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# The non-ribosomal peptide synthetase-independent siderophore (NIS) rhizobactin produced by *Caballeronia mineralivorans* PML1(12) confers the ability to weather minerals

Cintia Blanco Nouche, Cédric Paris, Tiphaine Dhalleine, Phil M. Oger,  
Marie-Pierre Turpault, Stéphane Uroz

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7 **AUTHORS :** Cintia Blanco Nouche<sup>1,2</sup>, Cédric Paris<sup>3</sup>, Tiphaine Dhalleine<sup>1</sup>, Philippe Oger<sup>4</sup>,  
8 Marie-Pierre Turpault<sup>2</sup>, Stéphane Uroz<sup>1,2\*</sup>

9 <sup>1</sup> Université de Lorraine, INRAE, UMR1136 « Interactions Arbres-Microorganismes », F-  
10 54000 Nancy, France

11 <sup>2</sup> INRAE, UR1138 « Biogéochimie des Ecosystèmes Forestiers », F-54280 Champenoux,  
12 France

13 <sup>3</sup> Université de Lorraine, EA 4367 « Laboratoire d'Ingénierie des Biomolécules », Ecole  
14 Nationale Supérieure d'Agronomie et des Industries Alimentaires (ENSAIA), F-54505  
15 Vandœuvre-lès-Nancy, France

16 <sup>4</sup> Université de Lyon, INSA de Lyon, CNRS UMR5240 « Microbiologie, Adaptation et  
17 Pathogénie », F-69621, Villeurbanne France

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21 **Running title:** Rhizobactin, a new NIS involved in mineral weathering

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23 **\* Corresponding author:** Mailing address: [stephane.uroz@inrae.fr](mailto:stephane.uroz@inrae.fr)

24 Université de Lorraine, INRAE, UMR 1136 “Interactions Arbres Microorganismes”, F-54000

25 Nancy, France. Phone: +33 (0)3 83 39 40 81, Fax: +33 (0)3 83 39 40 69.

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28 **SUMMARY**

29 To mobilize nutrients entrapped into minerals and rocks, heterotrophic bacteria living in  
30 nutrient-poor environments have developed different mechanisms mainly based on acidolysis  
31 and chelation. However, the genetic bases of these mechanisms remain unidentified. To fill  
32 this gap, we considered the model strain *Caballeronia mineralivorans* PML1(12) known to be  
33 effective at weathering. Based on its transcriptomics and proteomics responses in Fe-depleted  
34 conditions we pointed a cluster of genes differentially expressed and putatively involved in  
35 the production of siderophores. In this study, we report the characterization of this gene  
36 region coding for the production of a Non-ribosomal peptide synthetase (NRPS)-Independent  
37 Siderophore (NIS). Targeted mutagenesis associated to functional assays and Liquid  
38 Chromatography coupled to High-Resolution tandem Mass Spectrometry (LC-HRMS/MS)  
39 demonstrated the production of a single siderophore, identified as rhizobactin. This  
40 siderophore represents the first NIS containing malic acid in its structure. The evidence of the  
41 implication of the rhizobactin in mineral weathering was demonstrated during a hematite  
42 dissolution assay. This study provides the first demonstration of the synthesis of a NIS in the  
43 genus *Caballeronia* and its involvement in mineral weathering. Our conclusions reinforce the  
44 idea that strain PML1(12) is particularly well adapted to nutrient-poor environments.

45

46 **Keywords:** bacteria, mineral weathering, NRPS-independent siderophore, rhizobactin, malic  
47 acid

48

49

50 **Importance**

51 This work deciphers the molecular and genetic bases used by the strain PML1(12) of  
52 *Caballeronia mineralivorans* to mobilize iron and weather minerals. Through the combination  
53 of bioinformatics, chemical and phylogenetic analyses we characterized the siderophore  
54 produced by strain PML1(12) and the related genes. This siderophore was identified as  
55 rhizobactin and classified as a Non-Ribosomal Peptide Synthetase (NRPS)-Independent  
56 Siderophore (NIS). Contrary to the previously identified NIS synthetases that form  
57 siderophores containing citric acid,  $\alpha$ -ketoglutarate, or succinic acid, our analyses revealed  
58 that rhizobactin contains malic acid in its structure, representing therefore the first identified  
59 NIS with such acid and probably a new NIS category. Last, this work demonstrates for the  
60 first time the effectiveness at weathering minerals of a siderophore of the NIS family. Our  
61 findings offer relevant information for different fields of research such as environmental  
62 genomics, microbiology and chemistry, as well as soil sciences.

63

## 64 INTRODUCTION

65 Iron represents an essential micronutrient required for living organisms. This element is  
66 used as a cofactor by many enzymes and is involved in a wide range of physiological  
67 processes such as respiration, DNA synthesis, or photosynthesis [1]. While it is the fourth  
68 most abundant element in the Earth crust, it is mainly found under insoluble forms [2,3] in  
69 primary (*e.g.*, biotite, granite;  $\text{Fe}^{3+}/\text{Fe}^{2+}$ ) and secondary minerals (*e.g.*, hematite, goethite, and  
70 magnetite;  $\text{Fe}^{3+}$ ) [4]. In addition, iron is usually poorly bioavailable at neutral to basic pH and  
71 under oxygenic conditions. To deal with these limiting conditions and their physiological  
72 requirements, bacteria have developed a diversity of mechanisms to mobilize iron and other  
73 nutrients poorly available or entrapped into minerals and rocks, a global process termed  
74 mineral weathering (MWe).

75 In heterotrophic bacteria, two main MWe mechanisms have been evidenced: acidolysis and  
76 chelation [5]. The first mechanism is related to the action of the by-products of the primary  
77 and secondary metabolisms of bacteria. Indeed, by consuming carbon substrates bacteria  
78 acidify their local environment due to the production and accumulation of organic acids (*e.g.*,  
79 citrate, gluconate) and protons. Such acidification allows the dissolution of the minerals and  
80 the release of their nutritive content. The effectiveness of this mechanism is clearly associated  
81 to the carbon substrates consumed, with glucose usually giving the stronger acidification  
82 [6,7]. The second mechanism is based on the production of chelating compounds such as  
83 organic acids (*e.g.*, oxalate) and siderophores. Bacteria can weather minerals using one or  
84 both mechanisms (*i.e.*, acidolysis and chelation) depending on the bacterial strain, the nutrient  
85 availability, the type of mineral, and the ionic strength of the local environment (*i.e.*, buffering  
86 capacity).

87 Many microorganisms, such as bacteria and fungi, can produce one or more type(s) of  
88 siderophore. These particular metabolites are low-molecular weight compounds, characterized

89 by a strong affinity for iron [8], although they can also chelate other divalent and trivalent  
90 cations such as Al, Mg or Cr [9,10]. Siderophores are produced when microorganisms  
91 experience nutrient limitation and especially iron deficiency [11]. In this sense, concentrations  
92 up to 2 and 12 nM of siderophores have been measured in the solution of nutrient-poor soils  
93 [12]. To date, two classes of siderophores have been identified: i) the Non-Ribosomal Peptide  
94 Synthetase (NRPS) siderophores (*e.g.*, pyoverdinin), and ii) the NRPS-Independent  
95 Siderophores (NIS) (*e.g.*, aerobactin) [13]. The contribution of siderophores to MWe has been  
96 known for a long time for different types of molecules (*i.e.*, desferrioxamine) in abiotic  
97 experiments [14]. However, under such experimental conditions the use of relatively high  
98 concentrations of siderophore did not allow to appreciate how microorganisms produced and  
99 regulated this production according to their local environment (*i.e.*, pH, ionic strength,  
100 nutrient availability) [9,15]. Despite a constant increase in the number of new siderophores  
101 identified, their role in MWe has rarely been assessed in a cellular context [6,16,17]. Indeed,  
102 few studies have considered how the MWe ability of a mutant impaired in siderophore  
103 production compares to that of the wild-type strain and how the nutrient limitations impact the  
104 action of the siderophore-mediated weathering.

