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1 Discovery and characterization of UipA, a uranium- and iron-binding PepSY
2 protein involved in uranium tolerance by soil bacteria.

3
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16

17 **Author Contributions:** V.C. designed and supervised the study. V.C. and L.P. performed the uranium
18 exposure experiments and prepared the samples for proteomics. N.T., M.F. and L.F. performed
19 microscopic analysis and ICP-AES measurements. B.A.-B. acquired the proteomics data and N.G., B.A.-
20 B. and J.A. performed the proteomic analyses. P.O. and M.B. performed genomic and phylogenetic
21 analyses and constructed ORF databases for proteomics. A.M.A. established UipA topology *in vivo*. N.G.
22 purified the recombinant proteins and performed fluorescence titration experiments, assisted by N.B. N.G.
23 and D.L. performed native mass spectrometry experiments. N.G. acquired FTIR data under C.B.
24 supervision. N.G. prepared the samples for synchrotron-based analysis and participated in EXAFS data
25 acquisition. C.D.A. performed EXAFS data acquisition and processing. N.B. and N.G. performed
26 crystallization tests and obtained the protein crystals under supervision of P.A. P.A. and P.L. resolved the
27 UipA structure. N.G., B.A.-B., P.O., M.B., N.B., A.M.A., D.L., C.D.A., P.A., C.B., J.A. and V.C. analyzed
28 the data. N.G. and V.C. wrote the paper and P.O., M.B., L.F., D.L., C.D.A., P.A., C.B. B.A.-B and J.A.
29 edited it.

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32 **Abstract**

33 Uranium is a naturally occurring radionuclide. Its redistribution, primarily due to human activities, can have
34 adverse effects on human and non-human biota, which poses environmental concerns. The molecular
35 mechanisms of uranium tolerance and the cellular response induced by uranium exposure in bacteria are
36 not yet fully understood. Here, we carried out a comparative analysis of four actinobacterial strains
37 isolated from metal and radionuclide-rich soils that display contrasted uranium tolerance phenotypes.
38 Comparative proteogenomics showed that uranyl exposure affects 39-47% of the total proteins, with an
39 impact on phosphate and iron metabolisms and membrane proteins. This approach highlighted a protein
40 of unknown function, named UipA, that is specific to the uranium-tolerant strains and that had the highest
41 positive fold-change upon uranium exposure. UipA is a single-pass transmembrane protein and its large
42 C-terminal soluble domain displayed a specific, nanomolar binding affinity for UO_2^{2+} and Fe^{3+} . ATR-FTIR
43 and XAS spectroscopy showed that mono and bidentate carboxylate groups of the protein coordinated
44 both metals. The crystal structure of UipA, solved in its apo state and bound to uranium, revealed a
45 tandem of PepSY domains in a swapped dimer, with a negatively charged face where uranium is bound
46 through a set of conserved residues. This work reveals the importance of UipA and its PepSY domains in
47 metal binding and radionuclide tolerance.

48

49 **Introduction**

50 Uranium is a long-lived radionuclide naturally found in the earth's crust. Its redistribution in the
51 environment is mainly due to anthropogenic activities raising environmental and human health
52 concerns(1). Uranium toxicity stems from chemical toxicity rather than radiotoxicity(2). In the environment,
53 uranium has two major oxidation states, U(IV) and U(VI). The U(IV) form is found under anoxic conditions
54 and has a limited toxicity due to its low solubility. The U(VI) form is prevalent under oxic conditions and its
55 speciation is pH-dependent. The water-soluble uranyl cation UO_2^{2+} is the most toxic form of U(VI) and
56 dominates at $\text{pH} < 5$ (3). As a hard cation, U(VI) in the uranyl molecule forms complexes with oxygen
57 ligands such as hydroxyl, amide, phosphate and carboxylate groups and can therefore interact with many
58 biological molecules. For instance, uranyl can replace physiological cations such as Ca^{2+} and Fe^{3+} and
59 impair functional binding sites on proteins(4).

60 Soil bacteria play a crucial role in the functioning of soils and ecosystems. It has been shown that uranium
61 can affect the structure and activity of soil bacterial communities(5-7). At the same time, high bacterial
62 diversity has been reported in uranium-rich environments, from both natural and anthropogenic origins(8-
63 11), and these environments are reservoirs of uranium- and metal-tolerant bacteria(11-17).

64 Bacteria interact with uranium and can modulate its speciation, playing a major role in its mobility and
65 transfer(18). Bacteria can immobilize uranium by different mechanisms such as reduction, sorption,
66 precipitation and mineralization. Under anoxic conditions, some bacterial species mediate reductive
67 precipitation of uranium(19). Under oxic conditions, the biosorption of uranyl on chelating groups occurs
68 on bacterial surfaces(20, 21). Uranium mobility can also be reduced by a variety of bacterial strains via
69 bioprecipitation and biomineralization with inorganic phosphate(22). Both processes can take place intra-
70 or extracellularly and involve phosphatase activity(23-26). Metabolites such as siderophores may also be
71 involved in uranium sequestration(27, 28) . All of these mechanisms have been proposed to participate in
72 uranium detoxification(26, 29). In addition, transporters such as P_{IB} -ATPases and cation diffusion
73 facilitator (CDF) may participate in uranium tolerance(30-33). The ability of some bacterial species to
74 sequester uranium makes them promising candidates to develop bioremediation processes(34).

75 Although a large body of work has been dedicated to the mechanisms of uranium-bacteria interaction, less
76 is known about the cellular response induced by uranium exposure. Transcriptome and proteome profiling
77 has revealed various cellular responses, including general and membrane stress responses, DNA repair,
78 protection against oxidative stress, two-component signal transduction systems, efflux proteins, and iron
79 transport systems(27, 29, 35-40). In all of these studies, a large proportion of the identified genes/proteins
80 have no known function, indicating that there is still much to discover.

81 In an effort to uncover the molecular mechanisms of uranium stress response and tolerance, we have
82 isolated bacteria from metal- and radionuclide-rich soils(10, 41-43). One of the most uranium-tolerant
83 strains in our collection, *Microbacterium oleivorans* A9, has been subjected to a detailed analysis with a
84 multidisciplinary approach. Using tightly controlled exposure conditions, a 3-step process involving uranyl
85 sorption, release and biomineralization was evidenced(44). Using proteogenomics, we further showed the
86 high impact of uranyl on the proteome of this strain(45, 46). In the present work, we have examined three
87 additional *Microbacterium* strains for which we obtained the whole-genome(47), and subjected them to
88 comparative analysis (Fig. 1a). We first showed that these four strains differ in their uranium tolerance.
89 Key proteins involved in the uranium stress response were identified by comparative proteomics, which
90 led to the discovery of a protein named UipA specific to uranium-tolerant strains and highly up-produced in
91 response to uranyl. Next, we established that UipA is a single-pass membrane protein showing high
92 affinity for UO_2^{2+} and Fe^{3+} with dissociation constants in the nanomolar range. FTIR- and XAS-
93 spectroscopy showed that uranyl coordination involves carboxylate groups of Asp and Glu residues. The
94 three-dimensional structure of UipA, solved in its apo and uranium bound states, finally revealed two
95 PepSY domains with uranium binding sites located on a highly negatively charged face, and stabilized by
96 a set of conserved residues.

97 **Materials and Methods**

98 A detailed protocol is available in Supplementary Information.

99 ***Strains and culture conditions***

100 *Microbacterium* sp. ViU2A and *Microbacterium lemovicicum* ViU22^T strains were isolated from a natural
101 uranium-rich soil collected in Bessines, France(10, 42). The *Microbacterium oleivorans* sp. A9 strain was
102 isolated from a contaminated waste storage site in Chernobyl(43). The *Microbacterium* sp. HG3 strain was
103 isolated from black sand collected in Vik, Iceland(41). The bacteria were routinely cultivated in Luria Broth
104 (LB, Difco Laboratories) at 32°C with shaking.

105

106 ***Uranium tolerance assay***

107 Bacteria were cultured until the exponential growth phase and exposed to uranyl following a previously
108 described procedure(44). Briefly, cells were suspended in 0.1 M NaCl (pH 5.0) supplemented with 0, 10,
109 50, 100, 250 or 500 µM uranyl nitrate and incubated 24 h at 25°C. Subsequently, cells were washed,
110 deposited on LB agar plates and incubated for 48 h at 32°C to estimate cell survival.

111

112 ***Sequestration of uranyl by Microbacterium spp. and sample preparation for proteomics***

113 Bacteria were cultured until the exponential growth phase and exposed to 0 or 10 µM uranyl nitrate in 0.1
114 M NaCl (pH 5.0) as previously described(44). Samples were taken after 0.5, 2, 4, 6, 10 and 24 h
115 exposure. Cell pellets and supernatant were separated by centrifugation, and uranium concentration in the
116 supernatant was measured by inductively coupled plasma-atomic emission spectrometry. Controls without
117 bacteria demonstrated that uranium remains soluble and that no loss of uranium due to adsorption on the
118 vial wall occurred within the 24h exposure experiment. The fraction of uranium associated with the cells
119 was calculated as follows: $U_{\text{cells}} = (U_{\text{blank}} - U_{\text{solution}}) * 100 / U_{\text{blank}}$. Ultra-thin sections of bacterial cells exposed
120 to uranium for 24 h were analyzed by transmission electron microscopy as previously described(44). To
121 estimate cell viability, aliquots of cell suspensions taken at 0.5 and 24 h were diluted in LB and spread on
122 LB agar plates. Colony forming units (CFUs) were counted after 24 h at 30°C.

123 For the proteomic analysis, approximately 2 mg of cell pellets exposed or not to uranyl were collected at
124 0.5, 4 and 24 h by centrifugation and were immediately frozen in liquid nitrogen and kept at -80°C until
125 further processing. Four independent biological replicates were made.

