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1 Metabolomic study of the response to cold shock in a strain of *Pseudomonas syringae* isolated from cloud 2 water

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11 Keywords: NMR, MS, cloud, peptides, metabolomics, cold stress, Pseudomonas, oxidative stress.

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20 21 22 Abstract

Introduction

23 24 25 Active microorganisms have been recently discovered in clouds, thus demonstrating the capacity of microorganisms to exist in harsh environments, including exposure to UV and oxidants, osmotic and cold shocks, etc. It is important

26 to understand how microorganisms respond to and survive such stresses at the metabolic level.

27 Objectives

28 The objective of this work is to assess metabolome modulation in a strain of *Pseudomonas syringae* isolated from

- 29 30 cloud water and facing temperature downshift from 17 °C to 5 °C by identifying key molecules and pathways of the
 - response/adaptation to cold shock.

31 Methods

- 32 33 Bacterial extracts from suspensions of cells grown at 17 °C and further incubated in microcosms at 5 °C and 17 °C to
- mimic cloud conditions were analysed by combining LC-MS and NMR; the results were evaluated in comparison to
- 34 similar suspensions kept at constant temperature. The differences in the metabolome profiles were deciphered using
- 35 multivariate statistics (PLS-DA).

36 Results

37 Key cold shock biomarkers were observed, including cryoprotectants (trehalose, glucose, glycerol, carnitine, 38 glutamate), antioxidants (glutathione and carnitine) and their precursors, alkaloids (bellendine and slaframine) and metabolites involved in energy metabolism (ATP, carbohydrates). Furthermore, new short peptides (9 dipeptides and 39

40 a tetrapeptide) were found that have no known function.

41 Conclusions

1. Introduction

- 42 This study shows that in response to cold temperatures, *Pseudomonas syringae* PDD-32b-74 demonstrates numerous
- 43 metabolism modifications to counteract the impacts of low temperatures.
- 44 45
- 46 Microorganisms have been studied in different areas of the environment including soil as well as ocean and surface
- 47 waters; however, microorganisms alive in cloud water have been more recently examined (Desprès et al. 2012;
- 48 Sattler et al. 2001; Bauer et al. 2002; Amato et al. 2005; Amato et al. 2007a; Ahern et al. 2007; Kourtev et al. 2011;
- 49 Vaïtilingom et al. 2012; Šantl-Temkiv et al. 2013; Frölich-Nowoisky et al. 2016). Although only a fraction of the
- 50 total microbial communities can be detected by cloud water cultures (Amato et al. 2007b), measurements of anabolic

51 precursor incorporation rates by the biomass (Sattler et al. 2001; Vaïtilingom et al. 2011; Vaïtilingom et al. 2013) and 52 ATP quantification (Vaïtilingom et al. 2012; Vaïtilingom et al. 2013; Amato et al. 2007c; Hill et al. 2007) revealed 53 surprisingly high level of metabolic activity. Microbial metabolic activity in clouds is thus suspected to contribute to 54 atmospheric chemistry (Ariya and Amyot 2004; Delort et al. 2010); it also proves that microorganisms are able to 55 survive in stressful atmospheric conditions, such as temperature changes, UV radiations, evaporation-condensation 56 cycles, exposure to chemical mixtures with potentially toxic compounds, strong oxidants, and acidic pH 57 (Deguillaume et al. 2014). A recent study by Joly et al. (2015) suggested that the greatest stresses encountered in the 58 atmosphere by microorganisms are freeze/thaw and condensation/evaporation cycles, both of which are generally 59 linked with cloud temperature shifts.

