (2012). Growth on acetate was studied in more detail after inoculating cells of strain R10.79 into mineral medium (Błaszczuk 1993) containing 0.1% sodium acetate (see Sect. 2.5).

2.5 Emissions of volatile organic compounds during growth on acetate

Strain R10.79 was grown over night in R2 medium until stationary phase was reached. One mL of the stationary phase culture was added to a vial containing 100 mL of liquid Nitrate Mineral Salts Medium in phosphate buffer [pH 6.8, Whittenbury et al. (1970)], 10 mM sodium acetate and $1 \text{ mL} \times \text{L}^{-1}$ each of Vitamin Mix, Thiamin, Vitamin B12 and trace metal SL-10 solution (Widdel and Bak 1992). The OD of the culture was measured periodically with a spectrophotometer. Volatile organic compounds (VOCs) were monitored in a growing culture using a Proton Transfer Reaction Time-of-Flight Mass Spectrometer (PTR-ToF-MS 8000; Ionicon Analytic GmbH, Austria, for details see Graus et al. 2010). This instrument produces H_3O^+ primary ions with a hollow cathode discharge in water vapour that can react with compounds having proton affinities greater than that of water. Most constituents in air such as O_2 or NO_2 have lower proton affinities than water and can hence serve as buffer gas while the majority of VOCs can undergo charge-transfer reactions with the primary ions (Graus et al. 2010). During growth of the culture, the headspace of the culture vessel was flushed with filtered air at a constant flow of 210 mL/min. The removed air was led to the PTR-MS instrument, where its constituents were analysed. The flow reached the instrument through an inlet line that was heated to 348 K, while the drift temperature inside the PTR-MS amounted to approximately 332 K. The ratio between the electric field strength (E) inside the drift tube and the buffer gas number density (N) was kept at a constant level of 130 Townsend (Td), the pressure in the reaction chamber at 2.3 mbar and the voltage at 595 V. Mass spectra from mass to charge ratios (m/z) of 15–200 m/z were recorded with a 1 min time resolution and post-processed using the PTR-MS Viewer 3.2.12. A total of 81 significant peaks were identified by investigating the time evolution of peaks with signals above 1 count per second. The temporal evolution of these peaks was explored according to the growth phase of the culture (lag phase, exponential phase, stationary phase and late stationary phase).

2.6 DNA extraction, genome sequencing and analysis

Genomic DNA was extracted using a Powerlyser Powersoil DNA Isolation Kit (MoBio Laboratories) following the manufacturer's protocol. Indexed libraries were prepared using the Nextera XT DNA Sample Preparation and Sample Preparation Index Kits. Sequencing was performed on an Illumina MiSeq instrument using 600 cycles MiSeq v3 Reagent Kit (Illumina), which produces paired 300-bp long reads. Illumina FASTQ data were trimmed with Trimmomatic-0.33 (Bolger et al. 2014) to remove adapters and low-quality regions at the ends of reads. Reads were assembled using SPAdes 3.6.1 (Bankevich et al. 2012), set to six different k-mer sizes (21,33,55,77,99,127). After trimming and assembly, QUAST 3.1 (Gurevich et al. 2013) was used to determine the total length of the genome, the number of contigs, the average G + C content and the N50 value. Coverage, genome completeness and genome contamination were assessed using CheckM v 1.0.5 (Parks et al. 2015). Gene annotation was performed using PROKKA 1.11 (Seemann 2014). Syringomycin biosynthesis genes were identified using the C-domain detection function in NAPDOS (Ziemert et al. 2012). Stress-related genes were found using hmmscan version 3.1b2 (Eddy 1998) against profile HMMs from the Pfam database (El-Gebali et al. 2019).

2.7 Citrate synthase-based phylogenetic tree of *P. syringae* strain R10.79 and its closest relatives

As the 16S rRNA gene sequence did not contain sufficient information to resolve the phylogeny of *P. syringae* strain R10.79, the concatenated citrate synthase (*cts*) housekeeping gene was used instead. Sixty-eight *P. syringae* reference strains that represented all phylogroups and clades were used to classify strain R10.79 among the 13 *P. syringae* phylogroups (Figure S1), as suggested by Berge et al. (2014). Based on the partial *cts* genes (410 bp) (Berge et al. 2014), the *cts* housekeeping gene sequence of *P. syringae* strain R10.79 was trimmed and aligned with the DAMBE software version 5 (Xia 2013). The phylogenetic tree based on the partial *cts* genes was built with the maximum-likelihood algorithm using the MEGA7 software with 500 bootstraps (Kumar et al. 2016). The most similar phylogroup for the *P. syringae* strain

R10.79 was found based on the tree's clustering pattern.

2.8 Accession numbers

The genome of *P. syringae* strain R10.79 was deposited at DDBJ/ENA/GenBank under the accession NQWX000000000. The version described in this paper is version NQWX000000000.1.