126

127 **Shotgun proteomic analysis**

128 The cell samples were analyzed by shotgun proteomics according to the protocol described for strain A9
129 in (46). Peptides obtained after in-gel proteolysis of proteins with trypsin were characterized using a Q-
130 Exactive HF mass spectrometer (ThermoFisher) coupled to an UltiMate 3 000 LC system (Dionex-
131 LCPackings) operated according to a Top20 data-dependent acquisition method, as previously
132 described(48). Data were interpreted as described in (46) against the genome-annotated protein
133 sequence databases of the four *Microbacterium* strains(47). The statistical protein abundance variations
134 between the exposure conditions were compared using the T-Fold option of the PatternLab 2.0
135 software(49), taking into account the four replicates. The full proteomics data for each strain are available
136 in SI Tables S1-S4. The mass spectrometry proteomics data were deposited in the ProteomeXchange
137 Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository(50) with the
138 dataset identifiers: PXD020778 and 10.6019/PXD020778 for *Microbacterium* sp. HG3, PXD020767 and
139 10.6019/PXD020767 for *Microbacterium lemovicicum* ViU22, PXD020737 and 10.6019/PXD020737 for
140 *Microbacterium* sp. ViU2A, and PXD020998 and 10.6019/PXD020998 for *Microbacterium oleivorans* sp.
141 A9.

142

143 **Genomic context and sequence analysis**

144 Genomic analyses were performed with Genobrowser, an in-house bioinformatics tool for data
145 management. Sequence alignment of UipA proteins was calculated with Clustal W and visualized in
146 Jalview(51). A phylogenetic tree was constructed using 16S rRNA gene sequences from 482
147 Microbacteriaceae genomes available at GTDB (Genome Taxonomy Database)(52), identified only as
148 species representatives. A subtree of *Microbacterium* strains containing particular features was extracted
149 and analyzed with the ETE3 Python package(53). For this, alignment was made using MAFFT(54),
150 overhangs were trimmed with TRIMAL(55), and the tree was built with Fasttree(56). The final phylogenic
151 tree visualization with the *Uip* operon was made by Interactive tree of life (iTOL) tools(57). Synteny
152 analyses were performed with the MicroScope platform(58).

153

154 **Topology of UipA in vivo**

155 To determine *in vivo* the orientation of the UipA protein in the membrane, the pKtop plasmid encoding a
156 dual PhoA-LacZ α reporter was used(59). Three pKtop derivatives were constructed, in which *phoA-lac*
157 was fused in frame after the *uipA-ViU2A* codons R69, G96 and D281 (see SI). The constructions were
158 made by overlap extension PCR cloning according to the method described in (60). In a first step of PCR,
159 the inserts corresponding to the targeted regions of *uipA* and the overlapping regions of the pKtop plasmid
160 were amplified. The PCR products were purified and used as megaprimers to perform a second PCR with
161 the plasmid pKtop as matrix DNA. PCR products were digested by *DpnI* and transformed in *E. coli* DH5 α .
162 Transformants were selected on LB agar plates supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin and 0.2%
163 glucose. For protein topology assays *in vivo*, freshly transformed colonies were streaked on LB agar
164 plates supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin, 1 mM IPTG, 80 $\mu\text{g mL}^{-1}$ Red-Gal, and 100 $\mu\text{g mL}^{-1}$ BCIP.
165 Plates were incubated 24 h at 30°C and 14 h at room temperature.

166

167 **Production and purification of the extracellular domain of UipA recombinant proteins**

168 The DNA fragments coding for the soluble domain of UipA proteins were amplified by PCR
169 with primers that introduce the TEV protease recognition site upstream of the start codon (see SI). The
170 PCR products were ligated into the pQE30 plasmid to obtain the pQE-UipA recombinant plasmids for each
171 of the three homologs. The recombinant proteins were expressed overnight at 17°C after addition of 0.1
172 mM IPTG in *E. coli* M15Rep4. Cells were lysed and the soluble extract was loaded on a HiTrap HP
173 chelation column. The recombinant proteins were eluted from the resin with imidazole, desalted, and
174 incubated 48 h with TEV protease to remove the His tags. The samples were then loaded on a HiTrap HP
175 column, and recombinant proteins without the His tag were collected in the eluate fraction. A final
176 purification step was performed on an exclusion chromatography column to increase the purity. Protein
177 purity and integrity were checked by SDS-PAGE and mass spectrometry in denaturing conditions.

178

179 **Non-covalent mass spectrometry**

180 Non-covalent mass spectrometry analyses were performed on a MicrOTOF-Q (Bruker, Wissembourg,
181 France) with an electrospray ionization source. Protein-uranyl complexes were prepared, and the samples
182 were continuously infused at a flow rate of 7 $\mu\text{L}\cdot\text{min}^{-1}$. The mass spectra were recorded in the 50-4000
183 mass-to-charge (m/z) range. Data were acquired in the positive mode, and the calibration was performed
184 using a calibrating solution of ESI Tune Mix (Agilent) in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (95/5-v/v). The system was controlled
185 using the MicrOTOF Control 2.2 software package, and data were processed with DataAnalysis 3.4.

186

187 ***Metal-binding affinities of the UipA soluble domain measured by fluorescence titration***

188 The metal binding affinities of the three UipA_{ext} peptides for different metal cations (UO_2^{2+} , Fe^{3+} , Cu^{2+} ,
189 Ca^{2+} , Ni^{2+} and Zn^{2+}) were examined by monitoring the fluorescence intensity of tryptophan and tyrosine.
190 Fe^{3+} and Ca^{2+} were selected because they can be replaced by UO_2^{2+} on protein. The other metals were
191 selected as representatives of physiological metals with no known competition with UO_2^{2+} . To remove all
192 traces of metal, each protein solution was incubated with 3 mM EDTA and then washed. For fluorescence
193 titrations in the presence of uranyl or iron, iminodiacetate (IDA) was added to the peptide solution at a
194 IDA:peptide ratio of 10:1 to avoid the formation of hydroxo complexes, as previously described(61); this
195 was also done to control iron and uranyl speciation. Competition experiments between the UipA_{ext} proteins
196 and IDA were performed to determine the conditional dissociation constants of the protein-cation
197 complexes (uranyl or iron) at pH 6. IDA binds uranyl and iron with moderate affinity and forms three and
198 two complexes, respectively: UO_2IDA , $[\text{UO}_2(\text{IDA})_2]^{2-}$, $[(\text{UO}_2)_2(\text{IDA})_2(\text{OH})_2]^{2-}$ and $[\text{FeIDA}]^+$, $[\text{Fe}(\text{IDA})_2]$. The
199 stability constants at 25°C and 0.1 M ionic strength were obtained from (62). For iron-IDA complexes, the
200 stability constants were obtained from(63). The experiments were carried out with 10 μM protein in 20 mM
201 MES buffer (100 mM KCl; pH 6). Fluorescence was measured at 25°C using a 275-nm excitation
202 wavelength. Emission was recorded from 300 to 380 nm. The excitation and emission slits were 10 nm.
203 The reported dissociation constants are averages of three experimental values, including standard
204 deviation. The analysis of the multiwavelength data set was performed using the commercially available
205 ReactLab software(64). The goodness of fit was assessed by the convergence value σ and visual
206 inspection of the residuals.

207

208 ***Fourier-transformed infrared spectroscopy***

209 For Fourier-transformed infrared spectroscopy (FTIR), proteins were concentrated to about 500 μM in 20
210 mM MES buffer (pH 6). Uranyl or iron from stock solutions of uranyl nitrate and iron chloride at 50 mM in
211 2% HCl were added to reach a stoichiometric peptide:metal ratio of 1:1. In parallel, control samples were
212 prepared in 20 mM MES buffer (pH 6) by adding an equivalent volume of 2% HCl solution. Samples with
213 proteins were then deposited onto the ATR-FTIR crystal. For spectra acquisition, samples were first dried
214 for half an hour after deposition. The spectra were recorded on an IFS66 SX FTIR spectrometer (Bruker)
215 equipped with a KBr beam splitter, a DTGS-KBR detector, and an attenuated total reflection device (ATR;
216 SensIR Technologies, CT) equipped with a 4.3-mm diameter diamond 9-bounce microprism and ZnSe
217 optics. Each single spectrum corresponded to 50 co-added scans at a resolution of 4 cm^{-1} . All reported
218 frequencies have an accuracy of $\pm 1\text{ cm}^{-1}$. The spectra correspond to the average of data recorded with 2
219 to 4 replicates in the same conditions. To calculate the difference spectra between uranyl or iron-
220 containing samples and control samples, interactive subtraction was used to minimize the effect of small
221 differences in absorption between samples. These differences in total absorption have consequences on
222 bands associated with the buffer in the difference spectra (noted as * in the spectra). Interpretation of the
223 FTIR data was limited to bands that were reproducibly observed in spectra obtained with different
224 samples. IR bands of MES buffer and nitrate were identified according to (65) and (66) respectively.

225

226 ***Synchrotron-based analysis***

227 UipA-uranyl complexes were prepared by mixing UipA_{ext} proteins at 120 μM in 20 mM MES buffer 100 mM
228 KCl pH 6 with 100 μM uranyl nitrate. Next, UipA-uranyl complexes were loaded in a kapton-teflon liquid
229 cell for synchrotron analysis. EXAFS experiments were performed on the MARS beamline at the SOLEIL
230 synchrotron facility(67). Energy calibration was performed at the yttrium K edge at 17038 eV, and EXAFS
231 experiments at the U L_{III} edge. All measurements were conducted in fluorescence mode using a 13-
232 element high purity germanium detector (ORTEC) at room temperature. EXAFS data processing was
233 performed using the ATHENA code(68). The E₀ energy was identified at the maximum of the absorption

234 edge. Fourier transformation (FT) with k^2 weighting was performed between 2.8-11.8 \AA^{-1} with a Hanning
235 window. The fits were performed using the DEMETER code (Demeter version 0.9.25) in R space between
236 1 and 5 \AA . For EXAFS data fitting, one global amplitude factor S_0^2 and one energy threshold correction
237 factor E_0 were used for every path of the fits. The agreement factor r (%) and the quality factor (QF =
238 reduced χ^2) of the fits were provided directly by DEMETER. Phases and amplitudes were calculated using
239 the FEFF6 simulation code integrated in DEMETER based on the structural model of the uranyl-acetate
240 complex ($\text{UO}_2(\text{CH}_3\text{COO})_2$)(69). During the fitting procedure, the relative number of mono and bidentate
241 carboxylates was allowed to fluctuate, but the total number of O_{eq} was fixed to 5.

