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1 **Proteogenomic insights into uranium tolerance of a Chernobyl's**

2 ***Microbacterium* bacterial isolate**

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20 Keywords

21 Radionuclides, toxicity, proteogenomics, bioinformatics, toxicoproteomics, *Microbacterium*

22 **Abstract**

23 *Microbacterium oleivorans* A9 is a uranium-tolerant actinobacteria isolated from the trench T22
24 located near the Chernobyl nuclear power plant. This site is contaminated with different
25 radionuclides including uranium. To observe the molecular changes at the proteome level occurring
26 in this strain upon uranyl exposure and understand molecular mechanisms explaining its uranium
27 tolerance, we established its draft genome and used this raw information to perform an in-depth
28 proteogenomics study. High-throughput proteomics were performed on cells exposed or not to 10
29 μM uranyl nitrate sampled at three previously identified phases of uranyl tolerance. We
30 experimentally detected and annotated 1,532 proteins and highlighted a total of 591 proteins for
31 which abundances were significantly differing between conditions. Notably, proteins involved in
32 phosphate and iron metabolisms show high dynamics. A large ratio of proteins more abundant upon
33 uranyl stress, are distant from functionally-annotated known proteins, highlighting the lack of
34 fundamental knowledge regarding numerous key molecular players from soil bacteria.

35

36 **Biological significance**

37 *Microbacterium oleivorans* A9 is an interesting environmental model to understand biological
38 processes engaged in tolerance to radionuclides. Using an innovative proteogenomics approach, we
39 explored its molecular mechanisms involved in uranium tolerance. We sequenced its genome,
40 interpreted high-throughput proteomic data against a six-reading frame ORF database deduced from
41 the draft genome, annotated the identified proteins and compared protein abundances from cells
42 exposed or not to uranyl stress after a cascade search. These data show that a complex cellular
43 response to uranium occurs in *Microbacterium oleivorans* A9, where one third of the experimental
44 proteome is modified. In particular, the uranyl stress perturbed the phosphate and iron metabolic
45 pathways. Furthermore, several transporters have been identified to be specifically associated to

46 uranyl stress, paving the way to the development of biotechnological tools for uranium
47 decontamination.

48

49 **1. Introduction**

50 Radionuclides (RNs) are naturally present in the environment but are redistributed by anthropogenic
51 activities *e.g.* chemical nitrogen fertilizer use, weapons manufacturing, nuclear research and nuclear
52 fuel production. Several major nuclear accidents such as Chernobyl and Fukushima led to the
53 dispersion of several RNs through the atmosphere in microparticles [1] and in the soil [2]. In
54 Chernobyl, radionuclides-contaminated wastes were buried on site in about 800 trenches. Besides
55 ^{137}Cs and ^{90}Sr , other RNs are present in the Chernobyl trench T22, including ^{60}Co , ^{154}Eu , $^{238, 239, 240}\text{Pu}$,
56 ^{241}Am and $^{235, 238}\text{U}$.

57 Uranium (U) belongs to the actinide series. It exhibits both radiological and chemical toxicities, and a
58 long half-life, resulting in environmental and human health major concern [3]. As a hard Lewis acid
59 and according to the HSAB theory which qualitatively explains the stability of metal complexes [4],
60 uranyl tends to interact with hard Lewis base such as phosphate, sulfate, carbonate or amine by ionic
61 interactions. It can displace other hard Lewis acids like Ca^{2+} and Fe^{3+} in highly oxygenated site.
62 Moreover, different studies have confirmed the affinity between uranium and phosphate or
63 carbonate [5-9], or the replacement of iron by uranyl [10].

64 Bacteria can interact directly or indirectly with RNs and change their speciation, playing a major role
65 in their mobility and transfer in the environment. The use of both molecular- and culture-based
66 approaches to study bacterial diversity in the trench T22 demonstrated that RNs-contaminated soils
67 host an unexpected diversity of bacteria [11].

68 During their sampling campaign at Chernobyl, Chapon and colleagues have constructed a large
69 collection of cultured bacteria from the trench T22 soils offering the opportunity to study the RN

70 tolerance of these bacteria [11]. The strain *Microbacterium oleivorans* A9 (referred as
71 *Microbacterium* sp. A9_sp3_-1_2 in [12]) is one of the most uranium-tolerant isolate of this
72 collection. In a previous paper, the interactions between uranium and this bacterium have been
73 investigated and specific exposure conditions in which the bacteria were kept alive while being
74 exposed to soluble forms of uranium have been set-up. With this tightly controlled exposure
75 conditions, it has been shown that *Microbacterium oleivorans* A9 exhibit three sequential
76 mechanisms involved in uranium detoxification: a rapid metal removal within the first 30 minutes,
77 then an active U(VI) release in the exposure medium accompanied by a phosphate efflux and a final
78 biomineralization step of uranium in autunite-like mineral phases [12]. For this reason, it constitutes
79 a relevant model to study the mechanisms involved in uranium tolerance and in uranium-bacteria
80 interactions. In bacteria, survival in metal-contaminated environment is mainly achieved through
81 active efflux pumps [13]. If such systems are well-known for a wide variety of non-essential metals,
82 involvement of an efflux system in detoxification of uranium has not yet been described, although
83 up-regulation of genes encoding metal efflux pumps has already been reported for
84 *Desulfotomaculum reducens* exposed to U(VI) in anaerobic conditions [14].

85 The cellular response of *Escherichia coli*, *Acidithiobacillus ferrooxidans* and two *Anabaena* strains
86 upon uranium exposure has already been studied with 2D-PAGE based proteomic methodology [15-
87 17]. These studies highlighted an impact of uranium on the bacterial proteome, but the low
88 throughput of this approach allowed identifying only 11, 18, 45 and 27 proteins respectively. These
89 may represent only a partial view of the proteins modulated upon uranium stress. Two studies using
90 high-throughput tandem mass spectrometry have deepened the proteome changes occurring in two
91 other bacteria, *Geobacter sulfurreducens* and *Caulobacter crescentus* [18, 19]. These papers show
92 that resistance to uranium is a complex cellular response with induction of multiple stress response
93 systems. These four studies, based on gram-negative organisms, have used different uranium
94 concentrations (from 50 to 500 μ M) as well as different exposure conditions making any comparison
95 difficult.

96 Proteogenomics, the alliance of genomics and proteomics, has proved its efficiency in supporting the
97 existence of genes encoding hypothetical proteins or with unknown function, and even unannotated
98 in the genome for several organisms [20]. This methodology is even more appropriate when a quick
99 focus on mechanisms for which the main players are unknown is required [21-23]. Because,
100 proteogenomics is based on the assignment of MS/MS spectra with a six-frame translation of the
101 genome, time-consuming, resource-dependent and hard-working genome sequence completion
102 steps are bypassed. For example, the annotation of *Deinococcus deserti* by means of proteogenomics
103 has shown its added value to correct annotation errors in other *Deinococcus* sp. genomes [21, 24].
104 The exploration of the halotolerance molecular mechanisms of *Tistlia consotensis* by proteogenomics
105 based on a draft genome sequence straightforwardly highlighted major changes in response to hypo-
106 osmotic and hyper-osmotic conditions [22, 25].