3 Results and discussion

3.1 General genomic features, ice-nucleation and biosurfactants in *P. syringae* strain R10.79

The assembly of R10.79 genome resulted in 35 contigs. The final length of the draft genome was 5,846,635 bp with 99.1% estimated completeness. The genome has an average GC-content of 59.3% and contains 5062 protein-coding sequences (CDSs), which is within the range of CDSs commonly found in other *P. syringae* genomes. A summary of the genome assembly is provided in Table S1. *P. syringae* strain R10.79 clustered in phylogroup 2 of the *P. syringae* complex based on the comparisons of their partial *cts* gene sequences (409 bp). (Figure S1). This phylogroup 2 is the largest phylogroup of *P. syringae* and is formed by strains that are found ubiquitously in the environment spanning from plants to rain water. In agreement with 85% of the strains within this phylogroup (Berge et al. 2014), *P. syringae* strain R10.79 is an INA bacterium (Šantl-Temkiv et al. 2015; Ling et al. 2018). Its genome carries an *ina* gene. The closest relative of *P. syringae* R10.79 is *P. syringae* pv. *syringae* Cit7 that was isolated from a healthy orange tree leaf (Orser et al. 1985) and nucleates ice at -4°C (Berge et al. 2014). Similarly to 90% of phylogroup 2 members, the genome of strain R10.79 carries genes for the synthesis of syringomycin, a strong surfactant with the ability to reduce water surface tension by 55% (Hutchison et al. 1995). Syringomycin has a toxic effect on plant cells (Berge et al. 2014), as it dissolves plant membranes and produces pores by inserting into the membranes (Hutchison et al. 1995). *Pseudomonas* strains isolated from the atmosphere and cloud water have previously been reported to produce biosurfactants (Ahern et al. 2007; Renard et al. 2016), which are implicated in

atmospheric processes as they reduce the critical supersaturation at which aerosols attract sufficient water vapour to form cloud droplets (Li et al. 1998).

3.2 Physiological and genetic traits related to stress resistance in the atmosphere

The genome of strain R10.79 contains a *rpoS* gene that encodes a sigma factor (σ^S ; RpoS). RpoS is the main regulator of the stationary phase stress response or general stress response found in *E. coli* and other enteric bacteria (Chung et al. 2006). It controls the transcription of more than 35 genes and can be induced by a variety of stresses, such as starvation, hyperosmotic stress, high or low temperature as well as low pH (Chung et al. 2006). Most of these stresses are encountered by bacteria in the atmosphere. The ability of *E. coli* to survive the salt, acid and heat stresses has been related to the expression of RpoS and other stress response elements (Chung et al. 2006). Using gene-knockout mutants, Ng et al. (2017) demonstrated that the *rpoS* gene improved the survival of *E. coli* that were aerosolized at different humidity levels. RpoS may have a similar role during aerosolization of *P. syringae*. In addition, many of the general stress response proteins are controlled by RpoS; therefore, the presence of the *rpoS* gene in the R10.79 genome indicates the existence of numerous stress protective mechanisms.

3.2.1 Response of strain R10.79 to temperature shifts and freezing

Low temperature and rapid temperature changes are a common type of stress that cells experience in the atmosphere. The atmospheric temperature fluctuates rapidly due to the low heat capacity of air and is determined by factors such as relative humidity, convection, cloud formation and altitude. We observed that strain R10.79 grows at low temperatures (4°C , data not shown). This observation is in agreement with Amato et al. (2007), who reported that several *Pseudomonas* strains isolated from cloud water were able to grow at 5°C . Prior to that report, Sattler et al. (2001) established that cloud-borne communities can grow in supercooled cloud droplets. Very often sub-zero temperatures will induce freezing of water and due to fluctuations between above zero and sub-zero temperatures, airborne bacterial cells

may undergo repeated cycles of freezing and thawing. Freeze–thaw cycles can kill cells by disrupting the cell membranes (Sleight et al. 2006). Results of our freeze–thaw experiment showed that 15%–43% of strain R10.79 cells survived a single freeze–thaw cycle (Fig. 1a), suggesting the presence of mechanisms to mitigate freeze–thaw damage. A previous study by Joly et al. (2015) showed an even higher survival in two INA strains of *P. syringae* isolated from the atmosphere, with a fraction of surviving cells ranging from ~ 30 to ~ 90% cells after being exposed to a single freeze–thaw cycle. A metabolomic study on *P. syringae* demonstrated that in response to cold temperatures, the cells exhibited metabolism modifications, such as cryoprotectants and antioxidants, to reduce the effects of low temperatures (Jousse et al. 2018).

Strain R10.79 possesses two genes that encode cold shock proteins (CSPs), i.e. a *cspA* gene and a *cspD* gene (Fig. 2, SI-Table 1), and therefore has the genetic potential to tolerate temperature drops and freeze–thaw cycles in the atmosphere. When cooled, bacterial cells rapidly produce CSPs that protect the cells against the negative effects of temperature downshifts by their involvement in DNA replication, transcription, translation, RNA stabilization and ribosome assembly (Gualerzi et al. 2003). Expression of cold shock protein genes has been demonstrated for *E. coli* during aerosolization, which is likely associated with

changes in temperature due to evaporating droplets (Ng et al. 2018). Possession of CSP genes allows bacterial cells to adapt ribosomes to function optimally at low temperatures (Horn et al. 2007). Furthermore, two *capB* genes are present in the genome of strain R10.79 that encode cold acclimation proteins (CAPs). Michel et al. (1997) studied *Pseudomonas fragi* and found that the *capB* gene promotes the ability of cells to divide at low temperatures by upholding the synthesis of histone-like nucleoid-structuring protein. The genome of strain R10.79 further contains several genes that show increased expression during cold shock in *E. coli*: RNA helicase, ribosome-binding factor RbfA, polynucleotide phosphorylase (PNPase), transcription factor NusA, translation initiation factors IF1, and IF2, RecA, DNA gyrase, protein chaperones, trigger factor, HscB, ClpB, recombination factor, dihydrolipoamide transferase, and pyruvate dehydrogenase (Phadtare 2004; Phadtare and Severinov 2010). This suggested that strain R10.79 has access to a complex system of genes that allow the cells to regulate and remodel RNA for maintenance and growth at low temperatures.