242

243 ***Crystal structure of UipA***

244 UipA_{ext} proteins were concentrated to approximately 15 $\text{mg}\cdot\text{ml}^{-1}$. We tested the crystallization of all three
245 UipA_{ext} proteins by vapor diffusion using a TECAN robot and different commercial crystallization kits
246 (JCSG, Pact Premier and Stura (Molecular Dimensions)). Only crystals of $\text{UipA}_{\text{ext}}\text{-ViU2A}$ could be
247 obtained, which diffracted poorly (to 15-20 \AA at synchrotron sources). Different strategies were therefore
248 examined to improve diffraction. The screening of additives proved to be the most efficient, as formamide
249 improved the diffraction quality by up to 3.2 \AA when using a rotating anode. The final crystallization
250 conditions consisted in mixing 0.7mL of $\text{UipA}_{\text{ext}}\text{-ViU2A}$ with an equal volume of a reservoir solution
251 (100mM MES pH6, PEG6K 22%, formamide 6% and ZnCl_2 5-15mM). For phasing, one crystal was
252 soaked in a mother liquor directly supplemented with gadolinium powder (Gd-HPDO3A). Another crystal
253 was soaked in a mother liquor supplemented with 5 mM uranium nitrate ($\text{UO}_2(\text{NO}_3)_2$). All crystals were
254 briefly soaked with a mother liquor containing 5% glycerol before flash-cooling directly in liquid nitrogen
255 before data collection.

256 Diffraction data were collected on a PROXIMA-1 beamline at the SOLEIL synchrotron (France). Data were
257 processed with the XDS program(70) using XDSME scripts(71). The structure of $\text{UipA}_{\text{ext}}\text{-ViU2A}$ was
258 solved by the SAD method using the Gd derivative, and the gadolinium positions were found using the
259 SHELX C/D/E suite of programs(72). Structure was built semi-automatically using the ARP/wARP

260 program(73) and completed by rounds of model building using COOT(74), with refinements using
261 Refmac(75). Details of the crystallographic analysis are presented in Table S5.

262

263 **Statistical analyses**

264 All measurements were performed at least in triplicate. The symbol \pm corresponds to standard deviation.

265

266

267 **Results**

268 ***Microbacterium spp. strains show contrasted U tolerance phenotypes***

269 Bacterial culture collections were previously established from uranium-rich soil samples collected in
270 France(10), radionuclide-contaminated soil samples collected at Chernobyl(43), and volcanic black sand
271 collected in Iceland(41). A preliminary broad plate screening of these bacterial strain collections indicated
272 that four actinobacterial strains, namely *Microbacterium* sp. HG3, *Microbacterium* sp. ViU2A,
273 *Microbacterium oleivorans* A9 and *Microbacterium lemovicicum* ViU22, had contrasted uranium tolerance
274 capabilities (data not shown). To refine this result, the tolerance of these four strains against uranium was
275 evaluated by a drop test. As shown in Fig. 1b, the survival of strains HG3 and ViU2A was not affected by
276 uranium at 500 μ M, and strain A9 could tolerate up to 250 μ M of uranium. In contrast, the survival of strain
277 ViU22 was severely affected by 100 μ M of uranium.

278

279 ***Interactions of Microbacterium spp. strains with uranyl***

280 Bacteria remained viable after 24h in the controls without uranyl and with 10 μ M uranyl (Fig. S1). All
281 strains were able to deplete an important fraction of the metal from the solution (Fig. 1c) and to
282 biomineralize it within 24 h (Fig. 1d). The time evolution of uranium removal by the uranium-tolerant
283 strains ViU2A, HG3 and A9 had similar overall profiles, comprising 3 steps: a rapid removal of 80-90% of
284 uranium within the first 30 minutes, likely by biosorption; a release of uranium into the solution between
285 0.5 and 4 h; and a final slow uranium removal step leading to removal of more than 80% of the initial
286 uranium. The release of uranium by strain A9 was less efficient compared to the other two uranium-

287 tolerant strains (ViU2A and HG3). Regarding the uranium-sensitive strain ViU22, only 40% of uranium was
288 associated with the cells after 30 minutes, no uranium release step was observed, and uranium removal
289 after 24 h was approximately 60% of the initial amount of uranium.

290

291 ***U-stress molecular response of *Microbacterium* spp. strains***

292 Given the different behaviors of the *Microbacterium* spp. strains toward uranium, a comparative
293 proteogenomics approach was used to decipher the molecular changes occurring in these strains upon
294 uranyl exposure, and to highlight the key proteins possibly involved in uranium tolerance. We recorded
295 large shotgun proteomics datasets for each strain at three times after uranyl addition, corresponding to the
296 sorption, release and biomineralization phases. We interpreted these data with proteomes constructed
297 from the corresponding complete genome sequences (GenBank accession numbers CP031421,
298 CP031422, CP031338, and CP031423). Overall, a total of 2 038, 1 156, 1 523 and 1 312 proteins were
299 validated by at least two peptides for strains HG3, ViU2A, A9 and ViU22, respectively (Tables S1-S4).
300 These proteins represented between 29.7 and 53.4% of the proteins predicted by the genome, reflecting a
301 good coverage of the theoretical proteome. The functional classification of all proteins showed a
302 distribution between all clusters of orthologous genes (COG) similar to the distribution of Coding DNA
303 Sequences (CDS) predicted from the genomes (Table S6). As expected for the overlooked
304 *Microbacterium* genus, the “poorly characterized” proteins represented a large fraction, ranging from 38.9
305 to 48.4% of the total proteins.

306 In response to uranium, the abundance of proteins in strains HG3 (966 proteins), ViU2A (452 proteins), A9
307 (596 proteins) and ViU22 (535 proteins) was significantly modulated, with fold-change (FC) values varying
308 from -10.3 to +32.3 (\log_2 FC varying from -3.36 to 5.01; Fig. 1e). This represents 39.1-47.4% of the total
309 proteins detected, showing that uranium has a strong impact on the metabolism of the four strains,
310 regardless of their tolerance phenotype. The proteomics data shows that uranyl has an impact on
311 phosphate and iron metabolism in all strains (Table S7). In particular, several proteins were modulated by
312 uranyl, including proteins of the phosphate (Pho) regulon and the high-affinity phosphate transport system
313 (Pst) (Fig. S2), and proteins involved in siderophore transport (FepC, YclQ) and in iron-sulfur cluster

314 assembly (SufB, SufD). Several predicted transporters including ABC transporters were also modulated.
315 Noteworthy, the six proteins with the highest FC values are all predicted to be membrane proteins (Table
316 1).

317 To search for proteins potentially involved in uranium tolerance, we performed a comparative
318 proteogenomic analysis of the four strains. We identified only four proteins modulated by uranium that
319 were common to the tolerant strains, and which were absent or not modulated in the sensitive strain (in
320 bold in Table 1). One protein is annotated as YclQ. In response to uranium, its abundance increased in
321 the three tolerant strains (FC values: 8.4 and 7.1 in strain ViU2A; 2.2 and 2.1 in strain HG3 at 4 and 24 h,
322 respectively; and 1.4, 1.6 and 2.9 in strain A9 at 0.5, 4 and 24 h, respectively). In contrast, it was not
323 detected in the proteome of the uranium-sensitive strain ViU22, although the corresponding gene is
324 present in the genome. A second protein is annotated as DegP. This protein was more abundant in the
325 uranium-tolerant strains under uranium stress (FC values: 1.7 and 4.7 in strain ViU2A; 2.0 and 3.1 in
326 strain HG3; and 1.5 and 5.3 at 4 and 24 h in strain A9), and was either not modulated or less abundant in
327 ViU22 exposed to uranium (FC = -1.3 at 4h).

328 The two other proteins had no functional annotation and were named UipA and UipB for Uranium-Induced
329 Protein A and B. The FC values of UipB at 4 h were 2, 6.8 and 1.7 in strains ViU2A, HG3 and A9,
330 respectively (Table 1). The protein was detected in the proteome of strain ViU22, but its abundance was
331 not significantly modulated by uranyl exposure. UipB is predicted to be a membrane protein of 210 kDa
332 with most of it located outside of the cell. It displayed several conserved domains consisting of a cadherin
333 repeat (cd11304) and cadherin-like domains (pfam 17803), bacterial immunoglobulin domains (Big-9,
334 pfam17963), and fibronectin type III domains (Fn3, pfam00041). Finally, UipA was the most modulated
335 protein at 24 h in strain ViU2A (FC = 10.5), and at 4 and 24 h in strain A9 (FC = 11.5 and 32.3
336 respectively), and was the second-most modulated protein at 24 h in strain HG3 (FC = 8.62) (Table 1).
337 The corresponding gene was absent from the genome of the uranium-sensitive strain ViU22. Thus, the
338 UipA protein is specific to uranium-tolerant strains and is highly accumulated in cells in response to uranyl
339 exposure. This unique profile, which is expected for a protein involved in uranium tolerance, clearly sets
340 UipA apart from the other proteins. We therefore focused on this protein for a detailed characterization.

341

342 **Genomic context of *uipA***

343 Upstream of the *uipA*_{VIU2A} (CVS53_03692), *uipA*_{HG3} (CVS54_03678) and *uipA*_{A9} (BWL13_02796) genes,
344 we found two genes homologous to the two-component *czcRS* system involved in metal tolerance in
345 *Pseudomonas aeruginosa* and *Cupriavidus metallidurans*(76, 77). Based on this homology, these genes
346 were named *uipR* and *uipS* (Fig. S3A). The *uipA* genes are orphans with no homologs in any other
347 lineages than *Microbacterium*. A deeper analysis showed that the genomic context of *uipRSA* is
348 conserved in almost all *Microbacterium* strains with the *uipA* gene (Fig. S4). As was the case for the *uipA*
349 gene, the *uipR* and *uipS* genes were also absent from the genome of the U-sensitive strain ViU22.