107 Here, the molecular changes occurring in *Microbacterium oleivorans* A9 upon uranium exposure
108 were deciphered by means of a proteogenomics approach consisting in draft genome sequencing
109 and high-throughput proteome coverage by next-generation proteomics. Proteomic changes of cells
110 exposed to 10 μ M uranyl for 30 min, 4 h and 24 h were investigated. This experimental strategy
111 allowed identification without *a priori* of soluble and membrane proteins with significantly differing
112 abundances compared to controls, which could be players of the defense mechanism of
113 *Microbacterium oleivorans* A9 against uranium. Furthermore, these genome and proteome datasets
114 represent invaluable resources to gain insights into the physiology and role of *Microbacterium* in
115 soils.

116

117 2. Experimental procedures

118 2.1 Uranium exposure

119 The experimental procedure used to expose the bacteria to uranium is described in [12].
120 *Microbacterium oleivorans* A9 strain was routinely cultivated in 0.1 X Tryptic Soy Broth (TSB, Difco
121 Laboratories) at 30°C with shaking. Cells at exponential growth phase were harvested by
122 centrifugation for 10 min at 5000 *g*. From this stage on, samples were maintained at 25°C throughout
123 the experiment. The resulting cell pellets were washed twice in 0.1 M NaCl pH 5.0 and were re-
124 suspended at about 6×10^9 cells mL⁻¹ in 0.1 M NaCl pH 5.0 with 0 (control) or 10 μM U(VI). U(VI) was
125 added as uranyl nitrate UO₂(NO₃)₂·6H₂O (Sigma–Aldrich) from a 7.51 mM stock solution in 16 mM
126 HNO₃. No precipitate was observed along the exposure conditions. The nitrate concentration was
127 adjusted to 0.416 mM by adding NaNO₃ when needed. Bacteria exposed to U(VI) and controls were
128 incubated at 25°C with shaking. Four independent biological replicates were made for each condition
129 and for each time point. Fractions of 1 ml of cell suspension were taken after 0.5, 4, and 24 h of
130 uranium exposure. Samples were centrifuged for 5 min at 8000 *g*. The resulting supernatants were
131 removed and the pellets were conserved at -80 °C until proteomic analysis.

132 2.2 Genome of *Microbacterium oleivorans* A9

133 Genomic DNA was sequenced on a HiSeq 2000 sequencing platform (Illumina) by the GenoScreen
134 Company and genome *de novo* assembling was performed on the reads using ABYSS [26]. The Whole
135 Genome Shotgun project data have been deposited at DDBJ/EMBL/GenBank under the accession
136 number MTIO00000000. For each contig, the ORF translated sequences were extracted from stop
137 codon to stop codon in each of the six possible reading frames. The ORF-derived polypeptide list was
138 restricted to those exhibiting a length of at least 50 amino acids. Proteomic data and proteogenomic
139 procedures were then used to discriminate between false, correct but undetected, and correct and
140 detected protein sequences as described earlier [21, 27]. The ORF sequences for which at least one
141 spectrum has been assigned were kept to create a second database. This step allowed us to identify

142 more proteins and to improve qualitatively and quantitatively the MS/MS spectra attribution. The
143 protein sequences were given a cluster of orthologous groups (COG) assignment based on the
144 reference database [28]. Furthermore, enzymes annotation using PRIAM profiles [29] associated to
145 KEGG database [30] allowed the identification of metabolic pathways. An in-house batch program
146 was developed to emphasize metabolic pathways highlighted by proteomic analysis.

147 2.3 Proteome sample preparation and trypsin in-gel proteolysis

148 Cell pellets (between 2.0 and 4.6 mg, 3.1 mg average, wet weight) were dissolved in a given volume
149 (100 μ L for 1.7 mg of bacterial pellet) of 1X NuPAGE™ LDS Sample Buffer (Invitrogen) supplemented
150 with 2% β -mercaptoethanol. Samples were sonicated for 5 min in a transonic 780H sonicator and
151 subjected to a 5 min incubation at 99°C prior to SDS-PAGE. Samples were subjected to a 3 min short
152 migration in denaturing conditions as previously described [31]. For this, samples were loaded onto a
153 4-12% gradient 10-well NuPAGE Bis-Tris (Invitrogen) gel operated with NuPAGE MES (Invitrogen) as
154 running buffer at 200 V. After gel staining with Simply Blue Safe Stain, a ready-to-use Coomassie G-
155 250 stain from Invitrogen, the whole proteome content (comprising both soluble and membrane
156 proteins) from each sample was excised as a single piece of gel polyacrylamide band. The 24
157 resulting polyacrylamide bands were washed with MilliQ water and dehydrated with $\text{CH}_3\text{CN}:\text{NH}_4\text{HCO}_3$
158 50mM (1:1 v:v) before addition of 100% CH_3CN . Gel pieces were then dried for 2 to 5 min under
159 vacuum. For in-gel digestion, dry gel pieces were rehydrated with 100 mM NH_4HCO_3 containing 25
160 mM DTT and incubated for 10 min at 56°C. After removal of the solution, the gel pieces were further
161 treated with 100 mM NH_4HCO_3 containing 55 mM iodoacetamide at room temperature and dried
162 under vacuum as before. The gel bands were then processed for in-gel proteolysis with trypsin in
163 presence of 0.01% ProteaseMax (Promega) as previously described [32].

164 2.4 NanoLC-MS/MS analysis

165 NanoLC–MS/MS experiments for the 24 resulting peptide mixtures were performed using a Q-
166 Exactive HF mass spectrometer (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex-LC

167 Packings) in similar conditions as those previously described [33]. Peptide mixtures (10 μ l) were
168 loaded and desalted on-line on a reverse phase precolumn (Acclaim PepMap 100 C18, 5 μ m bead
169 size, 100 \AA pore size, 5 mm \times 300 μ m id) from LC Packings. Peptides were then resolved onto a
170 reverse phase Acclaim PepMap 100 C18 column (3 μ m, 100 \AA , 500 mm \times 75 μ m id) at a flow rate of
171 0.3 μ l/min with a 180 min linear gradient of $\text{CH}_3\text{CN}/0.1\%$ formic acid and injected into the Q-Exactive
172 HF mass spectrometer. The Q-Exactive HF instrument was operated according to a Top20 data-
173 dependent acquisition method as previously described [33]. Briefly, a scan cycle was initiated with a
174 full scan of peptide ions in the ultra-high-field Orbitrap analyzer, followed by selection of a single
175 precursor and its dissociation in high energy collisional mode, and MS/MS scans on the 20 most
176 abundant precursor ions. Full scan mass spectra were acquired with an Automatic Gain Control
177 Target set at 3×10^6 ions and a resolution of 60,000 from m/z 350 to 1,800. MS/MS scan was initiated
178 at a resolution of 15,000 when the ACG target reached 1×10^5 ions with a threshold intensity of
179 83,000 and potential charge states of 2+ and 3+. Precursor ions were selected with a dynamic
180 exclusion of 10 sec for increasing the reliability of spectral count measurements.