3.2.2 Resistance of strain R10.79 to desiccation and osmotic stress

Like freezing, desiccation increases solute concentration and results in reduced water activity. Desiccation

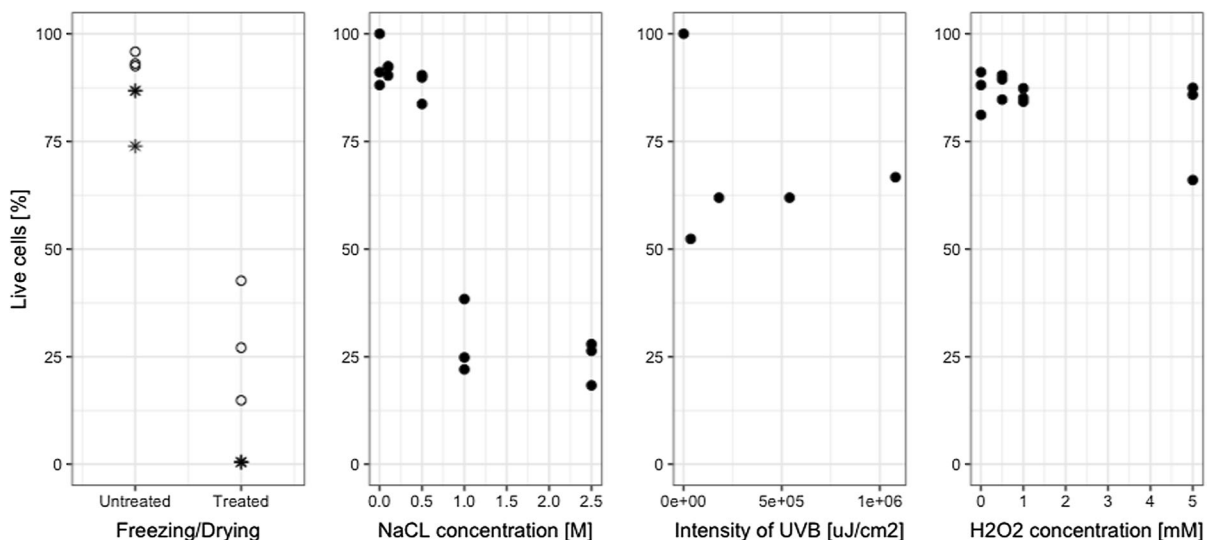


Fig. 1 Fraction of viable cells of *Pseudomonas syringae* strain R10.79 after exposure to different atmospherically relevant stressors: drying (asterisk)/freezing (circle) (a), osmotic stress (b), UVB radiation (c), oxidative stress (d)

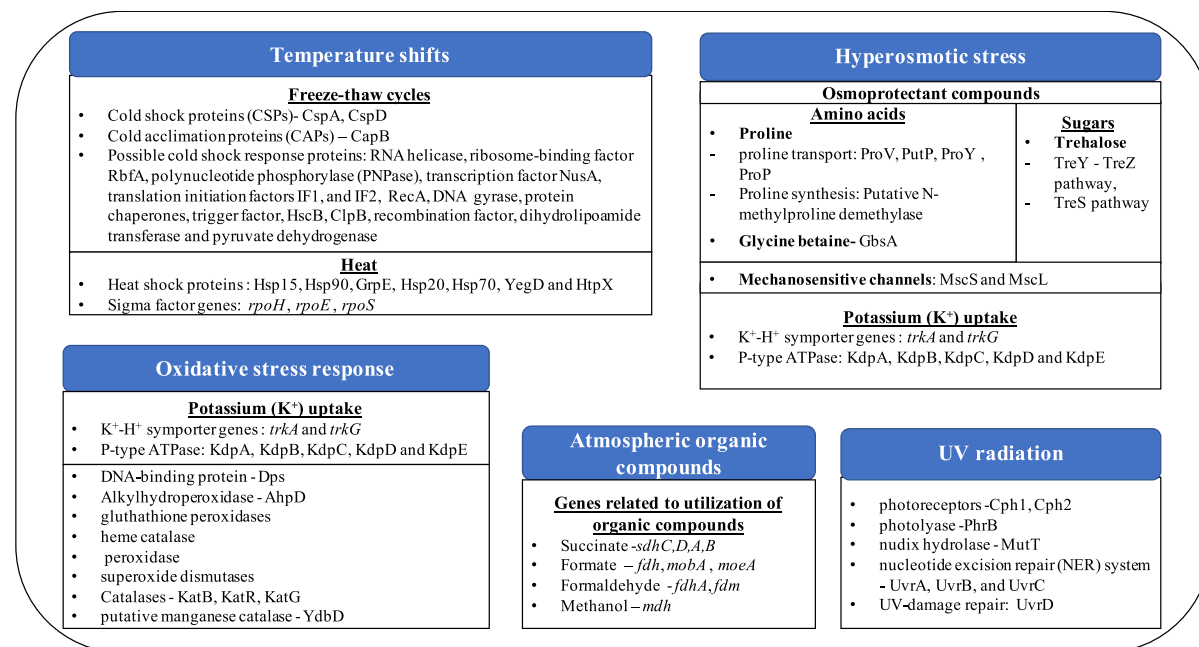


Fig. 2 Overview of stress response genes and genes involved in utilization of organic compounds that are present in the genome of strain R10.79 and that are potentially relevant for atmospheric