105 In this study we have focused on the model strain PML1(12) from the genus *Caballeronia*  
106 *mineralivorans* which has been isolated from a nutrient-poor soil and is known to be effective  
107 at MWe and at promoting plant growth [7,18]. However, the molecular basis of its MWe  
108 ability is not fully elucidated. The dual transcriptomic and proteomic analyses recently  
109 applied on mineral/bacteria interactions highlighted the specific increased expression of a  
110 cluster of genes related to iron mobilization and the production of a potential siderophore  
111 under Fe depleted conditions [7]. Among these genes the most highly expressed presented  
112 high homology with a putative diaminopimelate decarboxylase, cysteine synthase and  
113 IucA/IucC synthetase in conditions devoid of iron [7]. In this context, the objectives of this

114 study were to: i) characterize the cluster of genes conferring the ability to mobilize iron of  
115 strain PML1(12); ii) purify and characterize the siderophore produced by strain PML1(12);  
116 iii) quantify the contribution of siderophore production on mineral weathering; and iv) study  
117 the conservation of the genes involved in siderophore production in the *Burkholderia*,  
118 *Caballeronia*, *Paraburkholderia* (BCP) group. To do it, different bioinformatic tools were  
119 combined to characterize the cluster of genes related to the production of the NRPS-  
120 independent Siderophore (NIS) produced by strain PML1(12) and to determine the relative  
121 conservation of this cluster of genes in the genome of different strains from the BCP group.  
122 The use of a mutant impaired in its ability to produce the siderophore allowed to determine its  
123 role in hematite weathering. Ultra-High Performance Liquid Chromatography coupled with  
124 High-Resolution Mass Spectrometry allowed the identification of the chelating molecule.

125

## 126 **RESULTS**

### 127 ***A single genomic region is potentially involved in siderophore production***

128 The CAS assay performed on strain PML1(12) demonstrated its ability to mobilize  
129 iron as stated by the formation of a yellow halo around the colony, suggesting the production  
130 of one or several siderophores. An antiSMASH analysis of the PML1(12) genome identified a  
131 single siderophore production locus. This region is characterized by 10 genes, among which a  
132 single gene encoding a protein of 635 amino acids, we named RhiE, presenting the  
133 characteristic signature of the *iucA/iucC* domain. Such domain is associated to the synthesis  
134 of enzymes involved in the production of NRPS-Independent Siderophores (NIS). The other  
135 genes present in the region upstream and downstream of the *rhiE* gene could be involved in  
136 siderophore production (*rhiBCD*, intermediate production of the siderophore), transport (*rhiA*,  
137 TonB transporter; *rhiF*, MFS transporter; *rhiGHI*, ABC systems) and in the regulation of

138 siderophore production (*rhiJ*). The detail of the functions encoded by each gene of the  
139 *rhiABCDEFGHIJ* cluster is presented in Table 1.

140

141 ***The rhiABCDEFGHIJ region confers the ability to mobilize iron to strain***  
142 ***PML1(12)***

143 To investigate the implication of this region in siderophore biosynthesis, a *rhiE::Gm*  
144 knock-out mutant was constructed and tested on solid CAS assay to compare its siderophore  
145 production with that of the WT strain (Figure S1). After seven days of incubation, the WT  
146 strain presented a yellow halo of *ca.* 1.7 cm, while no halo was visible for the *rhiE::Gm*  
147 mutant strain. The lack of production of chelating molecules by the *rhiE::Gm* mutant strain  
148 was confirmed with the liquid CAS assay, which showed a very slight O.D.<sub>655nm</sub> decrease  
149 from  $0.48 \pm 0.01$  to  $0.43 \pm 0.01$  between the non-inoculated treatment and the *rhiE::Gm*  
150 mutant, when the decrease reached a final value of  $0.11 \pm 0.01$  for the WT strain indicative of  
151 a trapping of iron from the CAS-Fe(III) complex.

152

153 ***The rhiABCDEFGHIJ cluster permits the growth of strain PML1(12) in iron***  
154 ***deficient condition***

155 The growth capabilities of the WT and *rhiE::Gm* strains were measured in a medium  
156 devoid of iron (ABm-Fe) amended or not with various concentrations of iron (0, 0.5, 1, 5 and  
157 10 mg.L<sup>-1</sup> of Fe) (Figure 1). In absence of iron, the growth of the WT strain was delayed and  
158 reached a maximum O.D.<sub>595nm</sub> value of  $0.58 \pm 0.01$ . The growth rate in absence of iron was  
159 significantly lower ( $P < 0.05$ ) than that observed in the presence of iron at 5 or 10 mg.L<sup>-1</sup>  
160 concentration (O.D.<sub>595nm</sub> =  $0.88 \pm 0.01$ ). The *rhiE::Gm* mutant strain reached the same  
161 O.D.<sub>595nm</sub> as the WT strain when growing at the highest iron concentrations (*i.e.*, 5 and 10  
162 mg.L<sup>-1</sup>), but with an important growth delay. The maximal O.D.<sub>595nm</sub> was observed at 50h vs.



163 26h for the *rhiE*::Gm and WT strains, respectively. The largest growth differences (*i.e.*,  
164 maximal O.D.<sub>595nm</sub> and delay) between WT and *rhiE*::Gm strains were observed when iron  
165 concentrations were below 1 mg.L<sup>-1</sup> in the solution.

166

### 167 ***Inhibition of the siderophore production according to Fe concentration***

168 As the production of chelating molecules is usually dependent on Fe concentration in  
169 the medium, we assayed the production of siderophores in a range of concentrations of Fe  
170 from 0 to 5 mg.L<sup>-1</sup>. After a 3-day incubation period in ABm-Fe amended or not with iron (*i.e.*,  
171 FeCl<sub>3</sub>), the analyses done on the culture supernatant revealed the lack of chelating activity in  
172 the non-inoculated controls and in the WT cultures in presence of iron concentrations at 0.8  
173 mg.L<sup>-1</sup> (Figure 2), while this activity was detectable at 0.7 mg.L<sup>-1</sup> and below. Chelating  
174 activity reached its maximum in absence of iron in the medium. The same experiment  
175 performed with the *rhiE*::Gm mutant did not reveal any chelating activity whatever the iron  
176 concentration tested.

177

### 178 ***Hematite weathering***

179 The ability of the chelating molecule(s) produced by strain PML1(12) to weather  
180 minerals was evaluated considering hematite, an iron-rich mineral, as the sole source of iron  
181 in our experimental conditions. To limit the impact of acidification on the mineral weathering  
182 process, experiments were done in buffered ABm-Fe medium over 7 days of incubation. The  
183 pH of the medium was controlled before and after the experiments in presence/absence of  
184 bacterial inoculum and hematite and remained stable (pH = 6.1). In absence of hematite, the  
185 WT strain grew in the buffered ABm-Fe medium to an O.D.<sub>595nm</sub> = 0.47 ± 0.01. The presence  
186 of hematite in the medium significantly increased growth of the WT strain (O.D.<sub>595nm</sub> = 0.82 ±  
187 0.01), while hematite addition had no impact on growth of the *rhiE*::Gm mutant (O.D.<sub>595nm</sub> =

188  $0.45 \pm 0.01$  in presence vs.  $O.D._{595nm} = 0.44 \pm 0.02$  in absence of the mineral). The CAS assay  
189 confirmed that only the WT strain was presenting an active chelating activity in both  
190 conditions (*e.g.*, in the presence or in the absence of hematite). Quantification of the iron  
191 released in solution from hematite highlighted significantly higher concentrations of iron for  
192 the WT strain ( $[Fe] = 0.76 \pm 0.02 \text{ mg.L}^{-1}$ ;  $P < 0.05$ ) than for the *rhiE::Gm* mutant ( $0.04 \pm 0.02$   
193  $\text{mg.L}^{-1}$ ) and the non-inoculated control ( $0.06 \pm 0.02 \text{ mg.L}^{-1}$ ) (Figure 3).

