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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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21 **Abstract**

23 **Introduction**

24 Active microorganisms have been recently discovered in clouds, thus demonstrating the capacity of microorganisms
25 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 cloud water and facing temperature downshift from 17 °C to 5 °C by identifying key molecules and pathways of the
30 response/adaptation to cold shock.

31 **Methods**

32 Bacterial extracts from suspensions of cells grown at 17 °C and further incubated in microcosms at 5 °C and 17 °C to
33 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
39 metabolites involved in energy metabolism (ATP, carbohydrates). Furthermore, new short peptides (9 dipeptides and
40 a tetrapeptide) were found that have no known function.

41 **Conclusions**

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.

45 **1. Introduction**

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 Vaitilingom 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; Väitilingom et al. 2011; Väitilingom et al. 2013) and
52 ATP quantification (Väitilingom et al. 2012; Väitilingom 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 (Väitilingom 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**

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

82 83 **2.2. Microbial incubation and metabolite extraction**

84 *Pseudomonas syringae* PDD-32b-74 (GenBank accession number of the 16S rRNA gene sequence: HQ256872) was
85 originally isolated from cloud water sampled at the puy de Dôme summit (1465 m) in November 2009 (Väitilingom
86 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 °C and 17 °C). These extracts were treated for NMR or MS analyses (Figure S1, IV).

95 96 **2.3. LC-MS and LC-MS-MS analyses** (figure S1, IV and SM-§2)

97 98 *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 2.1 mm, 1.8 µm; Waters) and coupled to a MicroToF mass spectrometer in positive electrospray ionization mode (50-
102 1000 m/z; Bruker).

103 *UPLC-MS/MS analyses for structural identification*

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

107 **2.4. NMR analyses** (figure S1, IV and SM-§3)

108 *1D on flow NMR analyses for profiling*

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

114 *1D and 2D NMR analyses for structural identification*

115 The NMR spectrometer was an Avance 500 MHz equipped with a 5-mm inverse-triple tuned (TXI) $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ with
116 z-gradient coil probe (Bruker). One-dimensional ^1H -Spectra and 2D homonuclear (^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H -
117 ^1H JRES) and heteronuclear (^1H - ^{13}C HSQC) experiments were performed for structural identification.
118

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

120 *MS and NMR data treatments*

121 The MS raw data were exported using NetCDF format and extracted with the XCMS package (Smith et al. 2006) in
122 the R environment (version 2.15.3). Quadratic correction of the retention time was performed to consider the
123 chromatography time shift evolution in the analytical experiments. In-source fragmentation and adducts were
124 screened using CAMERA package (in the R environment; Kuhl et al. 2012) in order to reduce information
125 redundancy.
126

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.

131 *Statistical analyses*

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

137 *Databases*

138 Identification was completed using the metabolite and pathways databases [KEGG (Kanehisa and Goto 2000),
139 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

143 **3. Results and Discussion**

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

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

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
158 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
161 detailed list of the ¹H and ¹³C chemical shifts as well as the multiplicity and coupling constants of the identified
162 metabolites are given in the supplementary data (Table S3).

163
164 Table 2 reports the VIP values of the metabolites of interest as well as the ratio of relative intensities of a given
165 metabolite in cells incubated at 5 °C with respect to cells incubated at 17 °C, termed the stressed/reference (S/R)
166 ratio. The identification metrics proposed by Sumner et al. (2007, 2014) are used in this paper to assign an
167 identification score to each metabolite (as explained in SM-§5 and Table S1).

168 The comparison of the data presented in Table 2 clearly shows the complementarity nature of MS and NMR. On one
169 hand, all the peptides, most lipids and amines, as well as some specific compounds [phosphoric acid, slaframine,
170 gyromitrin, sarcosine, bellendine, 5'-desoxy-5'-(methylthio)adenosine] were only detected by LC-MS. On the other
171 hand, NMR was very useful to detect sugars and other metabolites such as valine, tyrosine and glycine, glycerol, O-
172 phosphocholine, ATP, acetate and lactate that could not be detected by MS.

173 Finally, it is worth noting that when molecules were detected by both techniques, the S/R ratios derived from MS and
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**

181 From the list of the biomarkers identified in Table 2 and referring to the KEGG databases and tools, the metabolic
182 pathways impacted by the cold shock imposed to *Pseudomonas syringae* PDD-32b-74 (see Figure 2 and 3) are
183 deciphered. The metabolites that are over-produced at 5 °C are indicated in yellow, while those that are under-
184 produced are indicated in blue; putative important metabolic intermediates not detected among the VIP ions are
185 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.