survival. The genes are grouped based on the type of trait. Details are reported in SI-Table 1

resistance is crucial for atmospheric dispersal, as all aerosolized cells are dried in the atmosphere, which is generally undersaturated with water. Desiccation may also lead to increased sensitivity to other stress factors in the atmosphere, as repair mechanisms are inhibited under dry conditions. Drying on surfaces was detrimental to cells of *P. syringae* strain R10.79 and only < 1% of the cells survived a single cycle (Fig. 1a). This is in agreement with a recent study that showed that the viability and the ice-nucleation activity of a *P. syringae* strain was critically affected by extended drying on a surface (de Araujo et al. 2019). We recently observed that the drying rate significantly affects the survival of strain R10.79 cells during aerosolization (Alsved et al. 2018). Thus, drying in nanoliter-sized droplets that took seconds significantly improved survival of strain R10.79 cells (~ 30% of cells surviving) compared to drying in microliter-sized droplets on a surface that took hours (Alsved et al. 2018).

Due to repeated condensation–evaporation cycles of droplets in clouds, airborne microbes are subjected to repeated incidences of osmotic shock (Joly et al. 2015). We tested osmoregulation of *P. syringae* strain R10.79 by subjecting cells to a pulse of increased

concentrations of NaCl (Fig. 1b), and found that strain R10.79 cells were not affected significantly by the presence of up to 0.5 M NaCl ($t = 0.3300$, $df = 4$, $p = 0.76$). Cell viability was significantly reduced at concentrations ≥ 1 M ($t = 10.0162$, $df = 4$, $p < 0.001$). However, 22–38% of the cells remained viable after 90 min of exposure. Even at 2.5 M NaCl, 18–28% of the cells remained viable, indicating a strong potential to withstand osmotic stress. We saw the same pattern when we tested growth in the presence of increasing concentrations of NaCl (Fig. 3). The minimal inhibitory concentration (MIC) of NaCl for strain R10.79 was determined to be 1.3 M. These results fit well with what Joly et al. (2015) found for two *P. syringae* strains, which were significantly affected by 2.5 M NaCl and had a MIC of 1.0–1.2 M. The mean bulk concentration of NaCl in cloud water is in the range between 30 and 300 μ M (Deguillaume et al. 2014). However, the solute concentration in cloud droplets varies over several orders of magnitude and evaporation may for example cause a four-orders-of-magnitude increase in solute concentration, when a droplet is reduced in size from a diameter of 20 μ m to a diameter of 1 μ m. Thus, NaCl

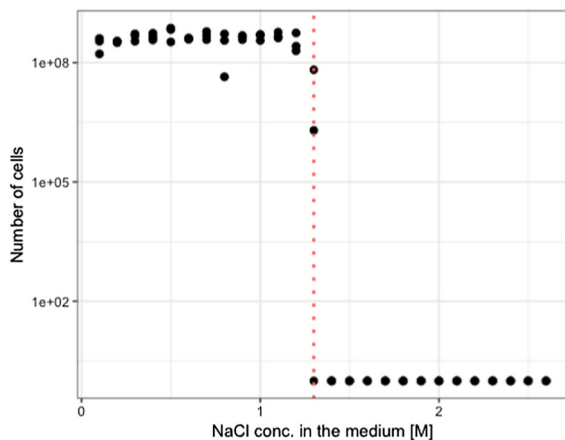


Fig. 3 Minimal inhibitory concentration (MIC) of NaCl for *P. syringae* strain R10.79. Shown is the number of cells that grew after 1 week of incubation in R2A medium as a function of the concentration of NaCl present in the medium. The red dotted line indicates the determined MIC concentration: 1.3 M NaCl

concentrations may often reach molar concentrations in the atmosphere.

Four different *kdp* genes (*kdpA*, *kdpB*, *kdpC*, *kdpD* and *kdpE*) and two *trk* genes (*trkA* and *trkG*), which facilitate uptake of K^+ either by a K^+-H^+ symporter (*trk* genes) or a P-type ATPase (*kdp* genes) (Sleator and Hill 2002; Wood 2015), were found in the genome of strain R10.79. Stimulation of potassium (K^+) uptake is a commonly found response to osmotic shock in bacteria, and Alsved et al. (2018b) suggested that increasing cytoplasmic K^+ concentration is also implicated in cell survival in strain R10.79 during drying. The genome of strain R10.79 contains several genes (*proV*, *proP*, *putP*, *proY* genes) encoding proteins that are involved in the transport of proline, which is known to be taken up as protectant against osmotic shock by several bacterial species, e.g. *Staphylococcus aureus*, *Lactococcus lactis* and *Bacillus subtilis* (Sleator and Hill 2002). A gene encoding a putative *N*-methylproline demethylase that synthesizes proline (Phillips et al. 1998) is also present in the genome of strain R10.79. Other mechanisms found in the genome of strain R10.79 that could protect cells experiencing osmotic shock are *gbsA*, involved in the synthesis of glycine betaine (Boch et al. 1996; Nau-Wagner et al. 2012) and *mscS/mscL*, involved in the release of compatible solutes from the cytoplasm into the environment (Booth and Blount 2012; Romantsov et al. 2009). Finally, the genome of strain R10.79

contains genes that encode for two production pathways of trehalose that has been demonstrated to accumulate in *P. syringae* under hyperosmotic (Kurz et al. 2010) and in cold conditions (Jousse et al. 2018) as well as promote growth in cells exposed to osmotic shock (Freeman et al. 2010). Thus, the trehalose biosynthetic pathways in strain R10.79 could increase the survival probability of cells that are confronted with rapid osmotic shifts in the atmosphere.