350

351 **Sequence analysis and topology of UipA proteins**

352 The genes *uipA*_{VIU2A}, *uipA*_{HG3} and *uipA*_{A9} respectively encode proteins of 281, 285 and 247 aa in length
353 (Fig. S3B-C), and share 36.6-74.6% sequence identity with each other. Two conserved PepSY domains
354 (Peptidase pro-peptide and YPEB domain; pfam03413) were predicted at their C-terminal part by
355 HHpred(78). UipA proteins are predicted to be single-pass transmembrane proteins with a cytoplasmic N-
356 terminal domain (38-75 aa in length), and an extracellular C-terminal domain of 185-186 residues
357 according to CCTOP(79). We obtained experimental validation of this predicted topology for UipA_{VIU2A}
358 using a PhoA-LacZ α reporter system(59) (Fig. S5).

359 Remarkably, these proteins have low pI values (3.86-3.99) due to their richness in negatively charged
360 residues (aspartate and glutamate), accounting for 18.6-20.7% of the total amino acids, as compared to
361 12.0% on average in the proteomes (Fig. S3B). Since carboxyl groups of aspartate and glutamate can
362 provide oxygen atoms for uranyl coordination(80), we speculated that the UipA extracellular domain could
363 bind uranyl with a high affinity. Thus, we investigated the ability of the recombinant extracellular domain of
364 UipA to bind uranyl using a combination of mass spectrometry in the native mode and spectroscopic
365 approaches.

366

367 **Stoichiometry of UipA_{ext} protein-(UO₂²⁺) complexes**

368 The extracellular domains of UipA proteins (named UipA_{ext}) were produced in *Escherichia coli* and
369 purified. The formation of UipA_{ext} protein-(UO₂²⁺) complexes was first analyzed by native mass
370 spectrometry. This method enables detecting the formation of protein-ligand complexes and assessing the
371 stoichiometry of these complexes. The spectra displayed peaks compatible with 1:1 and 1:2 UipA_{ext}-
372 (UO₂²⁺) complexes (Fig. S6).

373

374 ***Binding affinities of UipA proteins for uranyl and other metal cations***

375 The affinity of UipA_{ext} recombinant proteins for UO₂²⁺ was measured by fluorescence titration, taking
376 advantage of the presence of a conserved Trp residue on each of the proteins as well as an additional Tyr
377 on UipA_{ext}-HG3 and UipA_{ext}-ViU2A (Figs. S7-S12). In line with native mass spectrometry results, the best
378 fitting curves were obtained with the assumption of two uranyl binding sites, and K_d values were calculated
379 accordingly (Figs. S7, S9 and S11). As shown in Table 2, the conditional dissociation constants of the 1:1
380 and 1:2 UipA_{ext} - UO₂²⁺ complexes were within the nanomolar range, with K_d values ranging from 1.3 x
381 10⁻⁹ to 5.0 x 10⁻⁸ M. This indicates a high binding affinity of UipA_{ext} domains for the two uranyl molecules.
382 To determine whether UipA_{ext} proteins have a high affinity for other metals, the K_d values of complexes
383 between UipA_{ext} proteins and Ca²⁺, Fe³⁺, Ni²⁺ and Zn²⁺ were measured (Figs. S13-S33). The best fitting
384 curves of the three UipA_{ext} proteins and Fe³⁺ were obtained with the assumption of two metal binding sites
385 (Figs. S13, S15 and S17). The dissociation constants of the 1:1 protein - Fe³⁺ complexes were in the
386 nanomolar range, with K_d values ranging from 3.3 x 10⁻⁹ to 3.2 x 10⁻⁸ M. The dissociation constants of the
387 1:2 protein - Fe³⁺ complexes were more contrasted, with K_d values ranging from 2.3 x 10⁻⁸ to 4.4 x 10⁻⁵ M
388 (Table 2).

389 By contrast, the K_d values measured for Ca²⁺, Ni²⁺ and Zn²⁺ were at least three orders of magnitude higher
390 than the dissociation constants measured for UO₂²⁺ and Fe³⁺ (ranging from 1.7 x 10⁻⁵ to 2.5 x 10⁻³ M),
391 reflecting a much lower affinity of UipA_{ext} proteins for these divalent metal cations, as compared to UO₂²⁺
392 and Fe³⁺. It should be noted that for the three metal cations, two binding sites were inferred from the
393 fluorescence titrations for the HG3 and ViU2A strains. The 1:2 protein:metal complex was not detected on

394 the UipA_{ext}-A9 protein, unlike the other two proteins (Figs. S23, S28 and S33). This is probably due to the
395 lack of the Tyr residue in this protein, which could not be used as a spectroscopic probe.

396

397 ***Characterization of uranyl and iron coordination***

398 The coordination of UO₂²⁺ and Fe³⁺ by the UipA_{ext} domains was further studied by ATR-FTIR
399 spectroscopy. ATR-FTIR spectra were recorded for each UipA_{ext} protein with or without metal, and the
400 difference spectra were calculated (Figs. 2a and S34). In these spectra, positive and negative bands
401 represent vibration modes of functional groups that are subject to changes due to the coordination of
402 uranyl or iron by the protein. The FTIR spectra are dominated by contributions from the carboxylate
403 groups of Asp or Glu (ν_{as} and $\nu_s(\text{COO}^-)$ IR modes), which are highly sensitive to the coordination mode of
404 the carboxyl group to metal cations or uranyl(81-84). The spectra indicate uranyl coordination by
405 monodentate and bidentate carboxylate groups (Fig. 2a). The $\nu_{as}(\text{COO}^-)$ and $\nu_s(\text{COO}^-)$ IR modes of the
406 bidentate carboxylate(s) were assigned at 1 510 and 1 454 cm⁻¹ for UipA_{ext}-A9, and at 1 534 and 1 456
407 cm⁻¹ for UipA_{ext}-HG3 and UipA_{ext}-ViU2A. For the monodentate carboxylate groups, the $\nu_{as}(\text{COO}^-)$ modes
408 were observed at 1 627 cm⁻¹ for UipA_{ext}-A9 and at 1 606-1 610 cm⁻¹ for UipA_{ext}-HG3 and UipA_{ext}-ViU2A.
409 The frequency of the $\nu_{as}(\text{UO}_2^{2+})$ stretching mode also differed slightly for UipA_{ext}-A9 at 912 cm⁻¹, and for
410 the other strains at 916 cm⁻¹. A similar involvement of mainly carboxylate groups was observed upon iron
411 binding to the UipA_{ext} proteins (Fig. S34). As compared to uranyl, a greater number of bidentate ligands
412 appear to be present in the iron binding site, with a larger relative intensity of bands assigned to the
413 $\nu_{as}(\text{COO}^-)$ and $\nu_s(\text{COO}^-)$ IR modes of bidentate carboxylates at 1 513-1 526 and 1 441-1 441 cm⁻¹ (Fig.
414 S34). Note that for Fe³⁺, coordination is expected to involve 6-7 ligands in all directions, whereas for
415 uranyl, 5 to 6 ligands are expected to contribute in the equatorial plane.

416 EXAFS spectroscopy was performed as a complementary method to probe the uranyl coordination
417 environment within UipA_{ext} proteins. It can be observed that all spectra exhibit similar waves (Fig. 2b),
418 suggesting that the uranyl environment is very similar in all three proteins. Metrical parameters associated
419 with the fit of the EXAFS data are summarized in Table S8. All uranyl cations are made of 2 (fixed) oxo
420 bonds at 1.79 Å, in very good agreement with molecular uranyl in general(85) and close to solid state

421 uranyl acetate for instance(69). In all three proteins, the distance associated with equatorial oxygen atoms
422 is very similar, between 2.36 and 2.38 Å. Although the signal of the second coordination sphere is
423 relatively weak due to the presence of backscatters of low atomic number, best fits have been obtained
424 with a shell of C atoms (see Materials and Methods section), indicating that carboxylate functions (mono
425 and/or bidentate) are most likely involved in the uranyl coordination. Accordingly, a U-C single scattering
426 path accounting for the C atoms of bidentate functions and a U-O-C triple scattering path accounting for
427 the monodentate functions have been fitted. The values obtained for the U-O_{eq} average distance are
428 indicative of the presence of mostly monodentate carboxylate groups(84-87), although only average
429 values are discussed here. Regarding the carbon atoms, the U-C average distances reported in Table 1 in
430 the range of 2.9 - 3.0 Å also support the presence of bidentate carboxylate functions(82, 86). Therefore,
431 we propose the presence of approximately 2 monodentate functions (Table S8) and complementary
432 bidentate functions. It should be noted that the fit of the second coordination sphere in UipA_{ext}-A9 is
433 associated with very high uncertainties, and the values must be considered carefully. Therefore, we
434 propose the presence of at least one bidentate function (Table S8).

435

436 ***Crystal structure of UipA and the uranyl-binding site***

437 We tested the crystallization of all three UipA_{ext} proteins and obtained crystals of UipA_{ext}-VIU2A (hereafter
438 abbreviated as UipA) that diffracted up to 2.3 Å using a synchrotron source. The three-dimensional
439 structure was solved using the SAD method with a crystal soaked with Gd-HPDO3A (Table S5). Overall,
440 the three-dimensional structure of UipA (built from residues 143-280, the first 48 residues connecting the
441 membrane helix to residue 143 being disordered) displays a domain-swapped dimer, with each monomer
442 folding as two distinct domains (Fig. 3a).

443 Each domain adopts the same fold, a central α -helix packed on a 4-stranded antiparallel β -sheet (see Fig.
444 S35B for a superimposition of all individual domains) reminiscent of the PepSY (pfam03413) domain. In a
445 lipoprotein from *Clostridium difficile* (pdb code 4EXR; with 13% sequence identity), a distant 3D-
446 homologue found by threading, these two domains appear close together in a protein apparently forming a
447 monomer, while in UipA they are swapped in a dimer related by a crystallographic axis. However, the two

448 pseudo-monomers of UipA (the NTer domain of one monomer plus the CTer domain of the other
449 monomer and *vice versa*) superimpose very well with the lipoprotein, suggesting that the folding unit of
450 UipA is this pseudo-monomer (Fig. S35A). Although dimerization might be a crystallization artifact, it also
451 suggests that dimerization of UipA may be functional and could occur at high concentration, such as within
452 its membrane context, in which local protein concentration could be high due to 2D-limited diffusion. Since
453 the two α -helices of both domains of the pseudomonomer are on the same face, this arrangement creates
454 a six-stranded β -sheet concave surface which appears to be highly negatively charged (Fig. 3b). In the
455 native crystal structure of UipA, five zinc ions arise from the crystallization conditions, each of which were
456 located from an anomalous map collected at the Zn edge. Two of these zinc binding sites are on the α -
457 helical side and the other three are on the opposite concave face (Fig. S36).