181 2.5 Interpretation of mass spectrometry data

182 The recorded MS/MS spectra for the 24 samples were merged after being searched against the
183 home-made ORF database. First, peak lists were generated with the MASCOT DAEMON software
184 (version 2.3.2) from Matrix Science using the extract_msn.exe data import filter from the Xcalibur FT
185 package (version 2.0.7) proposed by ThermoFisher. Data import filter options were set as previously
186 described [34] at 400 (minimum mass), 5000 (maximum mass), 0 (grouping tolerance), 0
187 (intermediate scans), and 1000 (threshold). MS/MS spectra were searched with MASCOT against the
188 ORF database with the following parameters: tryptic peptides with a maximum of 2 miss cleavages
189 during proteolytic digestion, a mass tolerance of 5 ppm on the parent ion and 0.02 Da on the MS/MS,
190 fixed modification for carbamidomethylated Cys (+57.0215) and variable modification for oxidized
191 Met (+15.9949). All peptide matches with a peptide score above its query threshold set at $p \leq 0.05$
192 with the ORF database and rank 1 were parsed using the IRMa 1.31.1c software [35]. MS/MS spectra

193 assigned to several loci were systematically removed. The average error for the determination of
194 peptide mass is 0.59 ppm and the mean MASCOT score is 50.7. A protein was considered validated
195 when at least two different peptides were detected. False-positive identification of proteins was
196 estimated using a reverse decoy database as below 0.1% with these parameters.

197 The mass spectrometry proteomics data have been deposited in a public repository at the
198 ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner
199 repository [36] with the dataset identifier PXD005794 and project DOI 10.6019/PXD005794.

200 The number of MS/MS spectra per protein (spectral counts) was determined for the four replicates
201 of each of the three time points for both conditions. The protein abundances were compared for
202 each time point between the uranyl exposure and the control conditions. For each of these
203 comparisons, the list of non-redundant proteins detected among the six corresponding datasets was
204 established. The total spectral count of each polypeptide was used to rank the proteins from the
205 highest to the lowest detection intensities. The statistical protein variation among the four replicates
206 samples of the two specific exposure conditions compared was calculated using the T-Fold option of
207 the PatternLab 2.0 software [37]. This module allows normalizing the spectral count datasets,
208 calculating the average fold changes with statistics (t-test), and estimating the resulting theoretical
209 false discovery rate. MS/MS data were compiled in Excel (Microsoft) and converted for PatternLab
210 with an Excel home-designed macro. Normalization was done taking into account the total number
211 of spectral count for each sample, taking at least two readings per protein. A minimum value of 1 was
212 added systematically to all spectral count values in order to consider missing values as a standard
213 PatternLab normalization. Parameters for the comparisons were as follows: minimum fold change of
214 1.5, minimum p-value of 0.05 and BH-FDR Alfa of 0.15. Normalized spectral abundance factor (NSAF)
215 for each protein was calculated using the formula: (spectral counts / theoretical molecular weight) x
216 1000, see [34].

217

218 3. Results and discussion

219 3.1 *Microbacterium oleivorans* A9 genome annotation

220 The main characteristics of *Microbacterium oleivorans* A9 genome is described in [38]. Briefly, the
221 genome size is around 2.95 Mbp. The G+C content of the genome is 68.33%. COG assignment for the
222 resulting polypeptide dataset shows that two classes are predominant: proteins involved in
223 metabolism of amino acids (74 proteins) on the first hand, and those involved in metabolism of
224 carbohydrates (66 proteins) on the other. A search against the BacMet database [39], which
225 comprises antibacterial biocides and metal resistance genes, highlights the presence of 104 unique
226 metal resistance genes such as *actP* gene which encodes a copper-transporting P-type ATPase, *arsB*
227 gene which encodes an arsenic pump membrane protein or *yieF* gene which encodes a chromate
228 reductase. We found a 16S rRNA gene copy in the genome, exhibiting 99% sequence identity with
229 *Microbacterium oleivorans* 16S rRNA gene. Moreover, the assembling results are strikingly similar to
230 those obtained for *Microbacterium oleivorans* strain RIT293 (RefSeq NZ_JFY000000000.1) [40] with a
231 median total length of 2.9 Mbp, a G+C content of 69% and a total of 2,732 protein-coding genes. This
232 genome version comprises 11 contigs with a N50 parameter at 467,109 bp.

233 3.2 Shotgun proteogenomics of *Microbacterium oleivorans* A9

234 *Proteogenomics quick panorama*

235 **Figure 1** shows the strategy applied here for quickly deciphering the proteome dynamics from resting
236 cells exposed or not to 10 μ M uranium. A total of 1,221,282 MS/MS spectra were recorded. Most of
237 these spectra were of high quality as revealed by the high percentage of assignment obtained
238 (61.1%) at p-value 0.05. For interpreting MS/MS spectra, a six reading frame translated ORF database
239 was created. This proteogenomics database comprised a total of 30,853 putative polypeptide
240 sequences, totaling 4,903,573 amino acids with an average of 159 amino acids per putative
241 polypeptide and 2,064 as longest length. A total of 746,092 MS/MS spectra could be assigned in first
242 intention, allowing certifying the presence of 1,504 proteins detected with at least two different

243 peptides (Supplementary data Table S1). Because of the unusual large size of the six reading frame
244 ORF database(30,853 putative polypeptide sequences instead of 2813 annotated coding DNA
245 sequences), a second round of search was initiated after reducing the database to the proteins
246 detected in the first round as proposed previously for improving the sensitivity of proteogenomics
247 studies [41]. The second search led to the assignment of 747,621 peptide-to-spectrum matches,
248 certifying the detection of 17,027 unique peptide sequences and 1,532 non-redundant proteins
249 identified with at least two different peptides (Supplementary data Table S2). This two-round search
250 allows increasing the number of proteins validated with at least two different peptides (1532 instead
251 of 1504) and enhances the number of assigned MS/MS spectra (747 621 instead of 746 092). This
252 dataset compares favorably with previous studies of bacteria exposed to uranium, as a dataset of
253 less than 950 proteins were reported from *Caulobacter crescentus* [19] and 1,363 proteins were
254 listed from *Geobacter sulfurreducens* [18]. The proteomic dataset obtained here represents 54%
255 (including 15% of membrane proteins) of the predicted protein-coding genes found in the draft
256 genome of *Microbacterium oleivorans* A9 [38]. The distribution of the peptides detected during our
257 experiment along the chimeric genome of *Microbacterium oleivorans* A9 is uniform and confirm the
258 validity of this proteogenomics approach, as previously discussed for the proteogenomics mapping of
259 the alphaproteobacterium *Tistlia consotensis* [22].