60 At low temperatures, global cellular functioning is generally altered due to decreased molecular diffusion and 61 reaction rates as well as the rigidification of macromolecules and membranes. Various well-known responses in 62 microorganisms include the production or uptake of compatible solutes (e.g., Welsh 2000), modifications of the lipid 63 composition of membranes, production of chaperones and modified enzymes capable of functioning in the cold (e.g., 64 Feller and Gerday 2003), and adaptation of energy metabolism (e.g., Amato and Christner 2009). To better 65 understand how bacteria globally respond to such temperature shifts and resist atmospheric stresses, the response of 66 Pseudomonas syringae PDD-32b-74 to a cold shock was examined in this study. The choice of Pseudomonas 67 syringae 32b-74 as model strain is based on the abundance of Pseudomonas species in clouds at Puy de Dôme 68 (Vaïtilingom et al. 2012), in particular, *P. syringae*, and their capacity to be involved in atmospheric chemical (Amato 69 et al. 2007c) and physical processes (Attard et al. 2012; Joly et al. 2013; Amato et al. 2015; Renard et al. 2016). 70 Pseudomonas syringae 32b-74 is also of particular interest as it has a very efficient ice nucleation activity, which 71 could be advantageous to resist cold shocks (Joly et al. 2013). To study the impact of temperature on its metabolome, 72 a metabolomic approach combining LC-MS and NMR was used. Although it is still scarcely applied in the field of 73 environmental microbiology, metabolomics was shown to be a powerful tool to investigate the influence of 74 temperature changes on microbial metabolomes (Coucheney et al. 2008; Boroujerdi et al. 2009; Boroujerdi et al. 75 2012; Singh et al. 2011; Ye et al. 2012; Jozefczuk et al. 2010; Miranda et al. 2013; Azizan et al. 2012; Alreshidi et al. 76 2015). 77

78 2. Materials and Methods

Chemicals, reagents, standard solutions, sample preparations, and details on analytics (NMR, MS and statistics) are fully described in the supplementary material. A general outlook of the experimental design used for conducting the metabolomics experiment is also presented in the supplementary material (Figure S1).

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83 2.2. Microbial incubation and metabolite extraction

Pseudomonas syringae PDD-32b-74 (GenBank accession number of the 16S rRNA gene sequence: HQ256872) was
 originally isolated from cloud water sampled at the puy de Dôme summit (1465 m) in November 2009 (Vaïtilingom et al. 2012). First, the bacteria were cultured on agar plates (12 biological replicates) at 17 °C (Figure S1, I).

87 Second, bacteria collected from the plates were transferred in an aqueous medium (water) to mimic cloud water and 88 incubated for 14 hours at either 5 °C or 17 °C (Figure S1, II). The two temperatures were chosen according to the 89 average temperatures measured at the puy de Dôme summit where cloud water is sampled during winter (5 °C) and 90 summer (17 °C). In this way, two typical atmospheric scenarios can be mimicked. At the end of incubation in the 91 aqueous medium, the cell pellets were centrifuged and rinsed twice with sterile water. The metabolomes of the 24 92 bacterial pellets were extracted with 600 µL of a cold ternary solvent [H₂O:MeOH:CH₃CN, 1:2:2; (Lin et al. 2007; 93 Rabinowitz & Kimball 2007; Shin et al. 2010)]. Finally, 12 extracts of 600 µL were obtained for each incubation 94 temperature (5 $^{\circ}$ C and 17 $^{\circ}$ C). These extracts were treated for NMR or MS analyses (Figure S1, IV).

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2.3. LC-MS and LC-MS-MS analyses (figure S1, IV and SM-§2) 97

UPLC-MS analyses for profiling

99 Seventy-two profiles (3 x 20 µL aliquots of each extract) were analysed using the Metabolic profiler® platform

100 (Bruker). Briefly, a fast LC system (Agilent 1200 series) was equipped with an Acquity HSST3 C18 column (50 x

101 (Bluer). Briefly, a fast DC system (Agriefit 1200 series) was equipped with an Acquity HSS15 C18 column (50 x 2.1 mm, 1.8 μ m; Waters) and coupled to a MicroToF mass spectrometer in positive electrospray ionization mode (50-1002 m/z; Bruker).

UPLC-MS/MS analyses for structural identification

A few samples that were representative for the features in the statistical model (PLS-DA) were analysed in full scan mode and MS/MS on an LTQ Orbitrap Velos MS (ThermoFisher Scientific) with similar chromatographic conditions (gradient, column) as for profiling.

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108 **2.4. NMR analyses** (figure S1, IV and SM-§3) 109

1D on flow NMR analyses for profiling

111 Twenty-four one-dimensional ¹H NMR *noesygppr1d* experiments (*i.e.*, profiles) were done on a Metabolic Profiler® 112 equipped with an Avance III 500 MHz NMR spectrometer using a 3 mm FISEI z-gradient (¹H-¹³C) probe with a 60 113 μ L flow cell (Bruker).

1D and 2D NMR analyses for structural identification

The NMR spectrometer was an Avance 500 MHz equipped with a 5-mm inverse-triple tuned (TXI) ¹H/1³C/¹⁵N with z-gradient coil probe (Bruker). One-dimensional ¹H-Spectra and 2D homonuclear (¹H-¹H COSY, ¹H-¹H TOCSY, ¹H-¹H JRES) and heteronuclear (¹H-¹³C HSQC) experiments were performed for structural identification.

