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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.

12
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18 for her valuable technical assistance.

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).

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 Dalmaso 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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481
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483

484 **Figure legends**

485
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).

489
490
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.

498
499
500

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.

509

510

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).