194

### 195 ***Rhizobactin is the siderophore produced by strain PML1(12)***

196 Pre-purified supernatants from 3-day cultures of *rhiE::Gm* mutant and WT strains  
197 were analyzed by HPLC after purification. For this experiment, the growth was performed in  
198 ABs-Fe with succinate as sole carbon source to limit the production of exopolysaccharides,  
199 which are not compatible with the purification steps. The comparison of the HPLC  
200 chromatograms from the WT and *rhiE::Gm* strains as well as from the medium alone  
201 highlighted one major difference, a peak (peak #1) with a retention time (RT) of 0.84 min in  
202 the WT supernatant. This peak was 2.8 and 3.8 times more intense for the WT strain than for  
203 the *rhiE::Gm* mutant strain and the non-inoculated medium, respectively (Figure 4A). To  
204 correlate the HPLC chromatograms with the presence of chelating activities, the HPLC  
205 fractions were collected and assayed using the CAS assay. The CAS assay was positive for  
206 two fractions for the WT samples. The first positive fraction with the CAS assay ( $O.D._{655nm} =$   
207  $0.06$ ) was collected between 0.77 and 0.97 min corresponding to peak #1 region. The second  
208 positive fraction ( $O.D._{655nm} = 0.14$ ) was identified between 3.74 and 3.95 min. Detailed  
209 analysis of the HPLC profiles within this retention time highlighted the presence of a small  
210 peak (peak #2), only for WT strain (Figure 4B).

211 To identify the siderophore produced by strain PML1(12), we performed a HPLC-  
212 PDA-HRMS/MS analysis of the two fractions mentioned above. High resolution MS analysis

213 revealed the presence of a single molecule with the same exact mass of 377.1868 Da  
214 (monoprotonated ion seen at  $m/z = 378.1868$ ) in both active fractions and with the theoretical  
215 formula  $C_{15}H_{25}O_8N_3$  (Figure 5A). Such compound was found only in the WT fractions (and  
216 not in the *rhiE::Gm* strain, nor in the negative control). An additional MS signal with  $m/z =$   
217  $431.0982$  was also specifically observed in the two active WT fractions and was  
218 unambiguously attributed to the ferric complex  $[(C_{15}H_{25}O_8N_3-2H) + {}^{56}Fe(III)] + (m/z_{theo} =$   
219  $377.1788-2 \times 1.008 + 55.935 = 431.0978$ , with  ${}^{56}Fe = 55.935$  u and  ${}^1H = 1.008$  u). Indeed, the  
220 experimental isotopic pattern was in perfect agreement with the simulated one (Figure 6),  
221 thanks to the clear observation of the contribution of  ${}^{54}Fe$  isotope at  $m/z = 429.1029$   
222  $([(C_{15}H_{25}O_8N_3-2H) + {}^{54}Fe(III)]^+)$ . The previous mass spectrometric observations allow to  
223 conclude with certainty that compound of exact mass  $M = 377.1788$  Da can bind iron (III)  
224 efficiently and corresponds to the siderophore produced by strain PML1(12).

225         Such exact mass of 377.1868 Da has been previously associated to the siderophore  
226 rhizobactin by Smith *et al.* [19] in a strain of *Ensifer meliloti* and Kügler *et al.* [20] in the  
227 strain *Pseudomonas* sp. FEN. In order to elucidate the siderophore highlighted in PML1(12),  
228 a tandem mass spectrometry experiment (MS/MS) was performed (Figure 5B). Noticeably,  
229 our analyses identified the same four major product ions characterizing the rhizobactin  
230 described by Kügler *et al.* [20] (Figure 5C) as : i)  $m/z = 289.1351$  (formed as a result of loss  
231 of alanine), ii)  $m/z = 200.0882$  (formed as a result of loss of ethylenediamine and alanine and  
232 an additional carboxylic group), iii)  $m/z = 116.0681$  (identified as malic acid ion) and iv)  $m/z$   
233  $= 84.0790$  (identified as decarboxylated lysine ion). Overall, these analyses demonstrate that  
234 PML1(12) produces rhizobactin, a siderophore composed of a lysine, an alanine, an  
235 ethylenediamine group and malic acid. This work represents the first demonstration that  
236 rhizobactin is a NRPS-independent siderophore. Furthermore, it confirms that rhizobactin is

237 based on malic acid and not on other known NIS substrates (citric acid,  $\alpha$ -ketoglutarate, or  
238 succinic acid).

239

#### 240 *Comparative genomics of rhizobactin synthesis*

241 A BlastP search of the nr database using the RhiE protein as template allowed the  
242 identification of several homologous proteins presenting a percentage of identity ranging from  
243 64% to 85% and good sequence coverage (95 to 100%). The best hits appeared assigned to  
244 the genera *Paraburkholderia*, *Trinickia*, *Vogesella*, *Polaromonas* or *Pseudomonas*.  
245 Noticeably, a homologue was found in strain FEN of *Pseudomonas sp.* (63.5% homologue to  
246 RhiE; NCBI accession number WP\_191487624.1), a strain described previously for its ability  
247 to produce rhizobactin, but in which the synthesis genes had not been identified. Focusing on  
248 the *Burkholderiaceae*, we identified 38 proteins presenting a percentage of identity ranging  
249 from 34% to 85% and belonging to the genus *Paraburkholderia*, *Trinickia*, *Cupriavidus* and  
250 *Ralstonia*. Considering the BCP group, besides the homologues in *Caballeronia*, we found  
251 RhiE homologues only in 5 strains assigned to *Paraburkholderia* (*sp.* BL23I1N, *P.*  
252 *haematera*, *P. sp.* T12-10, *P. sp.* NMBU\_R16 and *P. sp.* Ac-20340), organized almost  
253 identically as in PML1(12) (*i.e.*, cluster *rhiABCDEFGJ*) (Figure 7), but with a variable number  
254 of genes. Noticeably, the ABC transport systems were not always present.

255 The phylogenetic analysis of NIS synthetases usually clusters them according to their  
256 substrate specificity [13]. To position the RhiE synthetase of strain PML1(12) in the NIS  
257 classification and to determine its relatedness to the known NIS synthetases, we constructed a  
258 N-J phylogenetic tree of a representative set of known NIS synthetases (Figure 8). A total of  
259 24 protein sequences of NIS synthetases which function, and substrate, are known were  
260 considered, as well as RhiE protein sequence of PML1(12) and the two homologous RhiE  
261 protein sequences identified in *Pseudomonas* FEN and *Ensifer (Sinorhizobium) meliloti* AK83

262 which are also potentially involved in rhizobactin production. Overall, our results confirm that  
263 NIS synthetases are distributed according to their substrate specificity [13,21-23]. The newly  
264 characterized RhiE NIS synthetase of strain PML1(12), as well as the two homologues from  
265 *Pseudomonas* and *Ensifer*, cluster in group A', forming a well-supported sub-branch within  
266 this group. Cluster A' is the only in the phylogeny which does not seem to follow substrate  
267 specificity. Indeed, as demonstrated in the current work, RhiE and homologues synthesize  
268 rhizobactin, which is based on the use of malic acid as substrate (see above), while others NIS  
269 from this cluster use citric acid to synthesize siderophores such as rhizoferrin. The low  
270 support of the tree at the base of this cluster (bootstrap values of 37 and 42) may indicate a  
271 problem of phylogenetic resolution using the current set of data.