3.2.3 UVB radiation-resistance of cells of strain R10.79

UV radiation is another common stress that microbial cells encounter in the atmosphere and that reduces cell survival due to DNA damage related to the formation of cyclobutane pyrimidine dimers and 6–4 photoproducts (Kim and Sundin 2001). Therefore, it is critical for aerosolized microbes to contain mechanisms that either protect from DNA damage or repair damaged DNA. We tested the persistence of *P. syringae* strain R10.79 to UV radiation by exposing cells to UVB radiation at 312 nm. We observed an initial drop in cell viability (reduction of viable cells by ~ 40%) already after subjecting cell suspensions to a small radiation dose (Fig. 1c). After this initial reduction, no significant further reduction was observed as consequence of an increasing UVB dose. It seems that the repair mechanisms were either not constitutively present in all cells or that cells were in different physiological stages and responded differently to the UVB stress. Joly et al. (2015) showed that the survivability of *P. syringae* isolates was largely unaffected by up to 10 h of simulated solar irradiation, thus demonstrating that strains of *P. syringae* are well adapted to survive radiation doses that they encounter in the atmosphere. Similarly, de Araujo et al. (2019) showed that two *P. syringae* strains were better adapted to UVA and UVB radiation than *E. coli*. Interestingly, the ice-nucleation activity was significantly reduced by radiation (de Araujo et al. 2019), which may have consequence for cell deposition rates as well as for cloud and precipitation development.

We found 19 phytochrome-like proteins of the types *cph1* and *cph2* in the genome of strain R10.79 (SI-Table 1). Phytochrome-like proteins act as UV photoreceptors that mediate the response to UV radiation and are involved in light-sensing (Ehling-Schulz and Scherer 1999). In addition, we found genes

involved in light-dependent photoreactivation, which is a major repair mechanism in microbes exposed to intense UV radiation: (1) the *phrB* gene encodes a photolyase that repairs pyrimidine dimers (Crowley et al. 2006; Osburne et al. 2010), (2) the *mutT* gene encodes a nudix hydrolase that prevents incorporation of 8-oxo-dGTP, thus counteracting mutations and preventing mispairing, and (3) the *uvrA*, *uvrB*, *uvrC* and *uvrD* genes are responsible for nucleotide excision repair system. Based on these results, we conclude that strain R10.79 is genetically well-equipped to counteract negative UV-radiation effects.

3.2.4 Oxidative stress resistance in cells of strain R10.79

Cloud droplets are an oxidizing and therefore cytotoxic environment, where solar radiation indirectly impacts cell survival by converting hydrogen peroxide (H_2O_2) into free oxygen radicals. These highly reactive oxygen radicals can cause DNA damage and can oxidize lipids, amino acids and carbohydrates that constitute bacterial cell walls and membranes. Therefore, the ability of airborne microbes to safely degrade H_2O_2 is crucial for their survival. We found that cells of strain R10.79 are highly resistant to H_2O_2 . Cell viability was not affected even by the presence of up to 5 mM H_2O_2 (Fig. 1d, $t = 0.9324$, $df = 4$, $p = 0.40$). At all tested H_2O_2 concentrations bubble formation was observed, indicating catalase activity converting H_2O_2 into O_2 and H_2O . A previous study Joly et al. (2015) reported that the survival of *P. syringae* isolated from cloud water was not affected by 0.1 mM H_2O_2 , a concentration typically found in cloud droplets. By performing experiments on cloud water samples, Vaitilingom et al. (2013) showed that cloud-borne microbial communities can degrade H_2O_2 , which indicates that they reduce the oxidative capacity of clouds. In follow-up study, Wirgot et al. (2017) demonstrated that in presence of H_2O_2 the average ATP concentration of the cloud microbiome was reduced. Due to the reduced energy level, the biotransformation rates of carbon compounds would be reduced, which implies an indirect impact of H_2O_2 on cloud chemistry.

Genes encoding the proteins superoxide dismutases and catalases are present in the genome of strain R10.79. In detail, its genome contains five different types of catalases: *katB*, *katR*, *katG*, a putative

manganese catalase (*ydbD*) as well as an unknown type of catalase. We also found several genes that encode for peroxidases, i.e. an alkylhydroperoxidase (*ahpD*), three glutathione peroxidases, a heme-type catalase and a peroxidase, that split peroxides into oxygen and water (Cabiscol et al. 2000). Finally, the genome of strain R10.79 encodes the *dps* gene, which is a DNA-binding protein that has been shown to protect bacterial DNA from oxidative damage during starvation (Frenkiel-Krispin et al. 2001). Expression of *dps* gene has been confirmed in *E. coli* during aerosolization and mutants with *dps* gene deletion had lower survival rates compared to wildtype *E. coli* strain (Ng et al. 2018). Based on this, Ng et al. (2018) proposed that the Dps has an ability to protect bacteria during aerosolization, as it reduces the risk of oxidative damage of the cells' DNA, which is increased with the exposure to the atmosphere.