458 To decipher the uranium binding site on UipA, we soaked a crystal in a mother liquor supplemented with
459 5mM uranyl nitrate. Since we collected data at a wavelength where U contributes to the anomalous signal
460 but not Zn, we were able to unambiguously determine the position of four uranyl binding sites, three of
461 which were located on the β -sheet concave face (Fig. 3b-d, Fig. S37). The highest peak (labeled U1) is
462 made of residues Glu190 and Glu192 from one monomer, and Ser205' and Asp207' from an adjacent
463 monomer (Fig. 3c). However, the side chains of these residues are not well-resolved (see Fig. S37 for the
464 refined $2F_{\text{obs}}-F_{\text{calc}}$ map), the distance to the uranium atom is quite long (3.7-4.8 Å), and the Asp207' side
465 chain is also involved in binding to a Zn ion. The second uranium binding site (U2) is contributed by
466 Glu192 and Glu180. The side chains of these residues are not well defined, and the distances to the
467 uranium atom are quite long (3.9 Å and 4.7 Å). The third uranium peak (U3) is surrounded by the side
468 chains of Ser170, Asp172, Asp244' and possibly Asp242'. This site is well resolved in the electron density,
469 and it was even possible to tentatively model one or two additional water molecules (although this could
470 be an over-interpretation, due to the medium resolution of 2.9 Å achieved by this crystal; see Fig. S37).

471 The distances of the oxygen atoms to the uranium atom ranged from 2.2-3.5 Å. Both carboxylates of these
472 aspartate residues are monodentate, as observed on all of the other sites, although the moderate
473 resolution obtained might not be sufficient to precisely determine their binding mode. The fourth uranium
474 site (U4) is contributed by the side chains of Glu152 and Glu197, with the uranyl to oxygen distances

475 ranging from 2.8-3.9 Å (Fig. S37). Sites U1 and U3 are remarkably similar, as they are made by four
476 residues distributed on two adjacent β -strands (Fig. S38). Finally, we found that within the crystal packing,
477 the dimers of UipA and the U and Zn binding sites create large tunnels of approximately 35 Å along the
478 P4₃ crystallographic axis that are lined with the concave acidic face of UipA, and where almost all of the
479 zinc and uranium ions bind (Fig. S39).

480

481 **Discussion**

482 *Microbacterium* is a widespread genus of actinobacteria that remains largely understudied. In this work,
483 we confirmed the ability of *Microbacterium* spp. A9, ViU2A and HG3 to tolerate uranium. This phenotype
484 has been reported for several other *Microbacterium* strains of diverse origin (11, 16, 17, 88). Our work has
485 shown that this trait is not strictly conserved across the *Microbacterium* genus, as *M. lemovicicum* ViU22
486 was shown to be sensitive to uranium.

487 When exposed to a non-toxic concentration of bioavailable uranyl, all strains were able to sequester the
488 metal by biosorption and intracellular biomineralization, regardless of their uranium tolerance phenotype.

489 Therefore, biomineralization, which has been proposed to contribute to uranium tolerance(26, 89), does
490 not appear to be sufficient on its own. Phosphatases, in particular acid and alkaline phosphatase, play a

491 major role in the biomineralization of uranium. Here, alkaline phosphatase was not detected in the

492 proteome in any of the four stains, while acid phosphatase was detected in the proteome of strains A9,

493 HG3 and ViU22, albeit with a very low abundance (0.006, 0.005 and 0.016% of the total proteins,

494 respectively; Tables S2, S3 and S4). The proteins were not modulated by uranyl, except in strain HG3 at

495 4h (FC = 3.7). In strain ViU2A, one gene annotated as acid phosphatase was detected in the genome but

496 the corresponding protein was not detected in the proteome, which can be due to the low abundance of

497 the protein. The contribution of constitutive phosphatases to uranium biomineralization has been observed

498 in *Serratia* sp. strain OT II 7(90) and *Caulobacter crescentus*(89).

499 Interestingly, we only observed a transitory step of uranyl release in the uranium-tolerant strains. A

500 uranium release step has been observed in other microorganisms (26, 37, 91, 92). Although the

501 mechanism of uranyl release is unexplained, it could involve an efflux transporter. Efflux is a conserved

502 mechanism that can mitigate the intoxication of a variety of physiological and toxic metals(93). Up-
503 regulation of genes encoding metal efflux pumps of the RND, CDF and PIB-ATPase families during
504 uranium exposure has been reported(31, 40, 94, 95), and horizontal gene transfer of PIB-ATPases has
505 been observed in bacteria isolated from uranium rich soils(30, 32). However, even though multidrug
506 resistance pumps have been induced in *C. crescentus* by uranyl exposure, none of them were found to
507 play a role in uranium tolerance(96). In fact, except for *C. crescentus*, in which the membrane transporter
508 RsaFa (involved in S-layers export) is proposed to be involved in uranium efflux, the molecular
509 mechanisms of uranyl trafficking across membranes remain largely unknown. We have shown that several
510 proteins of the ABC transporter family (YxdL, YclQ, GlnP, NatA, YbiT, PstA and PstC) were up-produced
511 by uranyl in at least one uranium-tolerant strain. YxdL is the only one of these proteins predicted to be an
512 efflux transporter, mediating the efflux of cationic antimicrobial peptides and drugs in *B. subtilis*(97) and
513 *Clostridium hathewayi*(98), respectively. Its potential role in uranyl efflux needs to be further examined.
514 Regarding the other ABC transporters, YclQ is a petrobactin-binding protein that is part of an ABC
515 transporter involved in iron transport in *B. subtilis*(99); GlnP is a permease responsible for glutamate
516 uptake in bacteria(100); and PstA and PstC form a membrane channel responsible for phosphate
517 internalization. We speculate that all of these transporters could mitigate uranyl intoxication by importing a
518 variety of ligands (siderophores, phosphate and glutamate), which can chelate the metal and thereby
519 reduce its bioavailability.

520 Among the proteins that are positively modulated by uranyl, only four were specific to the uranium-tolerant
521 strains: YclQ and DegP, and two proteins with no functional annotation, UipA and UipB. Interestingly,
522 these four proteins were among those with the highest FC values in at least one strain. The corresponding
523 genes have no relationship at the genomic level. All of these proteins are predicted to be located at the
524 cell membrane, showing that uranyl causes membrane damages, which is consistent with the literature
525 (reviewed in (2)). DegP, a serine protease, is involved in the degradation of misfolded and aggregated
526 proteins in the periplasm of Gram-negative bacteria(101). In Gram-positive bacteria, DegP is located at
527 the membrane and could also be involved in the envelope stress response, as recently suggested for

528 *Streptococcus gordonii*(102). In *Microbacterium*, DegP could alleviate the toxic effect of uranyl on
529 membrane proteins and participate in uranyl tolerance.

530 The protein UipB displayed several conserved domains such as a cadherin repeat, and cadherin-like, Fn3
531 and Big-9 domains. Cadherin repeats and cadherin-like domains are found in Ca^{2+} -binding proteins
532 involved in cell-cell interactions and adhesion. In bacteria, Fn3 domains could be involved in the anchoring
533 of extracellular glycohydrolases to their substrate(103). Big domains are proposed to be involved in
534 various functions such as adhesion and biofilm formation(104), and have been described as Ca^{2+} -binding
535 domains in a surface adhesin(105). Big-9 domains are located at the N-terminal part of MtrF, a membrane
536 cytochrome involved in the utilization of extracellular mineral forms of iron and manganese as respiratory
537 electron acceptors in *Shewanella oneidensis*(106). In addition, immunoglobulins have been identified as
538 uranyl-binding proteins in the human serum(107). These data suggest that UipB could be a modular
539 protein with adhesion and uranyl- and/or metal cation-binding properties.

540 The protein UipA stood out from the other proteins modulated by uranyl because it was specific to the
541 uranium-tolerant strains and had the highest FC values. Here, we established that UipA is a single-pass
542 transmembrane protein with a large extracellular C-terminal part, displaying two high-affinity uranyl-
543 binding sites with conditional K_d values in the nanomolar range. By comparison, a calmodulin-derived
544 peptide considered to be a strong chelator of uranyl has conditional K_d value for UO_2^{2+} in the vicinity of 30
545 nM (61). ATR-FTIR and EXAFS spectroscopy established that uranyl coordination involves monodentate
546 and bidentate carboxylate groups, in line with what is described in the literature(80). Crystallographic
547 analysis showed that the extracellular part of UipA is organized in two PepSY domains, forming a domain-
548 swapped dimer with a highly negatively charged concave surface. Four uranyl-binding sites were detected
549 by crystallography, three of which are located on the negatively charged surface of UipA formed by 2
550 PepSY domains. However, in two of these sites (U2 and U4), the uranyl coordination only involves two
551 ligands. This is unexpected for uranyl coordination in proteins(80), and could be an artefact due to the
552 high concentration of the uranyl solution used to soak the crystal. In contrast, the U1 and U3 binding sites,
553 which involve conserved D and E residues, are highly similar (Fig. 3c-d, Fig. S38) and may correspond to
554 the two binding sites identified by mass spectrometry and fluorescence titration.

555 PepSY domains (pfam 03413) are found in the N-terminal pro-peptide of the M4 peptidase family, where
556 they inhibit protease activity(108). However, PepSY domains have also been detected in several non-
557 peptidase proteins classified in different families, where they play an unknown function. Interestingly,
558 membrane proteins with PepSY domains have been reported as iron-regulated (109-111). Here, we
559 assigned for the first time a function to the PepSY domain in a non-peptidase protein by showing that it
560 can bind iron and uranyl with a very high affinity. Regarding uranium, UipA could participate in uranium
561 tolerance through metal capture by its PepSY domains and sequestration at the cell surface, consequently
562 limiting uranium entry into the cytoplasm. However, this sequestration would not completely prevent metal
563 internalization in our experimental conditions since biomineralization occurred in all strains. It should be
564 mentioned that uranium was not detected by TEM at the cell membrane. This indicates that if UipA-uranyl
565 complexes formed at the membrane, they were not detectable by microscopy, possibly because non-
566 precipitated forms of uranium are under the detection limit.