272 The comparison of the complete genomic region of the cluster of genes involved in  
273 rhizobactin transport and production (*rhiABCDEFGHIJ*) (Table 1) found in strain PML1(12)  
274 with the genomes of the strains already known to produce rhizobactin (*Ensifer meliloti* and  
275 *Pseudomonas* sp. FEN) highlighted a partial conservation of this cluster (*i.e.*, *rhiBCDEF*).  
276 The clusters differed however in several instances: i) the presence of a TonB system in the  
277 upstream region in strain PML1(12) and at the downstream end in strain AK83  
278 (AEG58212.1) and ii) the absence of two ABC-transport systems (*rhiHI*) and the  
279 transcriptional regulator (*rhiJ*) in strains AK83 and FEN (Figure 7).

280

## 281 **DISCUSSION**

### 282 **Rhizobactin identification**

283 Rhizobactin was first described in the plant-associated bacterial strain *Ensifer meliloti*  
284 DM4 (former *Sinorhizobium meliloti*) [19]. This first study provided a detailed chemical  
285 composition based on mass spectrometry and NMR analyses, revealing that this carboxylate  
286 siderophore was formed by an ethylenediamine group coupled with alanine, lysine, and malic

287 acid. The same siderophore was reported more recently for a bacterial strain isolated from a  
288 nutrient-poor environment, the strain *Pseudomonas sp.* FEN [20]. Both studies provided the  
289 chemical characterization of this molecule, but neither the genetic characterization nor the  
290 classification of rhizobactin. Interestingly, *Pseudomonas sp.* FEN can produce rhizobactin and  
291 rhizobactin B, rhizobactin B being the main siderophore produced. Structurally, these two  
292 siderophores differ only by the presence of a methyl group at the C2 position of malic acid,  
293 giving a citramalic acid in rhizobactin B instead of a malic acid in rhizobactin. A third  
294 rhizobactin type was identified in strain 1021 of *Ensifer meliloti* (i.e., rhizobactin 1021), but  
295 completely different in terms of chemical structure from the one described above. This  
296 hydroxamate siderophore of 530 Da is based on a citrate derivative with the carboxyl groups  
297 linked to two different side chains [23]. In our study, we identified the siderophore produced  
298 by strain PML1(12) using a combination of CAS assay, HPLC purification and MS/MS  
299 analyses. This molecule not only binds the different iron isotopes but also presented an exact  
300 mass (377.1868 Da) and a fragmentation pattern like that of the rhizobactin identified in  
301 *Ensifer meliloti* strain DM4 and *Pseudomonas sp.* strain FEN [19,20]. Our *rhiE::Gm* mutant  
302 of strain PML1(12), which is unable to chelate iron, proves the involvement of the *rhiE* gene  
303 cluster in the production of the unique siderophore molecule produced by strain PML1(12).  
304 Noticeably, the rhizobactin gene cluster appeared relatively conserved in the two strains  
305 already described for the production of rhizobactin, though the strain *Pseudomonas sp.* FEN  
306 presented an additional gene identified as a citrate lyase (WP\_191487621.1), which is the first  
307 gene of the cluster in this strain, upstream of the *rhiE* homologue. Citrate lyase, may be  
308 involved in the process of transformation of malic acid into citramalic acid, explaining why  
309 rhizobactin B is the main siderophore produced by strain *Pseudomonas sp.* FEN, while  
310 rhizobactin is produced by *Ensifer meliloti* AK83 and strain PML1(12).

311

## 312 **Rhizobactin as NRPS-Independent Siderophore**

313 Two groups of bacterial siderophores are currently described according to their  
314 biosynthetic pathway. The first pathway uses complex, modular, multidomain enzymes  
315 known as NRPS [6,13,24]. The second relies on NRPS-independent synthetases [13,25]. In  
316 comparison to the wealth of information available on the genetics and chemistry of the NRPS  
317 siderophores, NIS and their related biosynthetic pathways are still poorly documented [25].  
318 This is an important point because siderophores cannot be categorized as NIS or NRPS  
319 without a proper genetic characterization. In this sense, rhizobactin was discovered and  
320 chemically characterized by Smith *et al.* [19], as early as 1984, in strain *Ensifer meliloti* DM4,  
321 but could only be classified as a NIS thanks to the current study and its homology at chemical  
322 ( $C_{15}H_{25}O_8N_3$ ) and genomic level (gene cluster) to that of strain PML1(12). The strain  
323 PML1(12) presents a gene cluster of 10 genes (Figure 7) with the characteristic signature of  
324 the NIS synthesis pathway as identified for the first described NIS (*i.e.*, aerobactin) [26, 27].  
325 The aerobactin-synthetases, *iucA* and *iucC* became since that, the hallmarks of the presence of  
326 NIS in genomes [22], either in a single copy such as for the synthesis of rhizoferrin (*e.g.*,  
327 FslA) in *Francisella tularensis* [28] or rhizobactin in strain PML1(12) (*e.g.*, RhiE, this study)  
328 or either in several copies such as for the synthesis of aerobactin (*e.g.*, IucA and IucC) [27].  
329 Our findings will enrich the NIS database and improve the siderophore determination using  
330 AntiSMASH.

331

## 332 **Rhizobactin a new class of NIS?**

333 NIS synthetases traditionally separate in five phylogenetic groups (A, A', B, C and  
334 C'). This classification which was obtained on experimentally demonstrated NIS synthetases  
335 is congruent with their substrate specificity with: i) type A and A' specific for citric acid; ii)  
336 type B for  $\alpha$ -ketoglutarate intermediates; and iii) type C and C' specific for citryl or succinyl-

337 based intermediates [13]. The number of experimentally demonstrated NIS synthetases is  
338 limited and consequently their position in phylogenetic trees may change with the evolution  
339 of NIS studies. As an example, the SfaB and SfaD enzymes responsible for the synthesis of  
340 Staphyloferrin A which presents a citric acid in its structure [29] were initially included in  
341 group B, a group of NIS synthetases specific for  $\alpha$ -ketoglutarate. With the discovery of other  
342 novel citric acid-based and chiral siderophores (*e.g.*, rhizoferrin, legiobactin) SfaB and SfaD  
343 now cluster within a new group, named A'. Our phylogenetic tree agrees with those  
344 previously published [13,21,22], confirming that the NIS synthetases mostly cluster according  
345 to their substrate specificity. In this respect, the rhizobactin synthetase clusters with group A',  
346 which corresponds to citrate-based and chiral siderophores. However, rhizobactin and  
347 rhizobactin B synthetases form a well-supported branch within group A'. Malic acid  
348 (rhizobactin) and citramalic acid (rhizobactin B) have never been described previously as  
349 possible substrates for NIS synthetases. Therefore, the difference of substrate specificity  
350 between rhizobactin synthetases (malic for rhizobactin and citramalic acid for rhizobactin B)  
351 and synthetases from group A' (citric acid), and the absence of other already characterized  
352 synthetases with malic acid as substrate, allow us to propose a new group of NIS called D.  
353 The future characterization of new NIS synthetases that use malic/citramalic acid as substrate  
354 should make more robust the separation from A' group, as it happened previously for groups  
355 B and A' [29].