119 **2.5. Data analyses** (SM-§4 & 5) 120

MS and NMR data treatments

The MS raw data were exported using NetCDF format and extracted with the XCMS package (Smith et al. 2006) in the R environment (version 2.15.3). Quadratic correction of the retention time was performed to consider the chromatography time shift evolution in the analytical experiments. In-source fragmentation and adducts were screened using CAMERA package (in the R environment; Kuhl et al. 2012) in order to reduce information redundancy.

127 One-dimensional ¹H-NMR spectra were divided into 0.001 ppm wide buckets after reduction over the chemical 128 range of -0.5 to 10 ppm and considering the exclusion area around the residual water signal (4.6 to 5.1 ppm), using 129 AMIX software (Bruker). The signal intensity in each bucket was integrated using the sum of the intensities mode, 130 and the spectra were scaled to total intensity.

Statistical analyses

One matrix per analytical device [ESI(+)/MS or ¹H-NMR] was built and subjected to multivariate analyses (SIMCA-P v. 12, Umetrics). Principal component analyses (PCA) were conducted to check the population homogeneity and detect possible outliers. A supplementary variable ("Y matrix") corresponding to the two classes of bacteria populations (exposed and not exposed to cold shock; Figures S2 and S3) was considered for the Partial Least Squares (PLS) regression analyses.

Databases

Identification was completed using the metabolite and pathways databases [KEGG (Kanehisa and Goto 2000),
 MetaCyc (Caspi et al. 2014), HMDB (Wishart et al. 2013), and METLIN (Smith et al. 2005)] as well as relevant

- 140 publications (Nicholson et al. 1995; Fan 1996). MS/MS fragmentation patterns were compared to simulations with
- 141 the Massfrontier (v.7; Thermo Scientific) software.
- 142

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143 **3. Results and Discussion**

144 **3.1 Identification of cold shock biomarkers in** *Pseudomonas syringae* **32b-74**

145 146 Figure 1a presents the PLS-DA analysis performed with 72 samples based on the LC-MS analysis. The two 147 populations corresponding to the bacterial suspensions incubated at 17 °C and 5 °C are clearly separated. Using the 148 two first components, 62 % of the variability explanation was obtained. One outlier sample was removed from the 149 MS data. A list of 118 ion discriminating cells incubated at the 2 temperatures was obtained to compose the VIP list. 150 Thirty-five ions were identified due to their exact mass and fragmentation pattern (validated by complementary 151 Orbitrap experiments), the use of reference samples or by a validation based on their detection by NMR (Table 1). 152 The detailed list of the masses and retention times of the identified metabolites is given in the supplementary data 153 (Table S2).

155 (1a 154

155 Figure 1b shows the PLS-DA analysis performed with the results from 24 samples based on 1D ¹H NMR

- 156 experiments; the two populations corresponding to the bacterial suspensions incubated at 17 °C or at 5 °C are clearly
- 157 separated along the first axis, explaining 32.7 % of the data dispersion. From this statistical analysis, a list of 260
- discriminating buckets was obtained to compose the VIP list; 21 metabolites (including two glucose isomers) were
- 159 identified as biomarkers. Their identification was validated by confirmation of their structure by 2-D NMR spectra
- 160 (¹H-¹H JRES, ¹H-¹H COSY, ¹H-¹H TOCSY, ¹H-¹³C HSQC) or by a validation due to MS information (Table 1). The detailed list of the ¹H and ¹³C chemical shifts as well as the multiplicity and coupling constants of the identified
- 101 detailed list of the 'H and 'C chemical shifts as well as the multiplicity and coupling constants of the id 162 metabolites are given in the supplementary data (Table S3).
- 162
- Table 2 reports the VIP values of the metabolites of interest as well as the ratio of relative intensities of a given metabolite in cells incubated at 5 °C with respect to cells incubated at 17 °C, termed the stressed/reference (S/R) ratio. The identification metrics proposed by Sumner et al. (2007, 2014) are used in this paper to assign an identification score to each metabolite (as explained in SM-§5 and Table S1).
- The comparison of the data presented in Table 2 clearly shows the complementarity nature of MS and NMR. On one hand, all the peptides, most lipids and amines, as well as some specific compounds [phosphoric acid, slaframine, gyromitrin, sarcosine, bellendine, 5'-desoxy-5'-(methylthio)adenosine] were only detected by LC-MS. On the other hand, NMR was very useful to detect sugars and other metabolites such as valine, tyrosine and glycine, glycerol, Ophosphocholine, ATP, acetate and lactate that could not be detected by MS.
- Finally, it is worth noting that when molecules were detected by both techniques, the S/R ratios derived from MS and
 NMR were very consistent; common amino acids (Leu/Ile, Ala, Lys, Phe) and amines (putrescine) were decreasing
- 174 NMR were very consistent; common amino acids (Leu/Ile, Ala, Lys, Phe) and amines (putrescine) were decreasing 175 under cold shock while AMP and glutathione concentrations were increasing.
- 176 Other analytical techniques could use complementary information such as GC-MS and FT-ICR/FT-MS.
- 177 The detailed evolutions of the metabolite concentrations are fully described in the next section in relation to their role
- 178 in maintaining vital functions facing cold stress.
- 179