260 *Functional categorization of the detected proteins and relative abundances*

261 The dataset of 1,532 identified proteins were classified into 22 COG categories. **Table 1** shows the
262 functional classification of the mass-spectrometry certified global proteome. Most of the detected
263 proteins (50%) are distributed in five classes: hypothetical proteins (15%), amino acid metabolism
264 (11%), carbohydrate metabolism (9%), translation, ribosomal structure, and biogenesis (9%) and
265 transcription (6%). The remaining 17 classes represent less than 5% each (**Table 1**). The prevalence of
266 the same classes has been shown in previous studies [18, 19], but here, the large number of
267 uncharacterized proteins is indicative of an important lack of knowledge regarding soil gram-positive
268 bacteria. 36 proteins account for 25% of the total proteome detected, and thus are the key molecular

269 draft horses within the cells. These 36 most abundant proteins detected throughout the study as
270 evaluated with their normalized spectral abundance factor (NSAF) are given in Supplementary data
271 Table S2. The most abundant protein is the HU bacterial nucleoid DNA-binding protein involved in
272 DNA structuration. Two of the top five most abundant proteins are the chaperonin GroEL (1.28%)
273 and the co-chaperonin GroES (1.06%). These two proteins form the binary GroEL/GroES complex,
274 which mediates proper folding of many proteins. The sub-unit GroEL is specifically known for its
275 important role both in normal and stressful conditions as well as in the resistance towards toxic
276 metals [42]. As expected the ribosomal proteins are abundant since the cells were collected during
277 the exponential growth phase. The 100 most abundant proteins represent 46.1% of total NSAF.
278 Amongst these, 16 proteins (8.3% of total NSAF) are components of the small ribosomal subunit and
279 27 proteins (10.6% total NSAF) belong to the large ribosomal subunit. This abundance of ribosomal
280 proteins has already been observed [23, 34]. Within the most abundant proteins, several are
281 involved in energy metabolism such as the F₀F₁-type ATP synthase (0.78% and 0.57% respectively).

282 3.3 Proteome dynamics upon uranium stress

283 *Common differentially produced proteins over the time course of the experiment*

284 Proteins with significant abundance changes (fold change ≥ 1.5 and p-value ≤ 0.05) in uranium-
285 exposed condition versus control were listed at each time point. A total of 592 proteins met the
286 criteria: 391 at 0.5h, 294 at 4h and 122 at 24h. The decrease of differentially produced proteins over
287 time is due to the non-growing exposure conditions. **Figure 2** shows a Venn diagram representation
288 where the numbers of these proteins are reported for each condition, highlighting the proteins
289 specific of a given time point and those common in two or three time points. A relatively small set of
290 24 proteins were shared along the whole kinetic. Remarkably seven of these 24 proteins are
291 predicted to be ABC-type transporters. Their spectrum activities appeared to be broad as they share
292 significant sequence similarities with dipeptide/oligopeptide/nickel, Fe³⁺-hydroxamate, multidrug or
293 enterocholin transporters. A serine protease and a DNA-binding transcriptional regulator belonging

294 to the PadR family are also found more abundant in presence of uranium for the three time points:
295 1.7, 1.6 and 4.6-fold; 2.1-fold, 2.6-fold and 2.0-fold, respectively. Members of the PadR family
296 regulate different pathways such as multidrug resistance and detoxification [43].

297 A total of 128 proteins were found significantly more abundant in 0.5h and 4h samples of exposure
298 to uranium compared to controls. These 128 proteins covered a large variety of molecular functions
299 making difficult to draw specific uranium responsive pathways. Six ABC-type transporters were
300 observed among this large panel of up-regulated proteins. They are predicted to be involved in
301 amino acid, sugar, cobalamin, Fe³⁺ siderophore and glycerol-3-phosphate transport. These data
302 reinforce the hypothesis that bacteria deploy a rather large panel of transport machineries to
303 respond to uranium exposure. Four proteins involved in cell division are also significantly more
304 abundant at these two initial phases of uranium exposure: the cell division proteins FtsI and FtsQ,
305 and two chromosome partitioning ATPases. Such observation reinforces the idea that cells are viable
306 and active.

307 Once again, a link with iron metabolism is highlighted in the proteins common after 0.5h and 24h of
308 uranium exposure with the presence of ABC-type Fe³⁺/spermidine/putrescine transport system (2.0
309 and 1.8-fold), the ferredoxin-NADP reductase (1.9 and 1.9-fold), the deferrochelataase/peroxidase
310 EfeB (1.7 and 1.8-fold) and the NADPH-dependent ferric siderophore reductase (1.8 and 1.6-fold).

311 Interestingly, a protein predicted to be a DNA-binding transcriptional regulator belonging to the ArsR
312 family is found significantly more abundant at 0.5h but less abundant after 24h uranium exposure.
313 This regulator is known to repress expression of stress-induced operons involved in toxic metal ion
314 tolerance [44]. This suggests that *Microbacterium oleivorans* A9 first represses these genes when
315 cells are at the initial stage of uranium contact, but need the expression of these genes when the
316 uranium is intracellularly biomineralized and so less bioavailable.

317 A comparison of the significant changes of protein abundances between 4h and 24h of exposure
318 shows that 17 proteins are in common for these two time points. Three are related to
319 osmoregulation (DNA-binding response regulator of the OmpR family and of the AcrR family,

320 maltooligosyltrehalose synthase) which is consistent with the exposure condition used *i.e.* NaCl
321 0.1M.

322 *Proteins specifically modulated at a given time point*

323 A large set of proteins (391) has been found significantly modulated in terms of abundance after 0.5h
324 exposure to U(VI) as compared to the control. **Table 2** shows the proteins with the most important
325 changes (fold-change ≥ 3.5). The chorismate mutase exhibited the highest fold-change (5.8-fold),
326 while its homologue from *Geobacter sulfurreducens* has been previously shown to decrease in
327 response to uranium [18]. This protein is involved in phenylalanine and tyrosine synthetic pathway
328 through the conversion of chorismate in prephenate. As reported in **Figure 3**, five other proteins
329 from the same pathway have an increased abundance upon uranium exposure, including the
330 isochorismate synthase (2.6-fold) and the aminotransferase/4-amino-4-deoxychorismate lyase (2.0-
331 fold). A protein involved in ABC-type glycerol-3-phosphate transport, two proteins responsible of acyl
332 transfer to glycerol-3-phosphate and the CDP-glycerol glycerophosphotransferase were more
333 abundant in uranium condition. Glycerol-3-phosphate appears as an important metabolite which
334 may provide phosphate for complexing and immobilizing uranium [45]. Major changes affected the
335 phosphate metabolism as evidenced by the important number (59) of proteins related to this
336 metabolism. A large panel of phosphatases (16 polypeptides) is found in higher amounts after
337 uranium stress. These enzymes are involved in phosphate removal mainly from sugar (fructose,
338 trehalose) and from amino acids (serine, threonine).

339 Regarding the two other time points analyzed by high-throughput proteomics, the most modulated
340 protein detected after 4h is also the protein with the higher abundance after 24h of uranium
341 exposure compared to control (11-fold and 25-fold respectively). This protein shares some sequence
342 similarities (63% identity) to the uncharacterized Ykol membrane protein of *Microbacterium*
343 *oleivorans* LKL04 (**Table 2**). It presents a PepSY domain (pfam 03413) hypothetically involved in
344 inhibition of a peptidase activity. Based on the TMHMM predictive tool [46], one transmembrane
345 segment has been identified in the *Microbacterium oleivorans* A9 protein homolog from residues 39

346 to 61, suggesting that the longest part of this protein is extracellular (residues 62 to 247). Regarding
347 the synteny in *Microbacterium oleivorans* A9 genome, a signal transduction histidine kinase is
348 adjacent to this protein, indicating a possible involvement in signaling function. One of the four
349 proteins for which the abundance decreased at 4h of uranium exposure is the glutaredoxin which can
350 interact physically with a mercury/uranyl reductase (MerA) and plays a role in toxic metals resistance
351 [47]. After 24h exposure, 12 transporters involved in the transport of diverse compounds
352 (lipoprotein, Fe³⁺-hydroxamate, dipeptide/oligopeptide/nickel, enterochelin,
353 Fe³⁺/spermidine/putrescine) are more abundant in the uranium condition compared to the control
354 (**Table 2**). A DNA-binding response regulator of the NarL/FixJ family is also more detected (5.3-fold).
355 The homologous regulator from *Escherichia coli* has been characterized and found to control nitrate-
356 and nitrite-regulated gene expression. This is concordant with the lower detection (-2.1-fold) of a
357 ferredoxin subunit of the nitrite reductase. In the present exposure condition, nitrate has been
358 added in both control and uranium conditions and probably contributes to modify the production of
359 these two proteins.