567 The high binding affinity of UipA for iron raises many questions about its physiological role and its potential
568 functions in iron metabolism. Upstream of the *uipA* gene we found *uipR* and *uipS*, two genes predicted to
569 encode a two-component system. One attractive hypothesis is that UipRSA constitutes a novel system
570 involved in iron/uranium sensing (Fig. 4). UipR and UipS were either not detected (strain ViU2A) or not
571 significantly modulated by uranyl (strains A9 and HG3) in the proteomic analysis, but this may be due to
572 their very low abundance. Hence, the role of UipR and S as an iron/uranium sensing system is a working
573 hypothesis that will need to be further examined.

574 A striking comparison can be made between *uipRSA* and the uranium-induced operon CCNA_01361-
575 *urpR-urpS* in *C. crescentus*(112) and the ferrous iron-responsive operon *bqsPQRS* in *P. aeruginosa*(111).
576 In these systems, the expression of PepSY membrane protein genes (CCNA_01361 as well as BqsP and
577 Q) is mediated by a two-component system (RS proteins) in response to metal exposure. In *B. subtilis*,
578 Yycl (a PepSY-containing membrane protein) has been shown to modulate the kinase activity of the
579 YycFG two-component system through transmembrane domain interactions(113), and the same role has
580 been proposed for BqsP and BqsQ in *P. aeruginosa*(111). A similar role can thus be suggested for UipA in
581 modulating the UipRS two-component system in response to iron concentration. In addition, our results

582 suggest that UipA, with its nanomolar binding affinity for iron, captures the metal outside of the cell and
583 may participate in iron homeostasis. Because iron is a critical resource, one hypothesis is that UipA could
584 constitute an iron reservoir for the cell. In this scenario, UipA would likely interact with another protein for
585 iron transport. The crystallographic structure shows that UipA has a flat, negatively charged surface.
586 Based on this observation, we speculate that another possible function of UipA could be to promote
587 interaction with solid surfaces such as iron-containing minerals. More generally, the interplay between
588 uranium and iron in these environmental bacteria will have to be explored in more detail.

589

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599

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602

603

604

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894

895 **Table and Figure legends**

896 **Table 1.** Top10 proteins with the highest FC values and their homologs in the other strains after 0.5, 4 and
897 24 h of uranium exposure. The data are sorted in descending order of maximum FC value. For clarity, the
898 data are highlighted using the following color code. Blue: positive FC; red: negative FC; green: FC (below
899 1.5) or p-value (above 0.05) not significant (ns); orange: not detected in the proteome (nd); and grey:
900 absent gene (abs). Proteins specific to the uranium-tolerant strains are shown in bold.

901
902 **Table 2.** Macroscopic dissociation constants for 1:1 and 1:2 protein-metal complexes of UipA_{ext} proteins
903 and selected metals. Values are given in M.

904 ± corresponds to the standard deviation, calculated from 3 independent experiments.

905

906 **Figure 1. Interactions of *Microbacterium* strains ViU2A, HG3, A9 and ViU22 with uranium.** a)

907 Schematic workflow to monitor uranium tolerance, sequestration and stress response of strains ViU2A,
908 HG3, A9 and ViU22. b) Uranium tolerance assay: cells of strains ViU2A, HG3, A9 and ViU22 were
909 exposed to 0-500 µM uranium for 24 h and drops of cell suspension were spotted onto LB agar plates.
910 Cell growth was observed after 2 days at 25°C. c) Time evolution of the uranium (% of the initial
911 concentration) associated with the cells of strains ViU2A (red), HG3 (orange), A9 (blue) and ViU22
912 (green). The sampling times for proteomics analysis are indicated by the dotted lines. Error bars denote
913 the standard deviations of triplicate measurements. When not visible, error bars are smaller than the
914 symbols. d) TEM micrograph of cells exposed to 10 µM U(VI) for 24 h at 25°C. The needle-like structures
915 indicated by the arrows were previously shown to contain U, Ca, K and P in strain A9(44). Scale bars
916 correspond to 300 nm for ViU2A and HG3 and to 200 nm for A9 and ViU22. e) Volcano plot showing
917 proteomic data at 0.5, 4 and 24 h of uranium exposure for ViU2A (red dots), HG3 (orange dots), A9 (blue
918 dots) and ViU22 (green dots). The position of UipA protein plots at 24h is indicated by arrows.

919

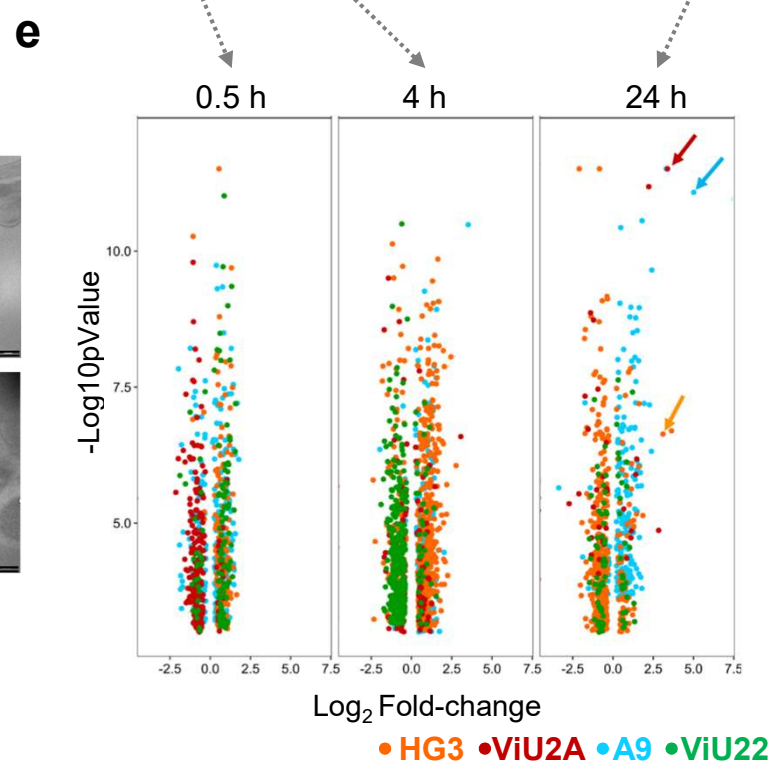
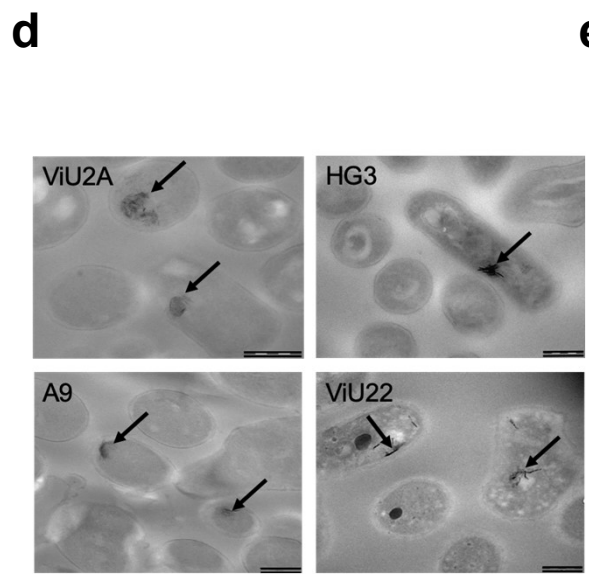
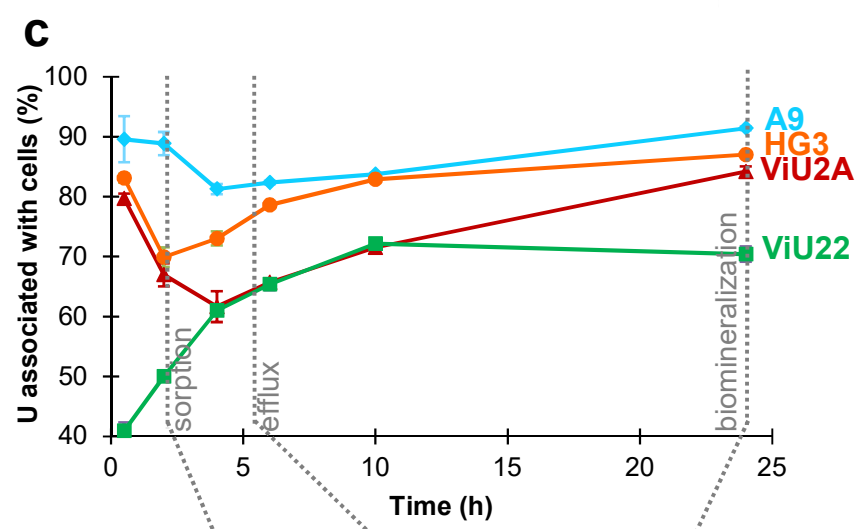
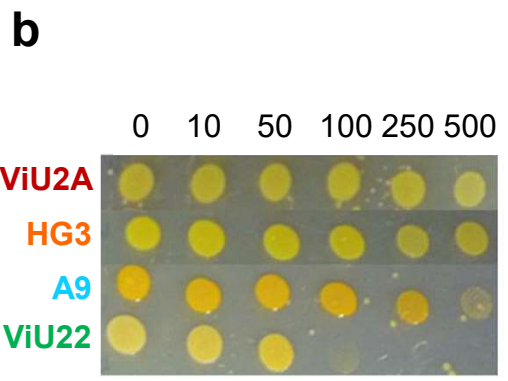
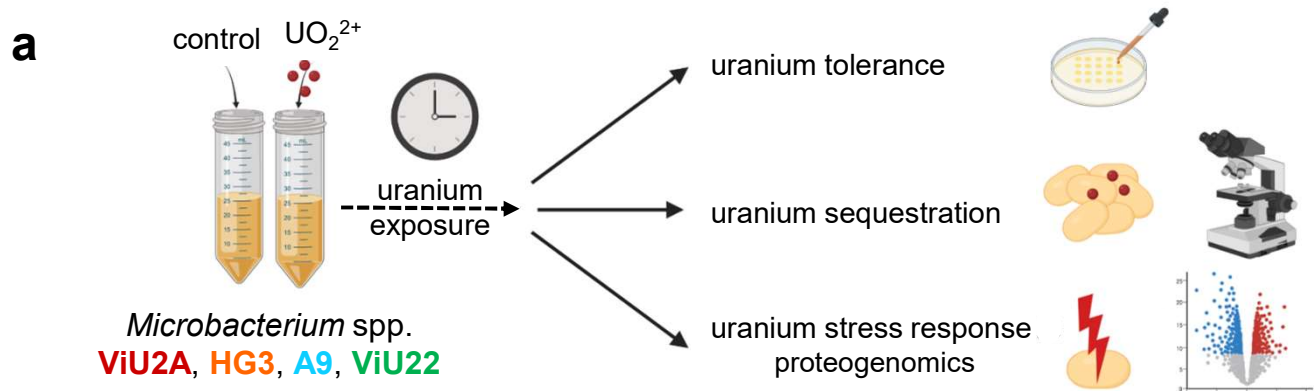
920 **Figure 2. Characterization of uranyl coordination by FTIR and EXAFS spectroscopy** a) FTIR