180 3.2 *Pseudomonas syringae* PDD-32b-74 adapts its metabolism to maintain vital functions facing cold stress

From the list of the biomarkers identified in Table 2 and referring to the KEGG databases and tools, the metabolic pathways impacted by the cold shock imposed to *Pseudomonas syringae* PDD-32b-74 (see Figure 2 and 3) are deciphered. The metabolites that are over-produced at 5 °C are indicated in yellow, while those that are underproduced are indicated in blue; putative important metabolic intermediates not detected among the VIP ions are indicated in white. The modulation of these metabolite concentrations reflects the adaptation of *Pseudomonas*

- 186 *syringae* PDD-32b-74 metabolism to maintain vital functions while facing cold stress.
- 187 Energy metabolism: At low temperatures, enzymatic activities and molecular diffusion are slowed down. To 188 maintain metabolic activity compatible with survival, some bacteria adjust their energy metabolism for increasing 189 ATP concentration notably (Amato and Christner 2009). As a consequence, a net increase of the energetic pool 190 constituted by AMP, ATP and Pi are observed when *P. syringae* PDD-32b-74 is exposed to cold shock (Figure 2). 191 This could result from the activation of carbohydrate metabolism and of the Krebs cycle as the glucose and trehalose 192 concentrations increased under cold conditions resulting from the degradation of glycogen.
- The decrease of acetic and lactic acid concentrations (Figure 2) might be consistent with the stimulation of respiration pathways and a slowdown of the fermentation metabolism. The decrease could also result from increased utilization. For example, a cold-adapted *Propionibacterium* species was shown to switch from aerobiose to anaerobiose when exposed to cold temperatures with the increased lactate uptake due to overexpression of the lactate dehydrogenase (Sardesai and Babu 2000). Proteomic studies of Proteobacteria species (*Rhizobium*, and *Psychrobacter*) exposed to cold shock also revealed overexpression of this enzyme (Bakermans et al. 2007; Dalmasso et al. 2012).
- 200

201 Membrane fluidity: When subjected to cold, bacteria have to deal with a very important issue: the rigidification of 202 their membrane. They need to re-establish membrane fluidity by adjusting the lipid composition to maintain vital 203 functions such as the proton motive force and other ion exchange processes. For instance, some bacteria modify the 204 balance phosphatidylcholine (PC) vs. phosphatidylethanolamine (PE; Fajardo et al. 2011) or increase the 205 concentration of unsaturated fatty acids (Arvizu-Gomez et al. 2013). In this case, lipid metabolism (Figure 2) was 206 globally slowed down as shown by the decrease of oleic and palmitoleic acid concentration (fatty acid metabolism) 207 and of the derivatives of phosphocholine and phosphoethanolamine (glycerophospholipid metabolism). However, 208 more lipid composition data would be needed to conclude the adjustment of membrane fluidity. In the future, more 209 specific extraction methods should be used to better characterize the lipid fraction.