360 *Phosphate metabolism*

361 In our previous study, we have evidenced a phosphate efflux concomitant to the uranium efflux
362 between 0.5h and 4h exposure and the intracellular formation of autunite, a mineral made of U, P
363 and Ca, suggesting a strong connection between phosphate and uranium [12]. Among these
364 proteins, the phosphohistidine phosphatase SixA is the most up-produced protein (3.5 fold-change at
365 0.5h and 4h). Its precise role is still unclear but its involvement in a signal transduction circuitry
366 through down-regulation of the ArcB-to-ArcA phosphorelay has been shown under anaerobic
367 conditions in *Escherichia coli* [56]. On the other hand, the increase abundance of the broad specificity
368 phosphatase PhoE only after 4h (2.3 fold-change) is congruent with the phosphate efflux observed in
369 our previous experiment [12]. Several components of an ABC-type glycerol-3-phosphate transport
370 system have also a significant positive fold-change. No such component is found more abundant
371 after 24h exposure probably because the phosphate is complexed with uranium leading to autunite

372 accumulation. The glycerol-3-phosphate has been demonstrated as a source of phosphate for the
373 precipitation of uranium [45]. A polyphosphate kinase shows an up-production after 0.5 h (fold-
374 change of 1.8). It can act as a defense mechanism by constituting a phosphate reserve that will be
375 used latter to form uranyl-phosphate complex. As reported in **Figure 4**, the genomic context of this
376 kinase reveals a locus with seven adjacent genes, possibly organized as an operon. These genes are
377 all related to phosphate metabolism. The operon comprises a transcriptional regulatory protein, the
378 phosphohistidine phosphatase SixA and four components of an ABC-type phosphate transport
379 system. Five of this six genes encode proteins which have been found more abundant in uranium
380 condition but with low statistical confidence (p-value above 0.05) or with significant but low fold-
381 change. Noteworthy, this cluster of genes is conserved amongst several *Microbacterium* species from
382 our collection (data not shown). In this work, several proteins involved in phosphate metabolism
383 were modulated upon uranium exposure. The relationships between uranium and phosphate
384 metabolism have also been evidenced by others [57-59].

385 Reminding that uranium can bind to highly oxygenated sites such as phosphate and carbonate, it is
386 interesting to point out that a component of an ABC-type nitrate/sulfonate/bicarbonate transport
387 system shows a significant increase in terms of abundance (1.9 fold-change) after an exposure of 4h,
388 in the same time as uranium and phosphate efflux occur. Several cations transporters (K^+ , Mn^{2+} , Zn^{2+} ,
389 Mg^{2+} , Co^{2+}) are also induced upon uranium exposure. One can speculate that one of these
390 transporters may be involved in uranyl efflux.

391 *Iron metabolism*

392 In our previous study and unlike to phosphate metabolism, we obtained no element suggesting an
393 effect of uranium on iron metabolism [12]. Here, however, a strong impact on iron metabolism is
394 highlighted through the large number of proteins related to iron metabolism more abundant under
395 uranyl stress (19 out of 43 proteins classified in the “Inorganic ion metabolism and transport”
396 category). Most of them are components of the siderophore iron uptake system, either ABC-
397 transport type subunits or siderophore modification enzymes. Among these 19 proteins, two ABC-

398 type Fe³⁺-hydroxamate transport systems and one ABC-type enterocholin transport system are found
399 systematically more abundant at the three time points (fold changes of 1.7, 2.1 and 2.8 respectively).
400 Hydroxamate and enterocholin are siderophores, low molecular compounds produced by bacteria to
401 scavenge iron (Fe(III)) in iron starvation conditions. The involvement of siderophores in tolerance to
402 toxic metals has already been demonstrated [48]. The ATPase component of an ABC-type
403 cobalamin/Fe³⁺-siderophore transport system is also found in higher proportion at 0.5h and 4h with
404 fold changes of 1.6 and 1.7 respectively. Moreover, the abundance of a NADPH-dependent ferric
405 siderophore reductase increased upon uranium exposure: fold change of a 1.8 and 1.6 at 0.5h and
406 24h, respectively. This protein is involved in the release of iron from siderophore. Involvement of
407 siderophores in the resistance to uranium has been poorly studied, but hydroxamate has been
408 shown to bind uranium and to be able to chelate more efficiently uranium with the carbonate form
409 [49]. In addition, induction of siderophore production in response to uranium stress has been shown
410 for the marine cyanobacterium *Synechococcus elongatus* BDU 130911 [50]. Taken together, these
411 data suggest that uranium could interact with siderophore in *Microbacterium oleivorans* A9. One
412 attractive hypothesis is that uranium could then enter the cells via this pathway.

413 Two components (EfeO and EfeB) of the EfeUOB transporter were also detected in higher amounts in
414 our dataset. This transporter is involved in iron uptake in *Bacillus subtilis* [51]. EfeB is a peroxidase
415 involved in oxidation of ferrous iron to ferric iron which allows its binding by the EfeO component.
416 This protein shows a regular increase at 0.5h, 4h, and 24h with fold changes of 1.7, 1.8 and 1.8,
417 respectively. Moreover, EfeB presents the ability to reduce the reactive oxygen species formed in
418 presence of ferrous iron and thus, confers protection to the cell. The prediction of both a cupredoxin-
419 like domain and an imelysin peptidase with a highly conserved HXXE motif confirm that we are
420 dealing with the EfeO component and not the EfeM for which the cupredoxin-like domain is absent
421 [52]. The EfeO component is a ferric iron binding protein and is able to transfer its substrate to the
422 permease EfeU. The variation in the abundance of EfeO during the experiment is not significant. The
423 EfeU component remained undetected despite its prediction in the genome. The EfeUOB transporter

424 of *Escherichia coli* has been shown to be induced under low pH condition [53]. This three-component
425 transporter is also found in *Bacillus subtilis* and was shown to be involved in high-affinity uptake of
426 both ferrous (Fe(II)) and ferric iron (Fe(III)) [51]. Taken together, these results suggest that uranium
427 exposure is perceived by *Microbacterium oleivorans* A9 as an iron starvation, thus enhancing the
428 synthesis of iron uptake systems. This result is in line with what has been shown for *Geobacter*
429 *sulfurreducens* in which uranium induces the Fur operon [18]. Other biological molecules involved in
430 iron metabolism, e.g. transferrin, have been shown to be able to link uranium and can constitute a
431 way for uranium to be transported in the cells [55].