356

### 357 **Role of rhizobactin in mineral weathering**

358 Iron is considered as an essential element and its deficiency alters the growth of  
359 bacteria [6,30,31]. Strain PML1(12) follows this rule, with a strong delay and decrease in  
360 growth in iron deprived conditions compared to non-limiting conditions. To adapt to low-iron  
361 conditions, bacteria can produce siderophores, whose production is regulated by the quantity



362 of iron available and stopped at a maximum of 5 mg.L<sup>-1</sup> of Fe [32]. Here, we showed that the  
363 production of rhizobactin by strain PML1(12) was detectable until an iron concentration of  
364 0.7 mg.L<sup>-1</sup>. Such iron regulation fits with the observations done by the dual transcriptomic and  
365 proteomic analyses on strain PML1(12) in the presence/absence of a mineral carrying iron  
366 (*i.e.*, biotite), which evidenced that the genes involved in siderophore production were only  
367 up-regulated in Fe-depleted conditions and in the absence of mineral [7]. Although minerals  
368 represent an important source of iron, the production of siderophores remains strongly  
369 determined by the iron bioavailability and the local conditions (pH, ionic strength). Several  
370 studies have demonstrated that in oligotrophic conditions minerals may be seen as a source of  
371 iron if siderophore-producing bacteria are present in the environment [33]. The effectiveness  
372 of siderophores at solubilizing iron from different minerals is known and demonstrated for a  
373 long time in abiotic conditions. As an example, desferrioxamine B was proved to weather Fe  
374 from several iron oxides [15]. Even more, rhizoferrin and desferrioxamine A and B are not  
375 only effective at mobilizing Fe from hydroxide minerals, but also other metals such as  
376 chromium [34,35]. However, such abiotic experiments were usually based on high  
377 concentrations of siderophores and did not permit to consider all the regulation occurring in  
378 bacteria according to nutrient availability, ionic strength of the medium and mineral type. In  
379 contrast, few studies investigated the ability of siderophore-producing bacteria to liberate  
380 nutrients trapped in minerals in a cellular context, considering mutants impaired in their  
381 ability to produce siderophores. Our study represents the first demonstration of the role of a  
382 NIS (*i.e.*, rhizobactin) produced in biotic conditions in the weathering of hematite. Such  
383 demonstration completes the list of siderophores already characterized for their role in  
384 mineral weathering [6,16,17,33].

385

386 **Occurrence of the Rhizobactin siderophore in the BCP group**

387           Among members of the same genus or species, the type of siderophore produced can  
388 strongly differ (*i.e.*, rhizoferrin biosynthesis) [36,37] or on the contrary be very conserved  
389 (*i.e.*, pyoverdine biosynthesis)[38]. Members of the *Burkholderia sensu lato* are well known  
390 for the different strategies they use to mobilize iron based on siderophore-independent (Ftr)  
391 [39] and siderophore-dependent (*e.g.*, ornibactin) [40,41] systems, but not yet based on a NIS.  
392 Our study represents the first evidence of the production of rhizobactin in this group. Based  
393 on this evidence, we evaluated the distribution of a homologue of the *Caballeronia*  
394 *mineralivorans* strain PML1(12) RhiE NIS synthetase and the conservation of the *rhi* cluster  
395 of genes. Our analysis revealed that homologues of RhiE can be found in many genera among  
396 Beta-proteobacteria, but the most represented remains the *Pseudomonas* genus. In this  
397 context, *Pseudomonas sp.* FEN is the only strain characterized for its ability to produce  
398 rhizobactin, while many *Pseudomonas* strains have been described for their ability to produce  
399 NRPS siderophores (*e.g.*, pyoverdin) [38]. Focusing on the BCP group (including  
400 *Burkholderia*, *Paraburkholderia* and *Caballeronia*), only five *Paraburkholderia* strains (*P.*  
401 *sp.* BL2311N, *P. haematera*, *sp.* T12-10, *P. sp.* NMBU\_R16 and *P. sp.* Ac-20340) presented  
402 the rhizobactin cluster, questioning its origin. In terms of ecology, many of the strains we  
403 identified to possess a rhizobactin synthesis locus homologue originate from the rhizosphere  
404 (*e.g.*, *Paraburkholderia sp.* T12-10, R16 and Ac-20340 and most of the members of *Trinickia*,  
405 *Vogesella*, *Polaromonas* or *Pseudomonas*) and nutrient-poor ecosystems suggesting a role in  
406 plant nutrition and adaptation to nutrient-poor conditions. The role of siderophore-producing  
407 bacteria in plant nutrition and growth promotion has already been demonstrated as in the case  
408 of the strains *Burkholderia pyrrocinia* JK-SH007 [40] or *Caballeronia mineralivorans*  
409 PML1(12) [42,43].

410

411           **Conclusion**

412 It is clearer and clearer that the ability of bacteria to weather minerals is finely  
413 regulated by local conditions and physiological processes. Nutrient availability, ionic strength  
414 of the medium and the type of mineral and of the carbon substrate metabolized, strongly  
415 determine the mechanism used by bacteria and the effectiveness at weathering. While the  
416 global mechanisms are identified (*i.e.*, acidification, chelation, oxido-reduction), the genetic  
417 and geochemical bases remain poorly documented. Demonstrations in a cellular context (*i.e.*,  
418 effect of a gene mutation) are essential to cover without *a priori* the diversity of mechanisms  
419 used by phylogenetically different bacteria and to decipher the regulations of these  
420 mechanisms occurring according to the mineral type and the local conditions. The  
421 combination of molecular and (geo-)chemical techniques used here not only allowed the  
422 identification of the siderophore produced by strain *Caballeronia mineralivorans* PML1(12)  
423 as rhizobactin, but also its classification as an NRPS-independent siderophore (NIS) and its  
424 contribution to mineral weathering. Our study provides new information regarding the  
425 complexity of mechanisms used by this model strain according to the local conditions and its  
426 metabolism. Our results highlight that siderophores play an essential role in mineral  
427 weathering under buffered and non-acidifying conditions, while organic acid and proton-  
428 based mechanisms represent the main mineral weathering mechanism used by this strain  
429 under non-buffered and acidifying conditions. The ability of rhizobactin to weather more  
430 complex and less weatherable minerals, with a lower iron content (*e.g.*, biotite) remains to be  
431 determined. Together, our results clearly highlight how the strain PML1(12) is well adapted  
432 to live in nutrient-poor environments and to develop complex and finely regulated  
433 mechanisms to mobilize nutrients from minerals.

434

## 435 MATERIALS AND METHODS

### 436 Bacterial strains and growth media

437 Bacterial strains and plasmid are listed in Table 2. The model *Caballeronia*  
438 *mineralivorans* strain PML1(12) was isolated from the ectomycorrhizosphere of *Scleroderma*  
439 *citrinum* associated with oak (for sampling details see Calvaruso *et al.* [44])

440 Strains, with the exception of *Escherichia coli*, were grown in AB medium [45]  
441 supplemented with mannitol (ABm) or succinic acid (ABs) (2 g.L<sup>-1</sup> final concentration) at 25  
442 °C. A version of the AB medium devoid of iron was also used (AB-Fe). *E. coli* strains were  
443 grown in Luria-Bertani (LB) at 37 °C. When required, gentamycin, kanamycin and ampicillin  
444 were added to the media at a final concentration of 20, 50 and 100 µg.mL<sup>-1</sup>, respectively.

445

446 *Preparation of bacterial inoculum of Caballeronia mineralivorans* strain PML1(12)  
447 and its mutant

448 For each of the assays presented in this work, the wild-type (WT) strain and the  
449 *rhiE::Gm* mutant strain were recovered from glycerol stock (-80 °C) and grown on solid ABm  
450 medium (25 °C) for 2 days. One isolated colony of each strain was inoculated in 10 mL of  
451 liquid ABm medium and incubated 3 days at 25 °C under 150 rpm agitation. Liquid cultures  
452 were centrifugated at 9000 g for 15 min at 4 °C and the pellet was washed three times with  
453 sterile MilliQ water. The pellet was resuspended in sterile MilliQ water, and the optical  
454 density was adjusted to O.D.<sub>595nm</sub> = 0.9 ± 0.03 (corresponding to 2.10<sup>9</sup> cell/mL).