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211 Protection against ROS (Reactive Oxygen Species): Glutathione was clearly at higher concentration at 5 °C as well 212 as other metabolites closely linked with it, including glutamate, glycine and 5'-desoxy 5'-methylthio(adenosine) 213 [analogue of 5'- methylthio(adenosine); Figure 3]. Glutathione is a tripeptide composed of glutamate, cysteine and 214 glycine; the increased glutathione concentration at low temperature is consistent with the concomitant increase of 215 glutamate and glycine concentrations. In parallel, carnitine concentration was also increased at 5 °C (Figure 3). 216 Glutathione and carnitine are well known key metabolites involved in oxidative stress metabolism (Davies 2000). 217 Many papers report a close relationship between cold stress and oxidative stress in bacteria. It was shown that the 218 production of free radicals $(O_2^{\circ} and OH)$ and H_2O_2 is increased when the temperature is decreased (Zhang et al. 219 2012). As a result, bacteria can increase the amount of detoxifying enzymes such as superoxide dismutase and 220 catalase for preventing the formation of radicals, or they can produce antioxidant compounds for reducing ROS 221 concentrations. Zhang et al. (2012) recently showed that GSH improves the resistance of Lactobacillus 222 sanfranciscensis to cold shock. Additionally, Arvizu et al. (2013) showed the induction of a gene encoding for 223 catalase in Pseudomonas syringae pv phaesicola when the temperature was decreased. In their metabolomic studies, 224 Singh et al. (2011) and Ye at al. (2012) suggested that the oxidative stress pathways could be impacted by 225 temperature shifts in L. monocytogenes and E. coli. In this study, the activation of the oxidative stress metabolism in 226 Pseudomonas syringae PDD-32b-74 is consistent with its high survival capacity under UV exposure and high 227 concentration of H_2O_2 (Joly et al. 2015).

229 Synthesis of cryoprotectants: To avoid disruptive freezing and maintain intracellular osmotic pressure, bacteria 230 synthesize small weight molecules called "cryoprotectants" or "compatible solutes" (e.g., polyols, polyamines, 231 carbohydrates and amino acids derivatives; see Shivaji and Prakash 2010). The most common cryoprotectants found 232 in bacteria are trehalose, glycine, betaine, glycerol and carnitine. These cryoprotectants are supposed to act as 233 "chemical chaperones" that prevent the denaturation and aggregation of proteins. In the case of Pseudomonas 234 syringae PDD-32b-74, we observed the accumulation of carbohydrates such as trehalose and glucose, amines such as 235 carnitine and amino acids such as glutamate and glycine, and glycerol (Figure 2 and 3). The same cryoprotectants 236 were found in metabolomic studies namely, trehalose, glucose, glycerol, glycine, carnitine and glutamate in the 237 psychrotolerant Listeria monocytogenes (Singh et al. 2011) and glycine in a Staphylococcus aureus strain exposed to 238 cold conditions (Alreshidi et al. 2015). Trehalose has already been reported as a cryoprotectant in other 239 Pseudomonas, in particular in different strains isolated from the rhizosphere of Himalayan plants (Bisht et al. 2013). 240 Glutamate and trehalose were shown to accumulate in Pseudomonas syringae when subjected to osmotic stress 241 (Kurz et al. 2010). 242

243 Alkaloid synthesis: Cold shock impacted the number of metabolites that are interconnected within the degradation 244 pathways of ornithine and lysine, including the unexpected alkaloids, bellendine and slaframine (Figure 3). 245 Bellendine was found at higher concentration in the cold condition; however, slaframine was present at lower 246 concentration. Bellendine has been described in plants while slaframin is a mycotoxin. Interestingly, in 247 a metabolomic study, slaframine concentration was shown to vary when the fungal plant pathogen Rhizoctonia solani 248 was grown in pure cultures or in dual-cultures with the mycoparasite Stachybotrys elegans (Chamoun et al. 2015). It 249 is worth noting that *Pseudomonas syringae* is from the phyllosphere, and some species can be plant pathogens 250 related to the presence of these alkaloids; although, they have not been described in such bacteria. 251

252 **Peptide synthesis:** The most striking result issued from this metabolomic study is the evidence of concentration 253 changes of specific short peptides (9 dipeptides and 1 tetrapeptide; Figure 2). Mass spectra analysis shows that these 254 peptides are linear, and their low number and specific composition exclude random protein degradation. 255 Interestingly, a parallel concentration change of the amino acids is present in these peptides. For instance, the 256 concentration of Ala-leu, Leu-Leu, Ala-Arg, Glu-Ala, Pro-Phe, Pro-Ile, Leu-Phe and Val-Val were decreasing at the 257 same time as that of Ala, Leu, Ile, Val and Phe. In contrast, the dipeptide Ser-Val and the tetrapeptide Gln-Ser-Ala-258 Gln were overproduced. A common amino acid to these two peptides is Serine; although, it could not be identified as 259 free in this dataset, and the synthesis of this amino acid is achieved from phosphoglyceric acid simultaneously as the 260 synthesis of glycine (Figure 2 and 3). Additionally, glutamine (a putative compound), which is present in the 261 tetrapeptide, is directly linked to glutamate that is also overproduced.