432 **4. Concluding remarks**

433 The high-throughput proteogenomic methodology applied to *Microbacterium oleivorans* A9 allows to
434 quickly identify a large number of proteins and highlights those that could be involved in uranium
435 tolerance. The proteome coverage obtained here is higher than previous proteomic studies devoted
436 at exploring the proteome under uranium stress [18, 19]. *Microbacterium oleivorans* A9 modifies
437 drastically its proteome upon uranium exposure. As previously hypothesized, proteins involved in
438 phosphate metabolism are found in larger abundances under uranium stress due to higher synthesis
439 or poorer degradation or both of these mechanisms. More surprisingly, proteins related to iron
440 metabolism were detected with significant changes in abundance. The detailed relationships
441 between uranium response and iron metabolism are unknown at the moment. However, based on
442 our current results it can be hypothesized that uranium may enter the cells via siderophore
443 transportation. This will be further investigated. The detection of 15% of functionally unassigned
444 proteins highlights the lack of knowledge regarding proteins with potentially key roles in response to
445 uranium stress. Focusing on the most interesting protein candidates could be done by a comparative
446 analysis using high-throughput proteogenomics between *Microbacterium oleivorans* A9 and other
447 *Microbacterium* isolates differing in uranium sensitivity such as *Microbacterium lemovicicum* ViU22
448 [60] which is uranium-sensitive.

449

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456 **References**

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617

618 **Table 1. Functional classification of the proteins detected by at least two peptides.**

COG category	Relative abundance (%)	Number of proteins
Hypothetical protein	14,6	223
Amino acid transport and metabolism	11,0	168
Translation, ribosomal structure and biogenesis	9,3	142
Carbohydrate transport and metabolism	8,8	135
Transcription	6,2	95
Energy production and conversion	5,5	84
Coenzyme transport and metabolism	5,3	82
General function	5,1	78
Lipid transport and metabolism	4,4	67
Nucleotide transport and metabolism	4,3	66
Posttranslational modification, protein turnover, chaperones	4,0	61
Cell wall/membrane/envelope biogenesis	3,9	60
Replication, recombination and repair	3,9	60
Signal transduction mechanisms	3,6	55
Function unknown	2,8	43
Inorganic ion transport and metabolism	2,8	43
Defense mechanism	1,3	20
Cell cycle control, cell division, chromosome partitioning	1,1	17
Secondary metabolites metabolism	1,0	16
Cell motility	0,5	8
Intracellular trafficking, secretion, and vesicular transport	0,5	8
RNA processing and modification	0,1	1

619

Table 2 - Key proteins with major changes after uranium exposure

ORFeome ID ^a	Functional annotation	Functional classification	Fold change ^b	p value	Best hit ^c
After 0.5h					
Contig10_25943_-2	Chorismate mutase	Amino acid metabolism	5.75	0.16E-03	CHMU_METJA
Contig1_343955_-2	Alpha-glucosidase, glycosyl hydrolase family GH31	Carbohydrate metabolism	4.50	0.02E-03	YCR2_ESCVU
Contig6_5891_-2	Acetyl-CoA acetyltransferase	Lipid metabolism	4.00	7.25E-06	BKTB_CUPNH
Contig11_20212_1	Beta-galactosidase/beta-glucuronidase	Carbohydrate metabolism	3.85	0.02E-03	BGAL_ARTSB
Contig3_31861_1	Sugar kinase of the NBD/HSP70 family	Carbohydrate metabolism	3.80	0.02E-03	XYLR_BACPZ
Contig3_278050_-1	DNA-binding transcriptional regulator, PadR family	Transcription	3.75	0.20E-03	YWZG_BACSU
Contig7_116064_-3	Dipeptidyl aminopeptidase/acylaminoacyl peptidase	Amino acid metabolism	3.75	0.15E-03	DAPB3_PSEMX
Contig8_12690_-3	tRNA A37 threonylcarbamoyladenine modification protein TsaB	Translation, ribosome	3.67	0.09E-03	Y3455_MYCBO
Contig2_261531_-3	Phosphohistidine phosphatase SixA	Signal transduction	3.50	0.10E-03	Y1276_MYCTO
Contig1_23131_1	Serine protease, subtilisin family	PTM, protein turnover	3.50	0.05E-03	HLY_NATA1
Contig8_9448_1	Hypothetical protein	Hypothetical	3.50	0.03E-03	Y3037_MYCTO
Contig1_334362_3	ABC-type glycerol-3-phosphate transport system	Carbohydrate metabolism	3.50	2.97E-03	YURO_BACSU
After 4h					
Contig11_87308_2	Uncharacterized membrane protein Ykol	Function unknown	10.75	3.99E-06	No results
Contig1_430924_1	D-arabinose 1-dehydrogenase, Zn-dependent alcohol dehydrogenase family	Carbohydrate metabolism	4.60	9.21E-03	ADH_SULTO
Contig3_147088_1	DNA-binding transcriptional regulator/sugar metabolism, DeoR/GlpR family	Transcription	4.58	1.72E-04	AGAR_ECO57
Contig5_94994_2	Hypothetical protein	Hypothetical	3.83	1.64E-03	No results
Contig11_20081_2	6-phosphogluconolactonase/Glucosamine-6-P isomerase/deaminase	Carbohydrate metabolism	3.80	2.68E-02	NAGB_STRCO
Contig5_120376_1	Hypothetical protein	Hypothetical	3.75	1.57E-02	No results
Contig5_105896_-2	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Coenzyme metabolism	3.55	2.92E-03	Y3329_MYCTU
Contig3_278050_-1	DNA-binding transcriptional regulator, PadR family	Transcription	3.50	5.59E-03	YWZG_BACSU
Contig2_261531_-3	Phosphohistidine phosphatase SixA	Signal transduction	3.50	2.28E-02	Y1276_MYCTO
After 24h					
Contig11_87308_2	Uncharacterized membrane protein Ykol	Function unknown	25	1.15E-04	No results
Contig5_20481_3	ABC-type lipoprotein export system	Cell wall/membrane	8.18	7.26E-07	Y1508_METJA
Contig5_94994_2	Hypothetical protein	Hypothetical	5.25	6.46E-03	No results
Contig5_161786_-2	DNA-binding response regulator, NarJ/FixJ family	Signal transduction	5.25	4.78E-04	DEGU_BREBE
Contig1_136842_3	Periplasmic serine protease, S1-C subfamily	PTM, protein turnover	4.33	8.18E-04	DEGP_SALTY
Contig3_196395_-3	Hypothetical protein	Hypothetical	4.28	2.74E-03	TITIN_MOUSE
Contig15_39811_-1	DNA-binding transcriptional regulator, AcrR family	Transcription	3.75	4.04E-03	MIFTR_MYCTO

^a ORFeome ID refers to Supplementary Data Table S2.

^b Fold changes represent the expression with uranium exposure normalized to expression without uranium.

^c The best hit correspond to the first hit obtained by BLASTp against Swissprot database.