455

#### 456 ***rhiE::Gm* mutant construction**

457 Total DNA was extracted from the WT strain PML1(12) using the protocol of  
458 Pospiech and Neumann [46]. To construct a targeted mutant of the gene homologous to  
459 *iucA/iucC* (*rhiE*), the total gene was amplified using a combination of primers including For-  
460 *rhiE* and Rev-*rhiE* for a complete amplification and Rev-*rhiE*\_SmaI and For-*rhiE*\_SmaI to  
461 insert a *SmaI* site (Table 2; Figure S2 for details). The insertion of a *SmaI* site in the middle of

462 the *rhiE* gene was done following 3 PCR rounds: i) For-*rhiE* with Rev-*rhiE\_SmaI* (967 bp),  
463 ii) For-*rhiE\_SmaI* with Rev-*rhiE* (966 bp) and iii) For-*rhiE* and Rev-*rhiE* (1908 bp). The PCR  
464 products of PCR1 and 2 were purified and used as matrix for PCR 3. After amplification and  
465 purification, PCR3 product was ligated into the pGEM-T Easy plasmid (Promega) resulting in  
466 the plasmid pGEM-*rhiE\_SmaI* (Table 2). The plasmid pGEM-*rhiE\_SmaI* was then digested  
467 with *SmaI* to introduce the *SmaI*-digested gentamycin (Gm) resistance cassette from plasmid  
468 pUC1318, resulting in the plasmid pGEM-*rhiE::Gm*. A fragment containing the *rhiE::Gm*  
469 cassette was then obtained by *EcoRI* restriction and cloned in the plasmid pK19mob, resulting  
470 in the pK19mob-*rhiE::Gm* plasmid. This construction was transferred to *E. coli* S17.1 $\lambda$ pir and  
471 then to strain PML1(12) by bi-parental conjugation.  $\Delta$ *rhiE* mutants were recovered after 5  
472 days of incubation at 25 °C on gentamycin-containing ABm plates. The transconjugants  
473 obtained were verified by PCR with For-*rhiE* and Rev-*rhiE* primers, to differentiate the single  
474 and double cross-over events. Several single and double-cross-over mutants were obtained  
475 and conserved. The experiments described in our study were done using a double cross-over  
476 mutant named *rhiE::Gm*.

477

#### 478 **Siderophore activity**

479 Siderophore activity was detected using the liquid and solid versions of the Chrome  
480 Azurol S (CAS) assay according to Schwyn and Neilands [47]. For both assays, the color  
481 change, sign of the chelating activity of the siderophore for the iron (III) of the CAS-Fe  
482 complex can be quantified by the presence of a halo on the solid CAS assay or through the  
483 measurement of the optical density (O.D.) at 655 nm for the liquid assay.

484 For the solid CAS assay, 5  $\mu$ L of the bacterial inoculum (both WT and mutant strains;  
485 corresponding to  $5.10^6$  colonies forming unit (CFU)) were inoculated in triplicate in agar

486 plates and incubated three days at 25 °C. The diameter of colony and the halo surrounding it  
487 were measured to determine growth and siderophore(s) production, respectively.

488 For the liquid CAS assay, 0.5 mL of the bacterial inoculum (both WT and mutant  
489 strains; corresponding to  $5 \cdot 10^8$  CFU) were inoculated in triplicate in 4.5 mL ABm-Fe. After 3-  
490 days incubation at 25 °C under agitation (150 rpm), the cultures were centrifugated at 9000 g  
491 during 15 min at 4 °C to recover culture supernatants. A volume of 100 µL of these  
492 supernatants were mixed with 100 µL of CAS-Fe(III) and incubated during 1h at room  
493 temperature in the dark. Then, the O.D. was measured at 655 nm using a microplate reader  
494 (Bio-Rad, model iMark). Decrease of absorbance at this specific wavelength is synonym of  
495 siderophore presence (change of media coloration from blue to yellow [6, 47]). Non-  
496 inoculated medium was used as negative control for the liquid assay.

497

#### 498 **Impact of iron concentration on the growth the WT and *rhiE*::Gm strains**

499 To determine if the concentration of iron and the mutation of the *rhiE* gene (*rhiE*::Gm  
500 mutant) affected the growth of strain PML1(12), growth was determined at different iron  
501 concentrations (*i.e.*, 0, 0.5, 1, 5, 10 mg.L<sup>-1</sup> of Fe). A bacterial inoculum of 10 µL  
502 (corresponding to  $6 \cdot 10^6$  CFU) was added to 190 µL of ABm-Fe and incubated in 96-well  
503 microplates for 5 days. This experience was performed in triplicate and conducted at 25 °C on  
504 orbital shaking in a microplate reader (Tecan infinite M200 pro). The absorbance was  
505 measured at 595 nm every 3 hours. To evaluate the growth of the strains, the slope of the  
506 exponential phase (*i.e.*, growth rate) was calculated for each condition and replicate.

507

#### 508 **Siderophore production inhibition**

509 Siderophore production is known to be regulated by the availability of nutritive  
510 elements in the culture medium, especially iron. To evaluate the concentration of Fe necessary

511 to inhibit the siderophore production, the WT and mutant strains were incubated at different  
512 iron concentrations. Non-inoculated conditions were used as negative controls for each  
513 concentration. A volume of 10  $\mu\text{L}$  of inoculum was added to 180  $\mu\text{L}$  of ABm-Fe  
514 supplemented with 10  $\mu\text{L}$  of different  $\text{FeCl}_3$  stock solutions ranging from 0 to 5  $\text{mg}\cdot\text{L}^{-1}$  of Fe  
515 to obtain the following final concentrations in the medium: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7,  
516 0.8, 0.9, 1, 1.5, 2, 2.5, 5  $\text{mg}\cdot\text{L}^{-1}$ . The 96-well microplates were incubated 3 days at 25 °C.  
517 Then, microplates were centrifugated at 3000 g during 15 minutes at 4 °C and 100  $\mu\text{L}$  of  
518 supernatant were mixed with 100  $\mu\text{L}$  of CAS-Fe(III). After 1 h incubation in the dark the  
519 plates were read at 655 nm using a microplate reader (Bio-Rad, model iMark) as described  
520 above.

521

## 522 **Hematite weathering assay**

### 523 *Hematite characteristics*

524 Hematite ( $\text{Fe}_2\text{O}_3$ ) is an iron-oxide mineral widely distributed in all soils [48] and  
525 characterized by a high iron content. The hematite used in this study comes from a batch  
526 extracted in Brazil. The chemical composition was determined on crushed powder by  
527 inductively coupled plasma-atomic emission spectrometry (700 Series ICP-AES, Agilent  
528 Technologies) as described in Picard *et al.* [49]. This analysis revealed that the hematite used  
529 is mainly composed of  $\text{Fe}_2\text{O}_3$  (99.3%) and also contains in small amounts: 0.40%  $\text{TiO}_2$ , 0.18%  
530  $\text{SiO}_2$ , 0.16%  $\text{Al}_2\text{O}_3$ , 0.10%  $\text{MgO}$ , 0.037%  $\text{MnO}$ .