This is the first clear report of such peptides as cold shock biomarkers; in addition, the presence of such linear short peptides was unexpected, as they have never been described before in *Pseudomonas* species. Nonlinear dipeptides have been described in *Pseudomonas* strains, and some cyclic dipeptides are involved in the regulation of quorum sensing (Degrassi et al. 2002); others such as NAGGN: N-AcethylGlutaminylGlutamine amide were shown to be 266 synthesized in response to osmotic stress (Smith and Smith 1989; Sagot et al. 2010). However, it is unlikely that all 267 these nonlinear dipeptides play the same role as the linear peptides detected in this study. The presence of linear short 268 peptides has only been reported in a few cases when bacteria are facing osmotic stresses. Using a metabolomic 269 approach, Kol et al. (2010) showed an upregulation of one dipeptide (Gly-Pro) and four tripeptides (Arg-Gly-Pro, 270 Lys-Gly-Pro, Glu-Gly-Pro and Ala-Gly-Pro), and in response to salt shock in Steptomyces coelicolor, these peptides 271 contained proline. The presence of short and linear proline-containing peptides has also been reported in other 272 microbial strains facing osmotic shocks, including Listeria monocytogenes (Amezaga et al. 1995), Lactobacillus 273 casei (Piuri et al. 2003), Oenococcus oeni (Le Marrec et al. 2007) and Bacillus subtilis (Zaprasis et al. 2013). The 274 intracellular accumulation of these proline-containing peptides is consistent with the known role of proline as an 275 osmoprotectant, which is obtained after peptide hydrolysis. However, these peptides may also have regulatory 276 functions, which are not yet understood, as observed in Oenococcus oeni (Le Marrec et al. 2007).

In this case, the function of the peptides over- or underproduced under cold shock remains to be discovered.

279 Relative importance of the resistant mechanisms to cold shock in Pseudomonas syringae:

The previous results show that many resistance mechanisms to cold shock are present in this strain. The VIP score values (Table 2) can be used to determine which mechanisms are the most important as these scores reflect the relative importance of each metabolite to discriminate the two temperatures. The higher the VIP value, the more discriminant it is. Based on the VIP score histograms from the MS and NMR results (Figure S4), three signal groups have been highlighted. The metabolites which present the highest VIP scores (group 1) are glutathione, trehalose, palmitoleic acid and (iso)Leucine (Table 2). The second group contains some amines and amino acids (piperidine, lysine and phenylalanine) and some dipeptides (Ala-Leu, Leu-Leu); the other metabolites are in the third group.

- These results suggest that the most important resistance mechanisms to this temperature change are the protection against oxidative stress (glutathione), the production of osmo/cryoprotectants (trehalose), and the adaptation of the membrane composition (Lipids). This result is consistent with the SEED subsystems annotated in the genome sequence of this strain that was recently published (Besaury et al 2017). From 181 SEED subsystems affiliated to stress response, the majority were assigned to the responses to oxidative stress (79), osmotic stress (32) and cold shock (5).
- 293

4. Conclusions

295 This is the first study reporting the metabolic response of a cloud bacterium to a cold shock, which is important to 296 the survival of bacteria in the atmosphere. The use of combined MS and NMR helped identify a number of 297 biomarkers and specify the metabolic routes potentially impacted by this stress. Pseudomonas syringae PDD-32b-74 298 could efficiently adapt its metabolism to re-establish some of its essential functions. It synthesized cryoprotectants 299 (trehalose, glutamate, carnitine and glycine) to maintain cellular structural and functional integrity. These 300 cryoprotectants could also act as osmoprotectants, which are also potentially important for survival in the 301 atmosphere. In clouds, bacteria are subjected to evaporation-condensation cycles inducing important osmotic 302 variations in cloud droplets. As a response to temperature shifts, P. syringae PDD-32b-74 is also reacted by 303 activating the metabolic route of glutathione. This indicates the high potential of this strain to protect against ROS. 304 Finally we showed the presence of unexpected peptides which synthetic pathways were impacted. The function of 305 these peptides remains unknown; however, new directions of research are introduced in the adaptation of 306 microorganisms to temperature changes and their general metabolic functioning. This study showed that P. syringae 307 32b-74 has a great potential to survive in clouds; it can adapt not only to cold stress but also to osmotic and oxidative 308 stresses. These stresses are actually concomitant in the atmosphere and might be related to the preponderance of the 309 genus Pseudomonas in clouds.