622 **Figure 1 – Shotgun nanoLC-MS/MS strategy used for the proteome analysis of *Microbacterium***
623 ***oleivorans* A9 under uranium exposure or not.** A total of 24 samples were processed: four biological
624 replicates for each time point at 0,5h, 4h and 24h of exposure. After lysis, the proteins were
625 subjected to a short SDS-PAGE migration before trypsin proteolysis in-gel. A Q-Exactive HF mass
626 spectrometer was used to identify the resulting peptides.

627 **Figure 2 – Venn diagram showing the number of proteins with significant abundance changes in**
628 **the three sampling time points.**

629 **Figure 3 – Example of the phenylalanine, tyrosine and tryptophan biosynthesis pathways.** The
630 boxes represent the branched metabolic pathways. The enzymes are identified through their EC
631 number. Proteins detected by proteogenomics are highlighted in red, proteins more abundant upon
632 uranium exposure are indicated in blue and proteins predicted in the genome but not detected by
633 proteomics are in grey. The genes' ID corresponding to the enzymes are as follow: 1.3.1.12:
634 MicroBact_A9DB#Contig4_204950_-2, 2.4.2.18: MicroBact_A9DB#Contig4_125788_-1, 2.5.1.19:
635 MicroBact_A9DB#Contig2_13726_-1, 2.5.1.54: MicroBact_A9DB#Contig4_169845_-3, 2.6.1.1:
636 MicroBact_A9DB#Contig12_70604_2, 2.6.1.9: MicroBact_A9DB#Contig7_122729_2, 2.7.1.71:
637 MicroBact_A9DB#Contig1_401394_-3, 4.1.1.48: MicroBact_A9DB#Contig1_371158_-1, 4.2.1.10:
638 MicroBact_A9DB#Contig1_399335_-2, 4.2.1.20: MicroBact_A9DB#Contig1_369105_-3, 4.2.1.51:
639 MicroBact_A9DB#Contig5_42426_3, 4.2.1.91: MicroBact_A9DB#Contig3_67122_3, 4.2.3.5:
640 MicroBact_A9DB#Contig1_401926_-1, 5.4.99.5: MicroBact_A9DB#Contig10_25943_-2, 6.1.1.1:
641 MicroBact_A9DB#Contig7_15747_3, 6.1.1.20: MicroBact_A9DB#Contig7_133127_2.

642 **Figure 4 – Genomic context of the polyphosphate kinase.** Fold-change (FC) are indicated for the first
643 time point of sampling (0.5h). Proteins for which the gene name is in bold were detected by
644 proteomics.

645

646

647 **List of supplementary material**

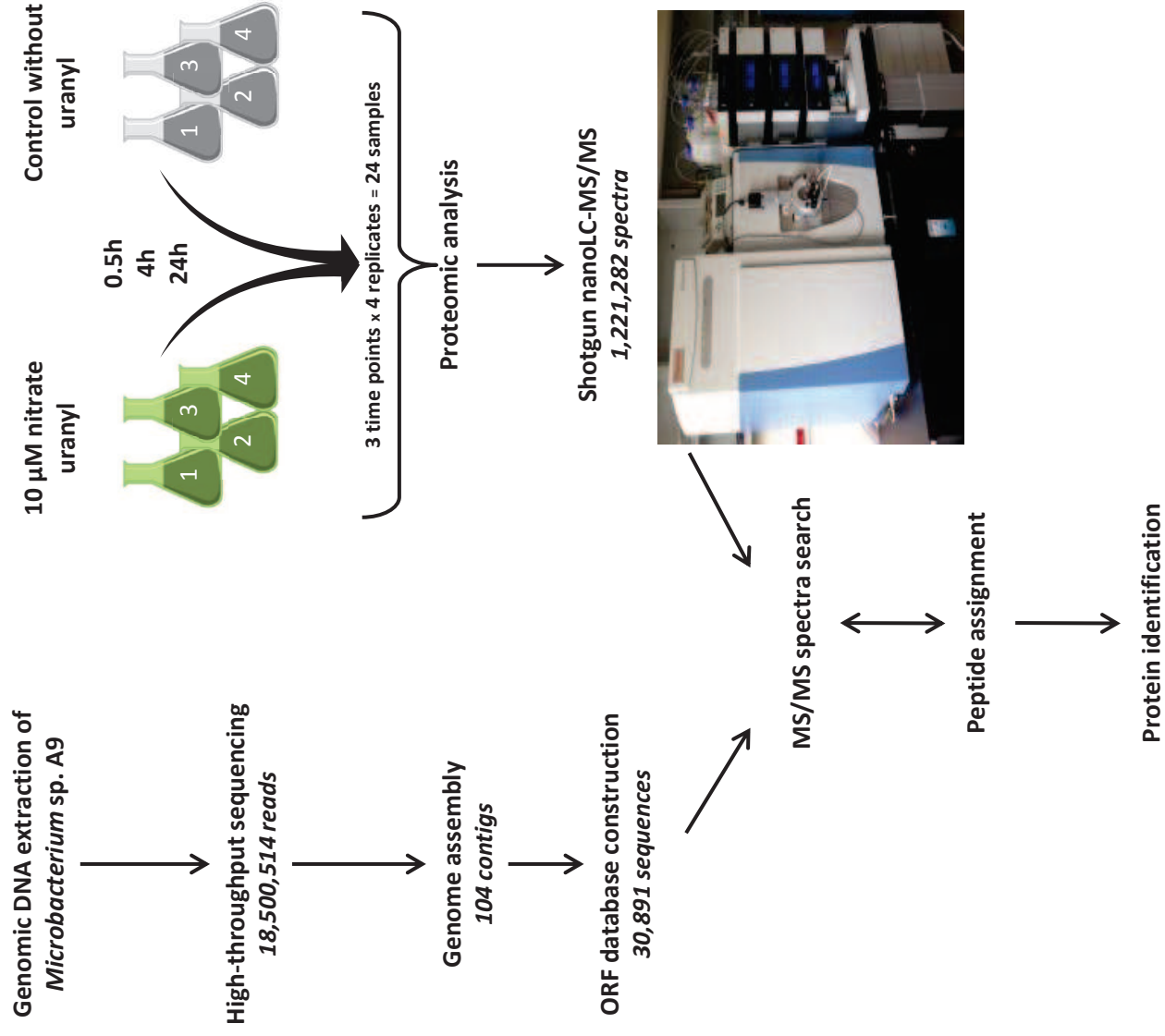
648 **Supplementary Table S1.** List of proteins and their spectral count in A9 samples in the first
649 proteogenomic search.