531

### 532 *Mineral weathering assay*

533 To avoid contamination by residues associated with glass tubes, all glassware was  
534 rinsed once with 3.6% HCl and then three times with MilliQ water. The tubes were filled with  
535 200 mg of hematite and 9 mL of ABm-Fe medium and autoclaved at 121 °C. To evaluate the

536 differences in the weathering ability of the *rhiE*::Gm mutant and the WT strains, 1 mL of each  
537 inoculum was added to the tubes (procedure performed in triplicate). Non-inoculated media  
538 with and without hematite were used as abiotic and sterility controls for weathering. Samples  
539 were incubated for 7 days under agitation at 150 rpm and 25 °C.

540 The cultures were used to determine: i) bacterial growth, ii) solution pH, iii)  
541 siderophore production and iv) iron concentrations. Bacterial growth was estimated directly  
542 on the liquid culture by measuring the O.D.<sub>595nm</sub> on 200 µL. The other measurements were  
543 done after centrifugation at 14000 g for 15 min to remove bacterial cells and hematite  
544 particles. A volume of 100 µL of supernatant was used to evaluate the level of siderophore(s)  
545 production using the liquid CAS assay according to the protocol described above. A volume  
546 of 180 µL of supernatant was used to measure the pH by mixing with 20 µL of bromocresol  
547 green (1 g.L<sup>-1</sup>) according to [18]. For the measure of the iron concentration, the supernatant  
548 was filtered at 0.22 µm (GHP Acrodisc 25 mm syringe filter; PALL) before mixing 180 µL of  
549 supernatant with 20 µL of ferrospectral. Both pH and iron determinations are based on optical  
550 density measurements at 595 nm using a microplate reader (Bio-Rad, model iMark) and after  
551 conversion with the calibration curves done on the bromocresol green and ferrospectral dyes,  
552 according to [18].

553

## 554 **Chemical characterization of the siderophore**

### 555 *Siderophore production and purification*

556 Volumes of 50 mL of ABs-Fe were inoculated with each strain (WT and *rhiE*::Gm)  
557 and incubated at 25 °C under 150 rpm shaking for three days. Succinic acid was used here as  
558 sole carbon source to limit the production of exopolysaccharides. A non-inoculated condition  
559 was also included as negative control. After the incubation time, cultures were centrifugated  
560 at 9000 g, during 15 min at 4 °C. The supernatant was filtered using 0.45 µm filters



561 (Millipore) and treated with a Sep pack Vac 6cc tC18 Cartridge (Waters) to pre-purify and  
562 concentrate the siderophore(s). The columns were first washed with 10 mL of 100% ethanol  
563 and then equilibrated with 10 mL of 0.01% trifluoroacetic acid (TFA). After this equilibration  
564 step, a volume of 25 mL of supernatant was added progressively to the column and finally the  
565 different fractions of the supernatant were eluted by the addition of 5 mL of 10% and then  
566 20% methanol in water. At each step the eluted volume (*ca.* 5 mL) was recovered and  
567 concentrated in the SpeedVac system. The dried samples were resuspended in 100  $\mu$ L of  
568 MilliQ water. These pre-purified fractions were used for further characterization using the  
569 liquid CAS assay to verify the presence of siderophore.

570

#### 571 *High Performance Liquid Chromatography (HPLC) analyses and fractionation*

572 The different pre-purified fractions recovered above (from WT, mutant and negative  
573 control samples) were analyzed by HPLC. A volume of 50  $\mu$ L of each extract was injected in  
574 a Gemini C18 column (150 x 3.0 mm, 5  $\mu$ m particle size, Phenomenex). Solvent A consisted  
575 of MilliQ water and solvent B consisted of acetonitrile plus 0.2% of TFA. A linear gradient  
576 elution from 2% to 40% of solvent B over 25 min at a flow rate of 1 mL.min<sup>-1</sup> was used to  
577 separate compounds. Absorbance was monitored at 210 nm.

578 Peaks of potential interest were identified by comparing the chromatogram of the WT  
579 sample with that of the *rhiE::Gm* mutant sample and the negative control. To determine the  
580 presence of the siderophore(s) activity, a fraction collection was performed in 96-wells  
581 microplates using a fraction collector system (FRC-10A fraction collector system, Shimadzu).  
582 Fractions of 200  $\mu$ L were recovered each 0.2 s using the same HPLC conditions as described  
583 before. Fraction collection was performed three times independently, and fractions with the  
584 same retention times were pooled. The fractions collected were then concentrated using the  
585 SpeedVac and resuspended in 100  $\mu$ L of MilliQ water. To determine the presence of

586 siderophore(s) activity, a volume of 25  $\mu\text{L}$  of the collected fractions was mixed with 25  $\mu\text{L}$  of  
587 liquid CAS and the absorbance at 655 nm was measured after overnight incubation.

588

589 *Qualitative HPLC-DAD-MS-MS/MS analysis of siderophores and ferrosiderophores*

590 A Thermo Scientific UHPLC-HRMS/MS system composed of a Vanquish™ liquid  
591 chromatography unit coupled to a photodiode array detector (PDA) and an Orbitrap ID-X™  
592 Tribrid™ high-resolution mass spectrometer operating in electrospray ionisation mode (ESI)  
593 was used to detect and identify the putative siderophores. Ten microliters of each bacterial  
594 extract were injected onto a Hichrom Alltima C18 column (150×2.1 mm - 5  $\mu\text{m}$ ) maintained  
595 at 25 °C. The flow rate was set at 0.2  $\text{mL}\cdot\text{min}^{-1}$  and mobile phases consisted in water modified  
596 with formic acid (0.1%) for A and acetonitrile modified with formic acid (0.1%) for B.  
597 Compounds of interest were eluted using a linear gradient from 5% to 20% of B for 10 min,  
598 then a 5 min isocratic step was applied at 98% of B to wash the column, before returning to  
599 the initial composition of 5% B for 6 min to reach the equilibrium. Mass analysis was carried  
600 out in ESI positive ion mode ( $\text{ESI}^+$ ) and mass spectrometry conditions were as follows: spray  
601 voltage was set at 3.5 kV; source gases were set (in arbitrary units. $\text{min}^{-1}$ ) for sheath gas,  
602 auxiliary gas and sweep gas at 35, 7 and 10, respectively; vaporizer and ion transfer tube  
603 temperatures were both set at 300 °C. Survey scans of precursors were performed from 150 to  
604 2000  $m/z$  at 60 K resolution (FWHM at 200  $m/z$ ) with MS parameters as follows: RF-lens,  
605 35%; maximum injection time, 50 ms; data type, profile; internal mass calibration EASY-IC  
606 TM activated; normalized AGC target: 25%. A top speed data dependent  $\text{MS}^2$  (0.6 sec for the  
607 whole cycle time) was carried out using a wide quadrupole isolation (1.5 Th), a HCD  
608 fragmentation with a stepped collision energy (20, 35 and 50%) and an Orbitrap measure at  
609 15 K resolution (high-resolution MS/MS analysis). Precursors with an intensity upper than  
610  $2\cdot 10^4$  were automatically sampled for  $\text{MS}^2$ . Dynamic exclusion was used, and the time of

611 exclusion was set at 2.5 s, with a 10 ppm tolerance around the selected precursor (isotopes  
612 excluded). Other MS<sup>2</sup> parameters were as follows: data type, profile; normalized AGC target:  
613 20%; AGC target, 10000.

614 Mass spectrometer calibration was performed by using the Thermo Scientific Pierce  
615 TM FlexMix TM calibration solution. MS data acquisition was carried out by using the  
616 Xcalibur v. 3.0 software (Thermo Scientific).

617

## 618 **Bioinformatic analyses**

### 619 *Genome analysis*

620 Genome analysis was performed using MicroScope MaGe (Magnifying Genomes)  
621 [50] and NCBI. The presence of *iucA/iucC* gene homologue was identified by antiSMASH  
622 [51] and confirmed by BLASTP analyses based on sequence homology [52].