3.3 Use of substrates that can be encountered in the atmosphere

Organic compounds such as methane, formaldehyde and methanol as well as the carboxylic acids formate, acetate, succinate and oxalate are present in the atmosphere at relatively high concentrations. Here, they can act as carbon and energy sources for microorganisms, as shown by Amato et al. (2007). Atmospheric microorganisms that can utilize these compounds as energy and carbon sources would have a competitive advantage, as they may use the conserved energy to activate the needed stress response. In numerous studies, it has been shown that bacteria have the capacity to grow in cloud droplets using these substrates as carbon and energy source (Sattler et al. 2001; Bauer et al. 2002; Vaitilingom et al. 2010; Temkiv et al. 2012; Šantl-Temkiv et al. 2013b). Furthermore, Krumins et al. (2014) have demonstrated that airborne cells of *S. aerolata* NW12 are metabolically active when supplied with gaseous ethanol or acetic acid. Using a Biolog assay, we showed that strain R10.79 can utilize 44% of the tested carbon sources including carbon compounds that constitute a major fraction of compounds found in the atmosphere, including carboxylic acids (SI-Table 1). Additional experiments confirmed that strain R10.79 can utilize succinate, formate and acetate as sole carbon and energy sources (data not shown). Studying several atmospheric strains of

Methylobacterium sp., which is also a typically epiphytic genus, it was found that these had a similarly broad substrate spectrum as *P. syringae* strain R10.79 (Temkiv et al. 2012). Such use of substrates was suggested to imply an opportunistic ecologic strategy, which may increase the probability of microbial activity in the atmosphere (Temkiv et al. 2012).

The genome of strain R10.79 contains genes encoding enzymes that are part of the succinate and formate degradation pathways. The *sdhA*, *B*, *C*, *D* genes that encode the succinate dehydrogenase as well as the iron-sulphur subunit of the enzyme were found in the genome of strain R10.79. Also, the three subunits of a formate dehydrogenase (FDH) as well as the *mobA* and *moeA* genes that encode for the synthesis of the molybdenum cofactor (Sun et al. 2011) were found. The genome of strain R10.79 also contains several genes associated with formaldehyde metabolism as inferred from the presence of two glutathione-independent formaldehyde dehydrogenase genes (*fdhA*) that are associated with the oxidation of formaldehyde to formate (Schütte et al. 1976). Another formaldehyde dismutase gene (*fdm*) encoding the Fdm enzyme was found in the genome. This enzyme catalyses a reversible reaction that involves the dismutation of formaldehyde to methanol and formic acid without an external electron acceptor (Kato et al. 1984). Finally, strain R10.79 possesses genes involved in methanol degradation such as gene (*mdh*) that encodes a NAD-dependent methanol dehydrogenase.

3.4 Emissions of volatile organic compounds during growth of strain R10.79 on acetate

In order to assess how bacterial cells may affect the concentration and composition of atmospherically relevant chemicals, we carried out a pilot study investigating VOCs emitted by *P. syringae* strain R10.79 during growth on acetate. Out of the 81 significant peaks, four distinct time trends could be determined throughout growth of strain R10.79 cells on acetate. Figure 4 illustrates the temporal evolution of the major compounds as inferred from the mass to charge ratios (*m/z*) as a function of time, during bacterial growth. We attribute the slightly elevated concentrations of *m/z* 69.0699 ((C_5H_8) H^+) and *m/z* 89.0597 (($\text{C}_4\text{H}_8\text{O}_2$) H^+) at the beginning of the incubation to a carry-over contamination present in the

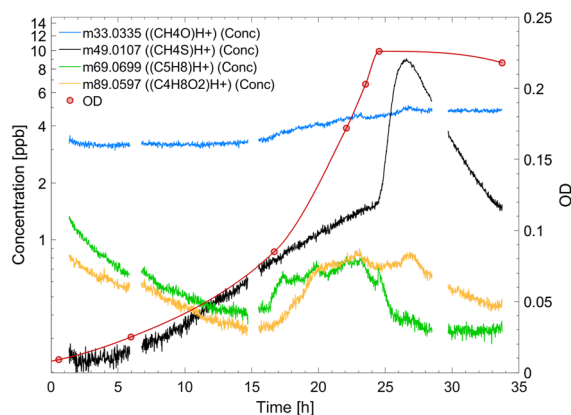


Fig. 4 Four representative volatile organic compounds are illustrated with different time evolutions (*m/z* 33.0335 in blue, *m/z* 49.0107 in black, *m/z* 69.0699 in green, *m/z* 89.0597 in yellow). The legend denotes the protonated *m/z*. Additionally, the OD (right y-axis in red) is shown to display the growth stage of the bacteria

inoculum. The protonated *m/z* 69.0699 ((C_5H_8) H^+) was found to initially decrease over time, followed by a clear increase after approximately 15 h, which lasted for the following 10 h. This increase occurred during the exponential growth phase and stopped when the stationary phase was reached. Most probably this *m/z* ratio is associated with isoprene (C_5H_8) that has previously been identified in emissions of several bacteria, commonly strains affiliated to the Actinobacteria and Proteobacteria phylum (McGenity et al. 2018). Isoprene is intensely studied by atmospheric chemists, because of its high abundance, its high reactivity and its contribution to forming secondary aerosols. The sudden increase in C_5H_8 points towards an emission caused by the bacteria during exponential growth. In *Pseudomonas* sp., isoprene is typically synthesized via the non-mevalonate pathway (Eisenreich et al. 2004), of which the first two enzymes, DXP synthase and DXP reductoisomerase, have been characterized in *Pseudomonas aeruginosa* (Altincicek et al. 2000). Genes encoding both of these enzymes and homologues of genes encoding the remaining enzymes in the pathway are present in the genome of strain R10.79. Several other organic compounds including alcohols and alkenes showed similar trends of emission during the different growth phases. The *m/z* 89.0597 (($\text{C}_4\text{H}_8\text{O}_2$) H^+) ratio increased after approximately 20 h and decreased later when the stationary phase was reached. This compound has the