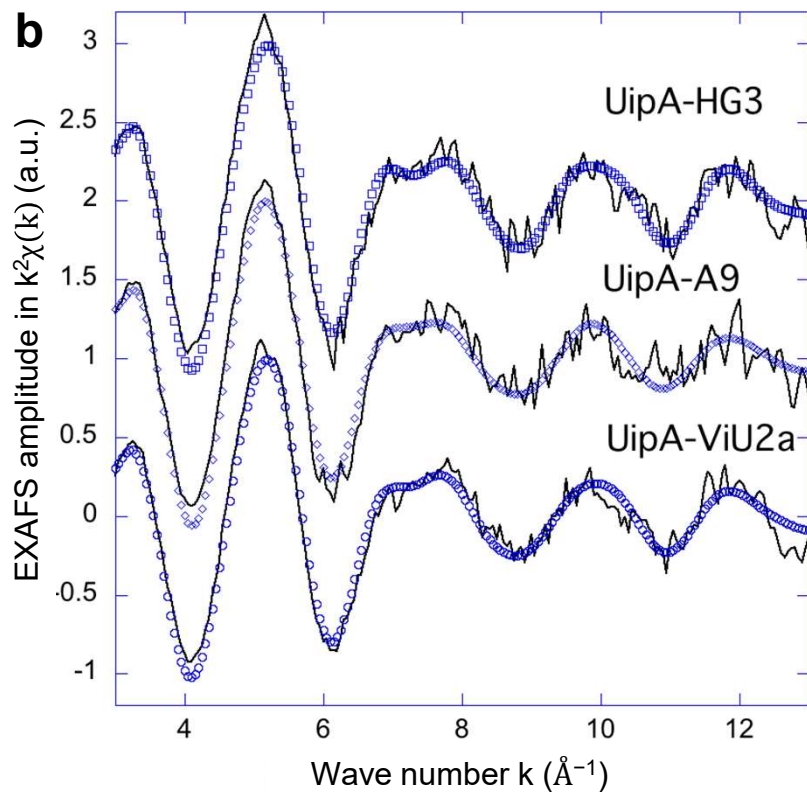
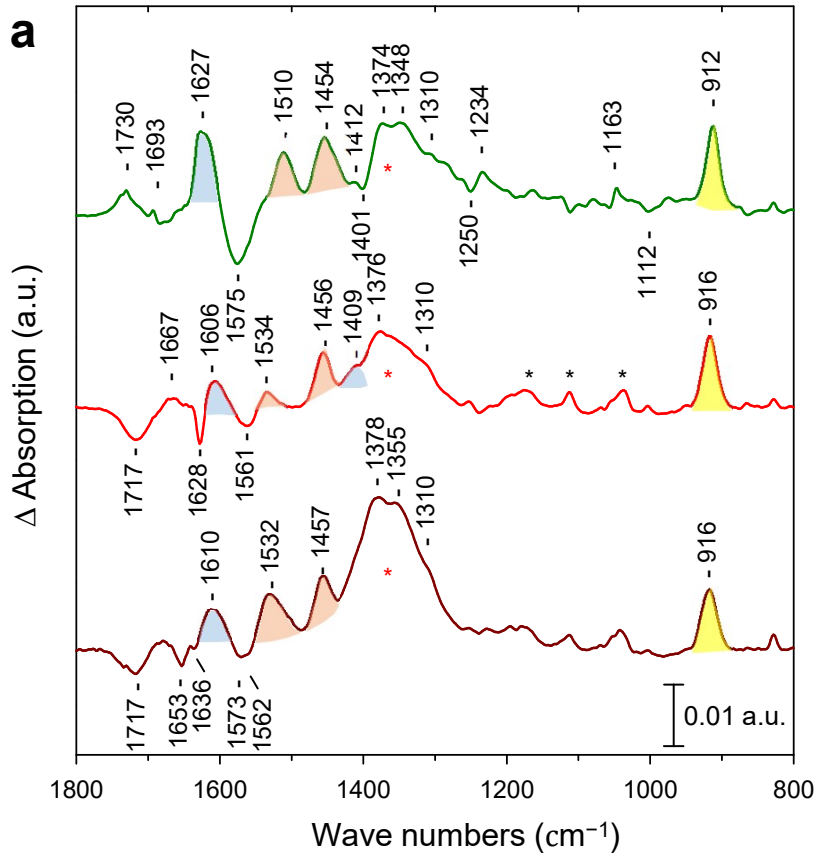
921 difference spectra recorded with UipA_{ext} with UO₂²⁺ – *minus* – without UO₂²⁺. Green line: UipA_{ext}-A9; red

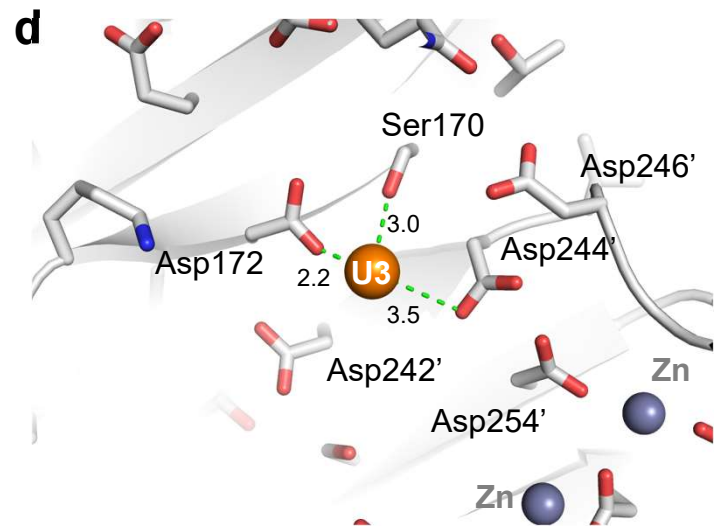
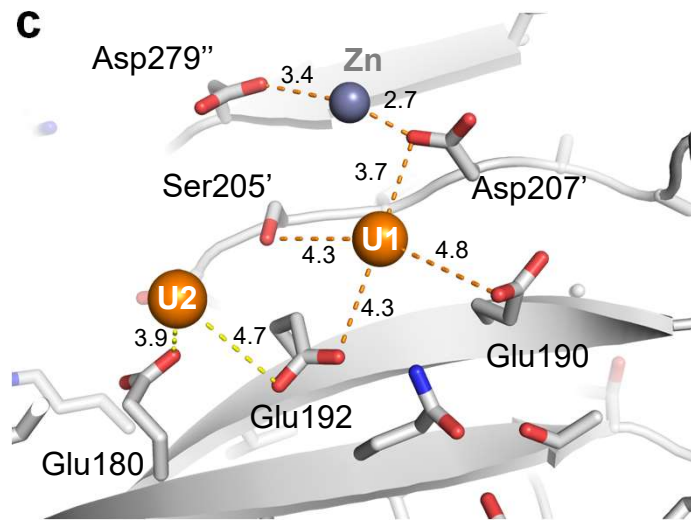
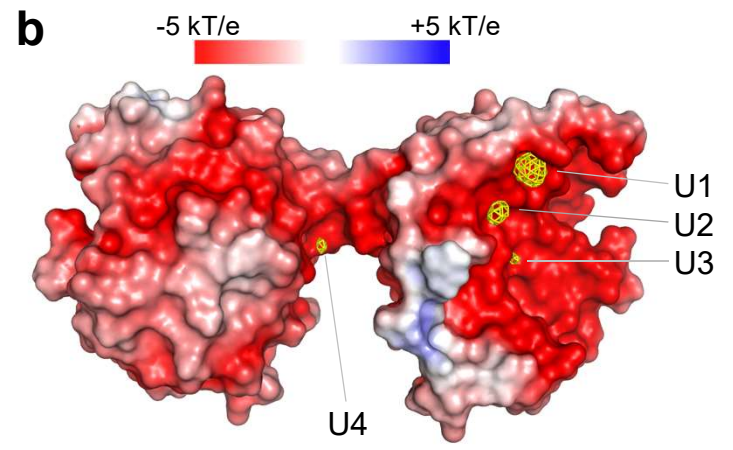
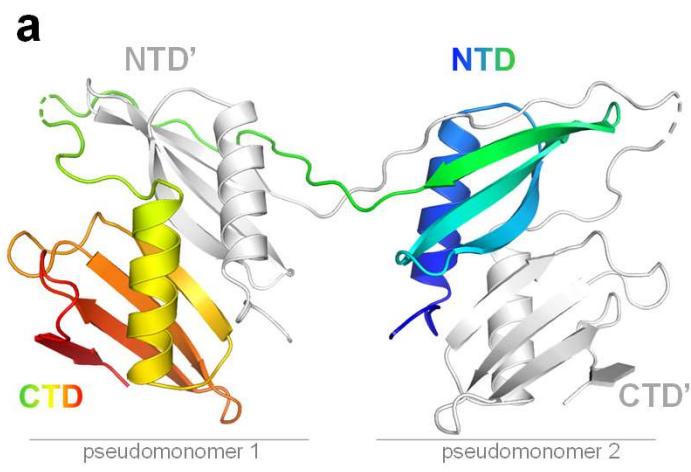
922 line: UipA_{ext}-HG3; brown line: UipA_{ext}-ViU2A. * corresponds to IR bands of the MES buffer and * to a large
923 band that is essentially due to the $\nu_{3as}(\text{NO}_3^-)$ IR mode. The bands assigned to the modes of monodentate
924 carboxylates are colored blue, and those assigned to bidentate carboxylates are colored orange. The
925 band assigned to the $\nu_{as}(\text{UO}_2)$ mode is colored yellow. b) EXAFS spectra at the U L_{III} edge of UipA_{ext}-HG3,
926 UipA_{ext}-A9 and UipA_{ext}-ViU2a. Experimental spectra = straight line, fit = blue forms.