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484 Figure legends

486 Fig. 1 a) Score plot from the PLS-DA of Pareto scaled LC-MS data (PC1 vs PC2 model). Blue triangles and red dots
487 are from extracts of bacteria incubated at 5 °C and 17 °C, respectively. In one group (5 °C), one sample is considered
488 as an outlier (out of Hotelling ellipse). b) Score plot from PLS-DA of Pareto scaled NMR data (PC1 vs PC2 model).

491 Fig. 2 Metabolic pathways (carbohydrates, amino-acids, lipids and peptides) impacted by exposure of *Pseudomonas* 492 syringae to cold shock. Metabolites that are overproduced at 5 °C are in yellow, and metabolites that are
 493 underproduced are in blue. Putative intermediates are indicated in white.

Fig. 3 Metabolic pathways of *Pseudomonas syringae* (glutathione, ornithine and lysine) impacted by exposure to cold. Metabolites that are overrepresented at 5 °C compared with 17 °C are in yellow, and metabolites that are underrepresented are in blue. Putative intermediates are indicated in white.

501 **Table 1:** Identification criteria for metabolites considering MS and NMR experiments, and according to relevant

502 databases (or standard when mentioned). MS experiments: TOF, accurate mass measurement on LC-ToF (Time of

503 Flight); HRMS, accurate mass measurement on LC-Orbitrap; MS/MS, fragmentation pattern on LC-Orbitrap; STD,

504 comparison using pure chemical standard (at least parent ion mass and retention time). NMR experiments (validation

505 mark for at least one result in the following experiment): ¹H, chemical shift on proton NMR experiment; Jres,

506 chemical shift and coupling constant on J-resolved experiment; COSY, chemical shift and correlation on COrrelation

507 SpectrocopY experiment; TOCSY, chemical shift and correlation on TOtal Correlation SpectroscopY experiment;

508 HSQC, chemical shift and correlation on Heteronuclear Single Quantum Correlation experiment

		MS EXP	ERIMENTS		NMR EXPERIMENTS				
Identification status	TOF	HRMS	MS/MS	STD	¹ H	Jres	COSY	TOCSY	HSQC
5'-Deoxy-5'-	Х	Х	Х						
(methylthio)adenosine									
Acetate					Х				Х
Alanine	Х				Х	Х	Х	Х	Х
Alanyl-Arginine		Х	Х	Х					
Alanyl-Leucine		Х	Х	Х					
AMP	Х			х			Х		Х
ATP							Х		
Bellendine	х								
Carnitine	Х	Х							
Feruloylputrescine	Х								
Gln-Ser-Ala-Gln		Х	Х						
Glutamate	Х			Х	Х	Х	Х		Х
Glutamyl-Alanine		Х	Х	Х					
Glutathione	Х				Х	Х	Х	Х	Х
Glycerol					Х	Х	Х	Х	Х
Glycine					Х				Х
Glycogen					Х	Х	Х	Х	Х
Gyromitrin	Х								
Isoleucine	X ¹				Х	Х	Х		Х
Lactate					Х	Х	Х		Х
Leucine	X1				Х	Х	Х	Х	Х
Leucyl-leucine	Х			Х					
Leucyl-Phenylalanine	Х			х					
Lysine	Х	Х			Х	Х	Х	Х	Х
LysoPC(16:1)	Х								
LysoPE 16:0	Х			х					
LysoPE 18:1	Х			х					
LysoPE(0:0/16:1(9Z))	Х				1				
Methionine	Х				Х	Х	Х	Х	Х
Oleic acid	Х								
O-phosphocholine					Х				х
Palmitoleic acid	Х			Х	1				
PE(P-16:0e/0:0)	Х				1				
Phenylalanine	Х	Х		Х	Х	Х	Х		Х

Phosphoric acid	Х	Х							
Piperidine	Х			Х					
Proline-(iso)Leucine		Х	Х	Х					
Proline-Phenylalanine	Х								
Putrescine	Х			Х			Х	Х	Х
Sarcosine	Х								
Serine-Valine		Х	Х	Х					
Slaframine	Х								
Trehalose					Х	Х	Х	Х	Х
Tyrosine					Х	Х	Х		Х
Valine					Х	Х	Х		Х
Valine-Valine		Х	Х	Х					
α-Glucose					Х	Х	Х	Х	Х
β-Glucose					Х	Х	Х	Х	Х



¹ MS data do not permit to differentiate leucine and isoleucine.