m/z ratio of acetoin (3-hydroxy-2-butanone), which is produced in bacteria via the α -acetolactate pathway (Xiao and Xu 2007). Genes encoding an acetolactate synthase that catalyses the first step in this pathway are present in the genome of strain R10.79. However, we could not find a homologue for the second step in this pathway, acetolactate decarboxylase, encoded in the genome. Instead, we found a gene encoding an enzyme with similarity to diacetyl reductase (based on similarity to KEGG K18009) that can lead to acetoin production following the spontaneous production of diacetyl from acetolactate (Bryn et al. 1971). Contrarily, m/z 49.0107 ($(\text{CH}_4\text{S})\text{H}^+$) slightly increased throughout the exponential growth phase and reached a peak during the stationary phase followed by a fast decay. This organosulfur compound is most likely methanethiol (CH_4S) that is known to be emitted by *P. syringae* (Misztal et al. 2018) and is often associated with the primary breakdown of organic matter (sulfur containing amino acids). No other m/z was found with a comparable time trend. Other *Pseudomonas* species have been shown to produce methanethiol during methionine degradation (Segal and Starkey 1969). Thus, the detection of methanethiol during stationary phase may indicate the transition from acetate as primary source of carbon and energy to cell necromass degradation, including amino acids. This reaction is typically mediated by the methionine γ -lyase (MdeA), an enzyme with a pyridoxal-5'-phosphate (PLP) cofactor (Inoue et al. 1997). The gene encoding MdeA is absent in the genome of strain R10.79 according to a reciprocal best BLAST hit analysis with the *mdeA* sequence from *Pseudomonas putida*. However, the genome of strain R10.79 contains six other genes encoding enzymes with the same PLP cofactor, as indicated by the presence of the Cys_Met_Meta_PP Pfam family. The most similar of these genes has a similarity of 63% to MdeA (PIO94629). This is annotated as encoding "O-succinylhomoserine sulfhydrylase" that can also catalyse the production of methanethiol from methionine (Flavin and Slaughter 1967). m/z 33.0335 ($(\text{CH}_4\text{O})\text{H}^+$), protonated methanol, is also a well-known microbial VOC (e.g. Misztal et al. 2018) that showed an increase after approximately 15 h (during the exponential growth phase), which becomes less pronounced during the stationary phase. Methanol production by *Pseudomonas* has been detected during the degradation of pectin (Schink and Zeikus 1980) and

aromatic acids (Donnelly and Dagley 1980). While no such compounds were included in the minimal medium used here, the degradation of compounds synthesized by the bacteria might explain the observed methanol production. Misztal et al. (2018), who investigated microbial VOCs during growth of *P. syringae*, detected similar compounds as those reported in our study, with the exception of isoprene (C_5H_8), which may be due to difference in medium composition and in the experimental setup. In contrast to the results reported by Misztal et al. (2018) that were based on growth on complex medium, we could show that the metabolism of a single simple carboxylic acid, acetate, can lead to the synthesis of a large assembly of volatile organic compounds. Thus, microbial metabolism in principle can account for a large variety of organics found in the atmosphere and other environments even when the initial substrate is as simple as acetate.

3.5 Comparison of strains of *P. syringae* isolated from the atmosphere and from terrestrial environments

The stress response genes found in the *P. syringae* strain R10.79 were compared to the stress response genes found in a closely related epiphytic strain with a sequenced genome, *P. syringae* pv aptata. Both *P. syringae* strains possess very similar stress response genes (SI-Table 1). Due to their lifestyle, which combines growth on plant surfaces with atmospheric dispersal, epiphytic bacteria may be adapted to stress encountered both in the phyllosphere and the atmosphere, such as high UV-radiation, low water availability and large temperature shifts (Morris et al. 2004; Temkiv et al. 2012). Being exposed to similar types of stress, this may explain why closely related strains with similar adaptations are found in both environments (Morris et al. 2004). Further studies are necessary to understand, which is the primary environment where these adaptations developed and whether survival in the atmosphere is the primary or secondary benefit of these traits.

4 Conclusion

Based on a phylogenetic analysis of partial *cts* genes, we show that *P. syringae* strain R10.79 belongs to

phylogroup 2 of the *P. syringae* complex, a complex that includes most of the ice-nucleation active *P. syringae* strains. We could show experimentally that strain R10.79 can resist some of the most common atmospheric stress factors. A large fraction of cells (30% to > 90%) could withstand oxidative stress (up to 5 mM H₂O₂), osmotic stress (up to 2.5 M NaCl), UVB radiation (up to 1.1 J/cm²) and freeze–thawing. Cells of strain R10.79 were more susceptible to surface drying than to fast drying in nanoliter-sized droplets. The strain possesses several stress-response and stress-tolerance genes to cope with the types of stress they encounter in the atmosphere such as the freeze–thawing, desiccation, UV radiation and oxidative stress. Using metabolic substrate tests, we could demonstrate that strain R10.79 has a broad substrate spectrum and can grow on several substrates of high abundance in the atmosphere. In addition, we found key genes of pathways that allow the cells to utilize abundant carbon compounds in the atmosphere. Finally, we show that cells of strain R10.79 growing on acetate produce a large assembly of volatile organic compounds, including isoprene and methanol, and could therefore influence atmospheric budgets of climatically relevant compounds. Most importantly, this study of *P. syringae* strain R10.79 that combined physiological and genomic data that are relevant for the strains' response to the atmospheric conditions provides the groundwork for focused follow-up studies of the strain under specific atmospheric conditions.