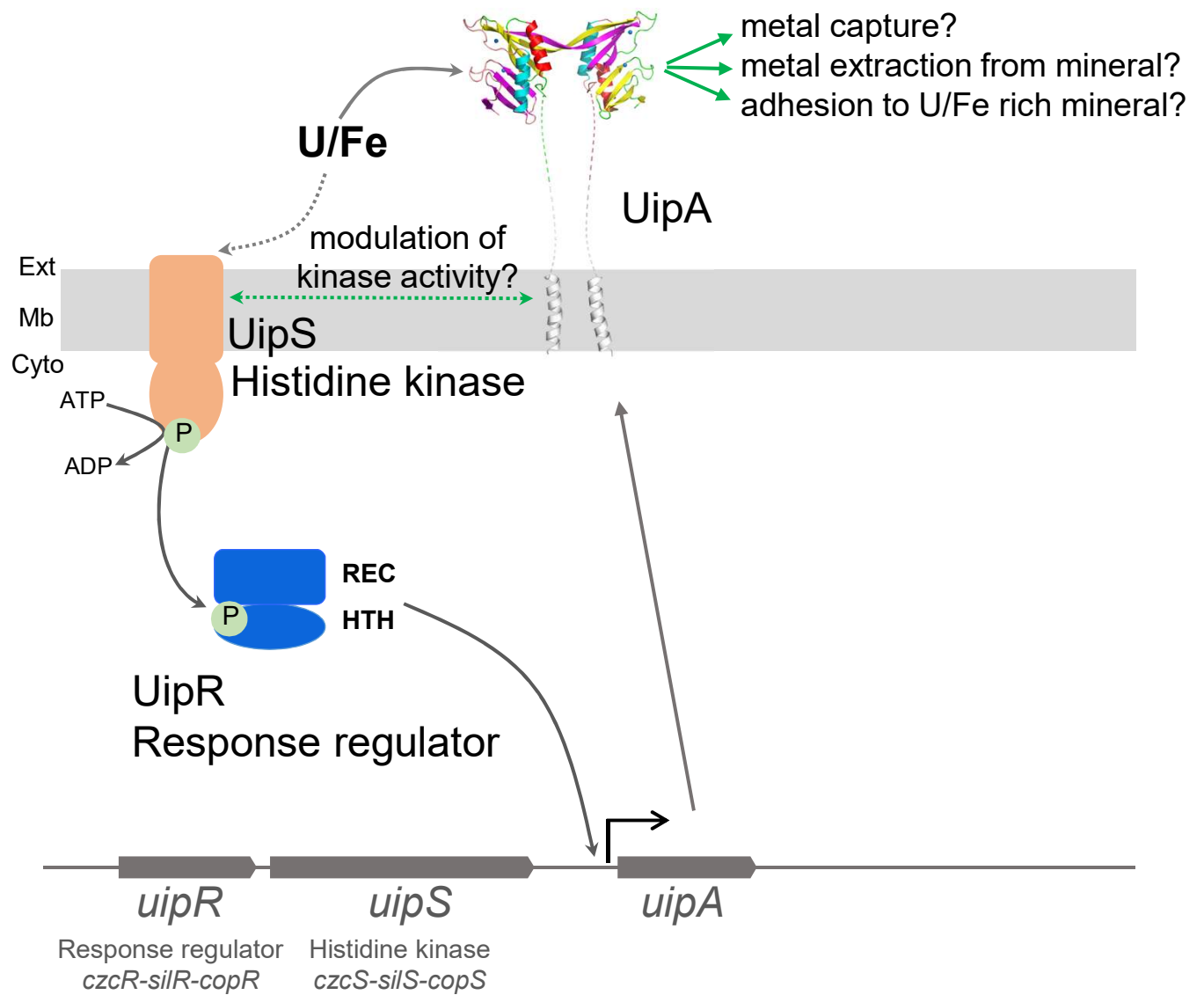
927
928 **Figure 3. Crystallographic structure of UipA_{ext}-ViU2A.** a) An illustration of the dimer structure, with one
929 monomer colored from the N-Terminus (blue) to the C-Terminus (red) and the second monomer colored in
930 grey. b) Molecular surface representation of the UipA_{ext}-ViU2A dimer (in the same orientation as in a)
931 colored according to its electrostatic potential using the APBS plugin in pymol. The surface is
932 superimposed with the anomalous map (yellow) collected at the uranium edge and contoured at 4σ all
933 around the dimer. At this contour level, there are four uranium binding sites, three of which are localized
934 on the negatively charged face of UipA (labeled U1-U3 as a function of peak height in the anomalous
935 electron density map). c) View of the two uranium binding sites U1 and U2. Ser205' and Asp207' indicate
936 residues that are contributed by the second monomer of the domain-swapped dimer. d) View of the third
937 uranium binding site (U3). In c and d, all of the residues are shown in stick form, and all distances from U
938 atoms to residues located in a 5 Å radius are shown. The $2F_{\text{obs}}-F_{\text{calc}}$ refined electron densities around the
939 three uranium binding sites are shown in Fig. S37.

940
941 **Figure 4. Working model showing the genomic organization of *uipRS* and *A* genes, and the**
942 **proposed mechanism of U sensing and the function of UipA.** The topology of UipS was predicted
943 using the CCTOP program. UipS is predicted to be a histidine kinase, which could be involved in Fe and U
944 sensing and activation of its cognate regulator, UipR (REC = receiver domain; HTH = Helix Turn Helix
945 domain), via phosphate transfer. The activated UipR would then induce *uipA* gene expression, leading to
946 overproduction of the protein UipA, formation of UipA dimers at the cell surface, and metal binding.
947 Hypothetical functions of UipA are proposed.









ViU2A			HG3			A9			ViU22			Functional annotation and subcellular localization
0.5h	4h	24h	0.5h	4h	24h	0.5h	4h	24h	0.5h	4h	24h	
1.4	ns	10.5	ns	ns	8.6	-1.3	11.5	32.3	abs	abs	abs	unknown function (UipA) - membrane
1.4	2	ns	ns	6.8	12	2.2	1.7	4.8	ns	ns	ns	unknown function (UipB) - membrane
nd	nd	nd	ns	ns	2	ns	3.2	9.9	nd	nd	nd	ABC transporter ATP-binding protein YxdL - membrane
ns	8.3	7.1	ns	2.2	2.1	1.4	1.6	2.9	nd	nd	nd	putative ABC transporter solute-binding prot YclQ - membrane
ns	ns	1.8	1.3	5.4	3	abs	abs	abs	abs	abs	abs	4-hydroxyacetophenone monooxygenase - membrane
ns	1.7	4.7	ns	2.0	3.1	ns	1.5	5.3	ns	-1.3	ns	Periplasmic serine endoprotease DegP - membrane
nd	nd	nd	ns	ns	ns	ns	2.8	5.2	nd	nd	nd	unknown function
ns	ns	ns	ns	4.7	-1.4	2	ns	ns	nd	nd	nd	unknown function
nd	nd	nd	ns	4.6	-2.0	ns	ns	1.7	ns	ns	ns	Putative SOS response-associated peptidase YedK
-1.7	ns	ns	ns	4.5	1.8	ns	-1.6	ns	ns	-1.7	ns	Protein RecA - cytoplasm

1

Metal	UipA _{ext} -ViU2A		UipA _{ext} -HG3		UipA _{ext} -A9	
	Prot-Me	Prot-Me2	Prot-Me	Prot-Me2	Prot-Me	Prot-Me2
Uranyl (UO₂²⁺)	$(4.2 \pm 1.8) \times 10^{-8}$	$(5.0 \pm 1.1) \times 10^{-8}$	$(3.1 \pm 2.5) \times 10^{-8}$	$(3.7 \pm 1.5) \times 10^{-8}$	$(1.3 \pm 0.6) \times 10^{-9}$	$(3.6 \pm 0.2) \times 10^{-8}$
Iron (Fe³⁺)	$(3.3 \pm 1.1) \times 10^{-9}$	$(2.3 \pm 0.1) \times 10^{-8}$	$(2.9 \pm 0.2) \times 10^{-8}$	$(2.6 \pm 0.4) \times 10^{-5}$	$(3.2 \pm 0.3) \times 10^{-8}$	$(4.4 \pm 0.1) \times 10^{-5}$
Calcium (Ca²⁺)	$(1.1 \pm 0.7) \times 10^{-4}$	$(6.9 \pm 0.7) \times 10^{-4}$	$(1.2 \pm 0.5) \times 10^{-4}$	$(2.5 \pm 0.2) \times 10^{-3}$	$(2.9 \pm 1.6) \times 10^{-4}$	-
Nickel (Ni²⁺)	$(1.7 \pm 0.3) \times 10^{-5}$	$(2.2 \pm 0.1) \times 10^{-5}$	$(4.0 \pm 0.9) \times 10^{-5}$	$(2.8 \pm 0.2) \times 10^{-5}$	$(1.8 \pm 0.8) \times 10^{-4}$	-
Zinc (Zn²⁺)	$(8.9 \pm 3.2) \times 10^{-5}$	$(1.3 \pm 0.1) \times 10^{-4}$	$(3.3 \pm 0.9) \times 10^{-4}$	$(6.9 \pm 0.4) \times 10^{-5}$	$(5.5 \pm 4.4) \times 10^{-5}$	-

2

3