Table 2: Identified metabolites according to MS and NMR experiments. All metabolites presented here are key biomarkers dicriminating the two bacterial populations incubated at 5°C (stress) and 17°C (reference). Statistical model provide a VIP value of metabolites' influence for each analytical technique. S/R (stress/reference) ratio represents the ratio of the means relative intensities measured on MS or NMR profiles of a given metabolite in cells incubated at 5 °C vs at 17°C.

Identified	Ν	MS NMR			ID metrics**			
VIP Metabolites	VIP score	S/R Ratio	VIP score S/R Ratio		class (score)			
Amino acids								
Valine			2.17-2.49	0.49	II (5)			
Leucine	2.01	0.501	3.38	0.51	II (9)			
Isoleucine	3.91	3.91 ¹ 0.59 ¹		0.38-0.5	II (5)			
Alanine	1.42	0.58	1.34-1.68	0.77-0.83	II (10)			
L-Lysine	2.76 ²	0.42 ²	1.18-1.49	0.72-0.77	II (10)			
Phenylalanine	2.91	0.53	1.09-1.37	0.72-0.74	Ι			
Tyrosine			1.03-1.08	0.49-0.52	II (5)			
Methionine	1.71	0.52	1.32-1.49	0.79-0.8	II (10)			
Sarcosine	1.62	0.52			III (1)			
L-Glutamate	1.222	1.18²	1.41-1.75	ND*	Ι			
Glycine			1.01	1.09*	II (2)			
	Sacch	narides						
Glucose			1.04-1.32	1.39-1.59	II (9)			
Trehalose			5.42-6.06	2.5	II (9)			
Glycogen			1.13-1.44	0.75-0.85	II (9)			
	Am	ines						
Putrescine	2.05	0.46	1.57-1.80	0.51-0.54	Ι			
Feruloylputrescine	1.64	0.28			III (1)			
Piperidine	3.41	0.5			Ι			
Carnitine	1.62	2.38			III (1)			
Lipids								
Palmitoleic acid	4.20	0.26			Ι			
Oleic acid	2.48	0.44			III (1)			
LysoPE 18:1	3.04	0.3			Ι			
O-phosphocholine			1.16	0.78*	II (2)			
LysoPE(0:0/16:1(9Z))	1.03	0.74			III (1)			
LysoPE 16:0	2.43	0.4			Ι			
LysoPC 16:1	1.66	0.37			III (1)			
PE(P-16:0e/0:0)	1.21	0.65			III (1)			
Miscellaneous								

Slaframine	1.78	0.11			III (1)			
Acetate			1.01-1.28	0.72-0.78	II (2)			
Lactate			1.76-2.04	0.67-0.78	II (5)			
AMP	1.49	1.28	1.03	1.11	Ι			
ATP			1.02-1.26	1.11-1.25	II (5)			
Phosphoric acid	1.42	1.45			III (1)			
Glycerol			4.40	1.45*	II (9)			
Gyromitrin	1.02	1.2			III (1)			
Bellendine	1.71	3.7			III (1)			
Glutathione	5.71	2	1.45-1.90	1.27-1.59	II (10)			
5'-Deoxy-5'-(methylthio)adenosine	1.09	2.5			II (2.5)			
Peptides								
Alanyl-leucine	2.78	0.35			Ι			
Leucyl-leucine	2.76	0.27			Ι			
Alanyl-arginine	2.11	0.22			Ι			
Leucyl-phenylalanine	1.72	0.3			Ι			
Glutamyl-alanine	1.61	0.44			Ι			
Prolyl-phenylalanine	1.40	0.22			III (1)			
Prolyl-(iso)leucine	1.37	0.22			Ι			
Valyl-valine	1.06	0.33			Ι			
Seryl-valine	1.34	1.75			Ι			
Gln-Ser-Ala-Gln	1.1	5.88			II (3)			

517 518 519 ¹ not differentiable through MS experiments; ² information about L/D conformation is not available through MS experiments; * quantifications are given according to ¹H-NMR buckets with peak overlaps. ** Classes and scores are according to Sumner et al. (2007; 2014; See Table S1).