193 The decrease of acetic and lactic acid concentrations (Figure 2) might be consistent with the stimulation of
194 respiration pathways and a slowdown of the fermentation metabolism. The decrease could also result from increased
195 utilization. For example, a cold-adapted *Propionibacterium* species was shown to switch from aerobiose to
196 anaerobiose when exposed to cold temperatures with the increased lactate uptake due to overexpression of the lactate
197 dehydrogenase (Sardesai and Babu 2000). Proteomic studies of Proteobacteria species (*Rhizobium*, and
198 *Psychrobacter*) exposed to cold shock also revealed overexpression of this enzyme (Bakermans et al. 2007;
199 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.

210
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 $^{\circ}OH$) 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 *phaeolicola* when the temperature was decreased. In their metabolomic studies,
224 Singh et al. (2011) and Ye et 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).
228

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.

262 This is the first clear report of such peptides as cold shock biomarkers; in addition, the presence of such linear short
263 peptides was unexpected, as they have never been described before in *Pseudomonas* species. Nonlinear dipeptides
264 have been described in *Pseudomonas* strains, and some cyclic dipeptides are involved in the regulation of quorum
265 sensing (Degraassi et al. 2002); others such as NAGGN: N-AcetylGlutaminylGlutamine 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 *Streptomyces 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* (Zapras 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).
277 In this case, the function of the peptides over- or underproduced under cold shock remains to be discovered.

278 279 **Relative importance of the resistant mechanisms to cold shock in *Pseudomonas syringae*:**

280 The previous results show that many resistance mechanisms to cold shock are present in this strain. The VIP score
281 values (Table 2) can be used to determine which mechanisms are the most important as these scores reflect the
282 relative importance of each metabolite to discriminate the two temperatures. The higher the VIP value, the more
283 discriminant it is. Based on the VIP score histograms from the MS and NMR results (Figure S4), three signal groups
284 have been highlighted. The metabolites which present the highest VIP scores (group 1) are glutathione, trehalose,
285 palmitoleic acid and (iso)Leucine (Table 2). The second group contains some amines and amino acids (piperidine,
286 lysine and phenylalanine) and some dipeptides (Ala-Leu, Leu-Leu); the other metabolites are in the third group.
287 These results suggest that the most important resistance mechanisms to this temperature change are the protection
288 against oxidative stress (glutathione), the production of osmo/cryoprotectants (trehalose), and the adaptation of the
289 membrane composition (Lipids). This result is consistent with the SEED subsystems annotated in the genome
290 sequence of this strain that was recently published (Besaury et al 2017). From 181 SEED subsystems affiliated to
291 stress response, the majority were assigned to the responses to oxidative stress (79), osmotic stress (32) and cold
292 shock (5).

293 294 **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**

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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).

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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.

494
495 **Fig. 3** Metabolic pathways of *Pseudomonas syringae* (glutathione, ornithine and lysine) impacted by exposure to
496 cold. Metabolites that are overrepresented at 5 °C compared with 17 °C are in yellow, and metabolites that are
497 underrepresented are in blue. Putative intermediates are indicated in white.

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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 COReLation
507 SpectroscopY experiment; TOCSY, chemical shift and correlation on TOtal Correlation SpectroscopY experiment;
508 HSQC, chemical shift and correlation on Heteronuclear Single Quantum Correlation experiment

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

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

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

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

Identified VIP Metabolites	MS		NMR		ID metrics**
	VIP score	S/R Ratio	VIP score	S/R Ratio	class (score)
Amino acids					
Valine			2.17-2.49	0.49	II (5)
Leucine	3.91 ¹	0.59 ¹	3.38	0.51	II (9)
Isoleucine			1.99-2.19	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	I
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	1.22 ²	1.18 ²	1.41-1.75	ND*	I
Glycine			1.01	1.09*	II (2)
Saccharides					
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)
Amines					
Putrescine	2.05	0.46	1.57-1.80	0.51-0.54	I
Feruloylputrescine	1.64	0.28			III (1)
Piperidine	3.41	0.5			I
Carnitine	1.62	2.38			III (1)
Lipids					
Palmitoleic acid	4.20	0.26			I
Oleic acid	2.48	0.44			III (1)
LysoPE 18:1	3.04	0.3			I
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			I
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	I
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			I
Leucyl-leucine	2.76	0.27			I
Alanyl-arginine	2.11	0.22			I
Leucyl-phenylalanine	1.72	0.3			I
Glutamyl-alanine	1.61	0.44			I
Prolyl-phenylalanine	1.40	0.22			III (1)
Prolyl-(iso)leucine	1.37	0.22			I
Valyl-valine	1.06	0.33			I

Seryl-valine	1.34	1.75			I
Gln-Ser-Ala-Gln	1.1	5.88			II (3)

516
517 ¹ not differentiable through MS experiments; ² information about L/D conformation is not available through MS
518 experiments; * quantifications are given according to ¹H-NMR buckets with peak overlaps. ** Classes and scores are
519 according to Sumner et al. (2007; 2014; See Table S1).