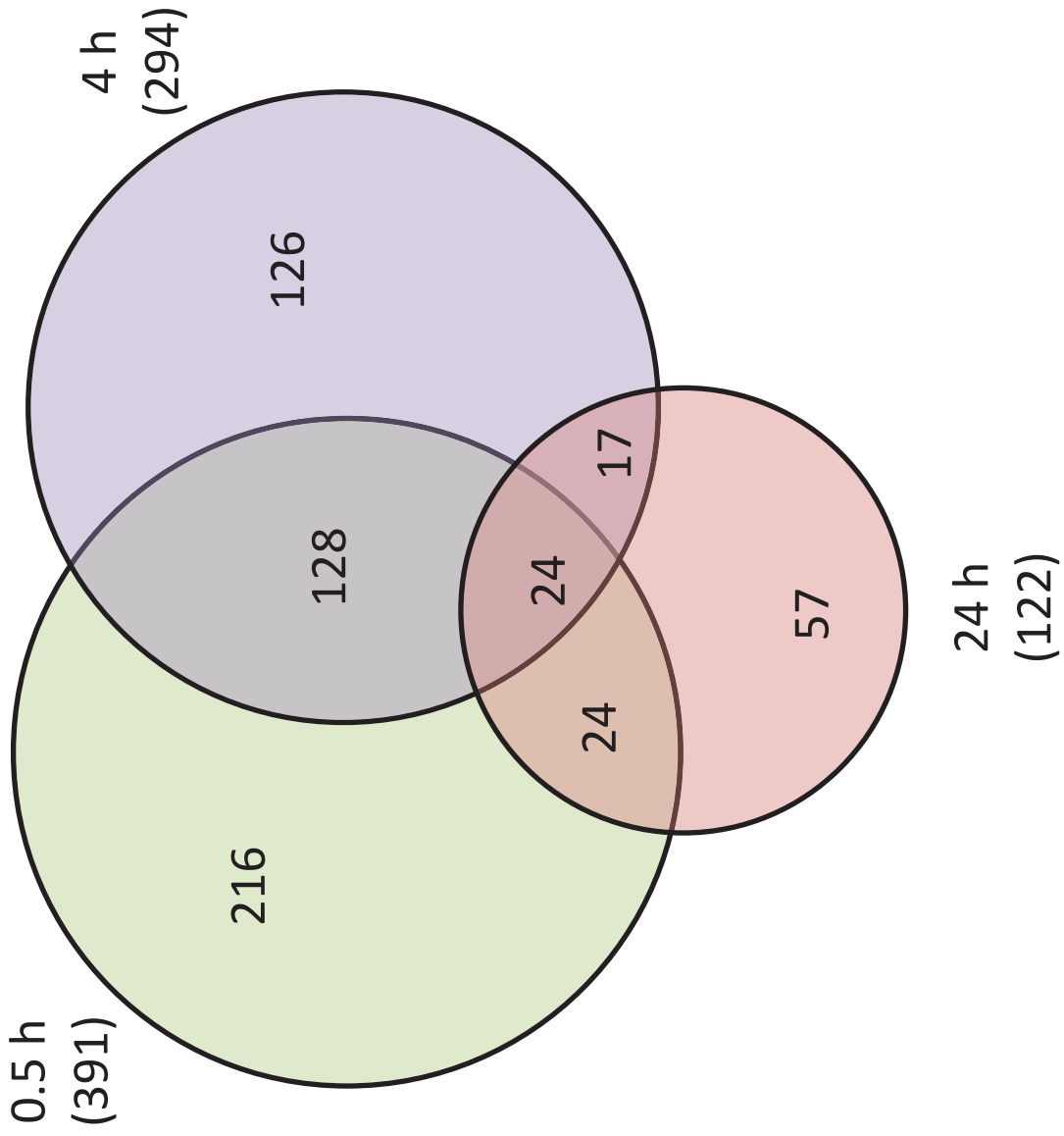
650 **Supplementary Table S2.** List of proteins and their spectral count in A9 samples in the
651 second round search. NSAF: Normalized Spectral Abundance Factor; SC: Spectral Count

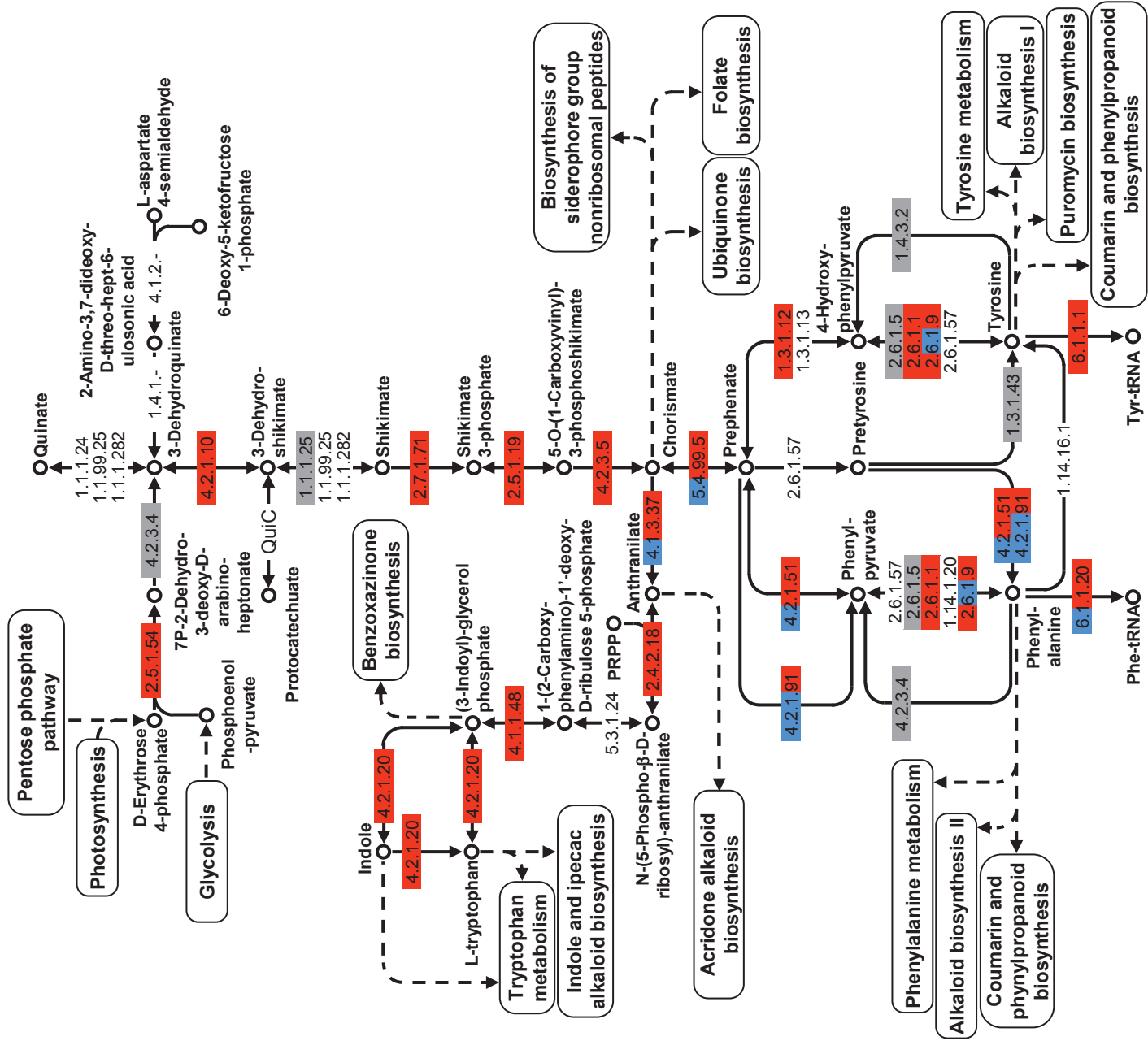
652

Figure

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Microbacterium oleivorans A9 chromosome