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Author contributions ML, TB, TŠ-T and KF designed the study, ML carried out the bioinformatics analysis and TŠ-T carried out the experiments. IM and LS assisted in the genome assembly. BR performed the analysis of PTR-TOF-MS data. ML and TŠ-T prepared the manuscript with contributions from all co-authors.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Properties relevant to atmospheric dispersal of the ice-nucleation active *Pseudomonas syringae* strain R10.79 isolated from rain water

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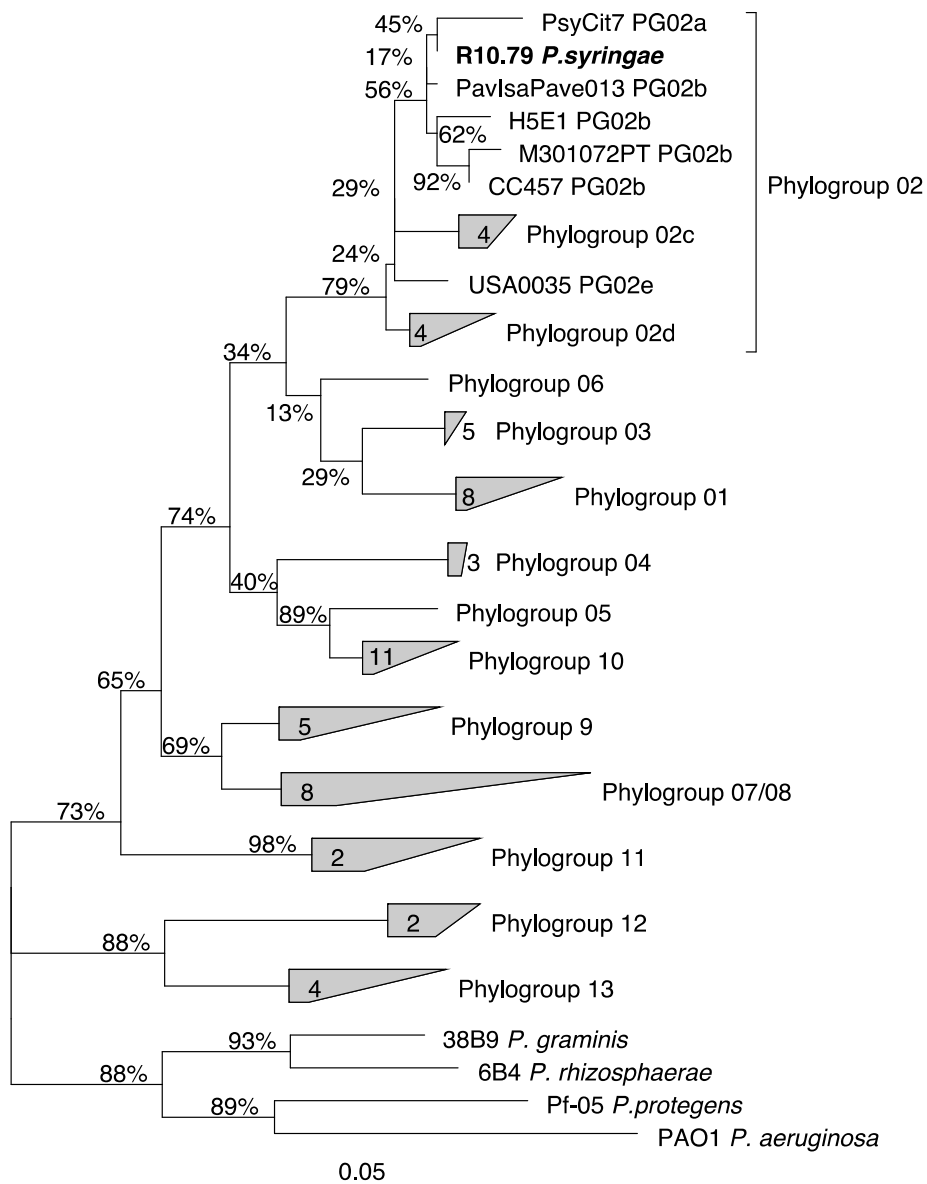


Figure S1: The phylogenetic position of strain *P. syringae* strain R10.79 in relation to 68 *P. syringae* reference strains was determined based on partial citrate synthase (*cts*) gene sequences (409 bp) (Figure 1). The citrate synthase gene was used because it has been shown that it represents the most reliable housekeeping gene to resolve phylogenies within the phylogroup of *P. syringae* (Berge et al. 2014). Comparative molecular phylogenetic analysis of the partial *cts* gene of *P. syringae* strain R10.79 and of 68 *P. syringae* reference strains by Maximum Likelihood method. Bootstrap percentage support for each split shown.