623

### 624 *Phylogenetic analyses*

625 A total of 27 protein sequences (Table S1) homologous to IucA/IucC, including the  
626 homologue of PML1(12) (RhiE), were recovered from NCBI to build a phylogenetic  
627 Neighbor-Joining (N-J) tree using the Seaview platform (version 5.0.4)[53]. For this analysis,  
628 the protein sequences considered were: i) sequences which role in siderophore biosynthesis  
629 were experimentally demonstrated; ii) sequences based on their homology with the RhiE  
630 protein identified in strain PML1(12). Protein sequences were aligned using MUSCLE  
631 algorithm and then a N-J tree with 100.000 iterations was constructed.

632

## 633 **Statistical analyses.**

634 Statistical analyses were performed in R software (Tidyverse packages)[54]. Triplicate  
635 samples were used in all experiments. Differences between samples means (pH, growth, CAS  
636 assay and released iron) or growth slopes were analyzed by ANOVA and TukeyHSD tests.

637

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647

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801  
802

803 **Figure Legends.**

804

805 **Figure 1. Growth assay.** The growth of the WT strain (A) and *rhiE*::Gm mutant (B) was  
806 monitored in presence of different iron concentrations (*i.e.*, 0, 0.5, 1, 5, 10 mg.L<sup>-1</sup> of Fe) in AB  
807 medium depleted of iron. The growth was studied under orbital shaking at 25 °C for 90 h. The  
808 absorbance was measured at 600 nm every 3 hours. Each dot is the mean of independent  
809 triplicates.

810

811 **Figure 2. Impact of increasing concentrations of iron on the chelating activity of the WT**  
812 **and mutant (*rhiE*::Gm) strains.** To determine the concentration of iron inhibiting  
813 siderophore production, liquid cultures of WT (red) and *rhiE*::Gm (blue) strains were  
814 performed with different concentrations of iron (*i.e.*, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9,  
815 1, 1.5, 2, 2.5, 5 mg.L<sup>-1</sup>). Pure water (grey) was used as a negative control. After 3 days, the  
816 absorbance at 655 nm was measured. The decrease of the measured absorbance means the  
817 presence of chelating molecules since the media coloration changes from blue to yellow and  
818 the absorbance measured at 655 nm measures blue color intensity.

819

820 **Figure 3. Hematite weathering potential of strain PML1(12) and its *rhiE*::Gm mutant.**

821 The weathering ability was evaluated by the measure of iron released from hematite in ABm  
822 medium devoid of iron, after 7 days of incubation at 25 °C under agitation (200 rpm). (A)  
823 **Concentration of iron released in solution.** The iron released from hematite was measured  
824 by ferrospectral determination (optical density measured at 595<sub>nm</sub>). Abbreviations were used  
825 as follows: WT + H, wild type strain plus hematite; WT, wild type strain; *rhiE*::Gm + H,  
826 mutant strain plus hematite; *rhiE*::Gm, mutant strain; abiotic + h, non-inoculated condition  
827 plus hematite; abiotic, non-inoculated condition. Non-inoculated conditions (abiotic control

828 with and without hematite) were used as controls. Samples with the same letter indicate no  
829 significant difference ( $P < 0.05$ ). **(B) Siderophore activity.** The siderophore activity of the  
830 different samples was determined using the CAS method. The yellow color indicates  
831 siderophore activity. For each measure, the results are the mean of independent triplicates.

832

833 **Figure 4. HPLC analysis and UV detection.** The pre-purified supernatant of the WT strain  
834 (red), *rhiE::Gm* mutant (blue) and non-inoculated medium (grey) were monitored at 210 nm  
835 after HPLC separation using a Milli-Q water / acetonitrile + 0.2% TFA gradient for 25 min at  
836  $1 \text{ mL} \cdot \text{min}^{-1}$ . To determine the presence of siderophore activity, fractions were collected during  
837 the HPLC run and tested using the liquid CAS assay. Positive fractions for siderophore  
838 presence are highlighted with black arrows. **(A)** A major peak (peak 1) at retention time of  
839 0.84 min was identified. **(B)** A second peak (peak 2) of lower intensity, with a retention time  
840 of 3.88 min, was identified after CAS test.

841

842 **Figure 5. Identification, fragmentation profile and chemical structure of rhizobactin.** **(A)**  
843 Mass spectrum of HPLC positive fractions showing the parent ion of the putative chelating  
844 molecule at  $m/z = 378.1868$  (raw formula:  $\text{C}_{15}\text{H}_{25}\text{O}_8\text{N}_3$ ). **(B)** Fragmentation pattern of the  
845 chelating molecule seen at  $m/z = 378.1868$ , revealing a perfect agreement with previous  
846 experimental  $\text{MS}^2$  results done on the rhizobactin identified in *Sinorhizobium meliloti* DM4  
847 by Smith *et al.* (1985) and in *Pseudomonas sp.* FEN by Kügler *et al.* (2020). The major  
848 product ions are: 289.1351 (in blue, consecutive to alanine loss), 200.0882 (in yellow, formed  
849 as a result of loss of ethylenediamine and alanine and an additional carboxylic group),  
850 116.0681 (in red, corresponding to malic acid ion -  $\text{H}_2\text{O}$ ) and 84.0790 (in green,  
851 corresponding to decarboxylated lysine ion) **(C)** Chemical structure (according to Smith *et al.*,  
852 1985) and fragmentation pattern of the rhizobactin. Same colors as in panel A are used to

853 highlight the correspondence of the chemical moieties of rhizobactin and product ions. Dotted  
854 boxes are used to highlight lost ions while undotted boxes mark the remaining structure of the  
855 molecule.

856

857

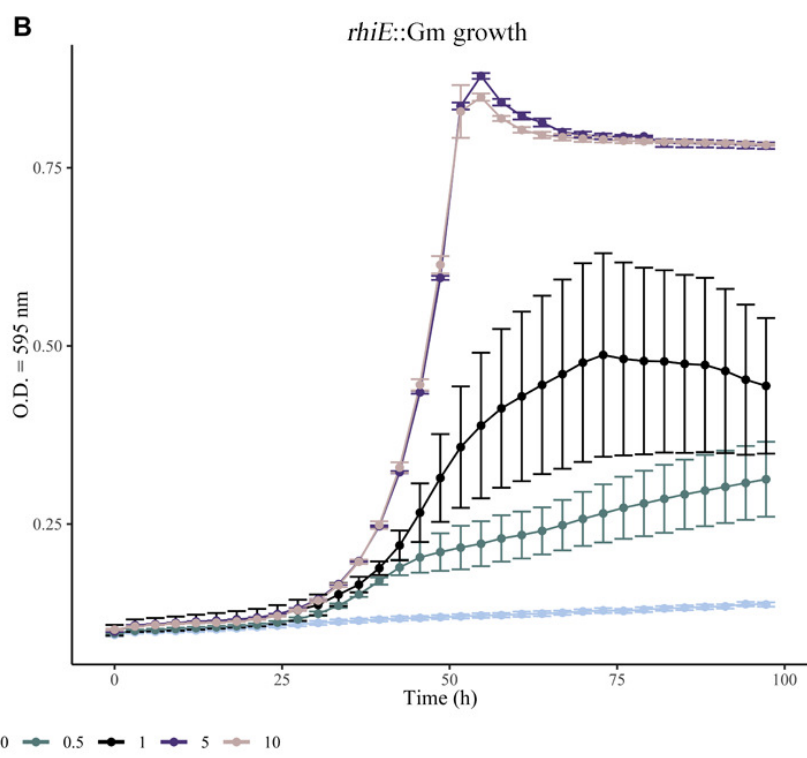
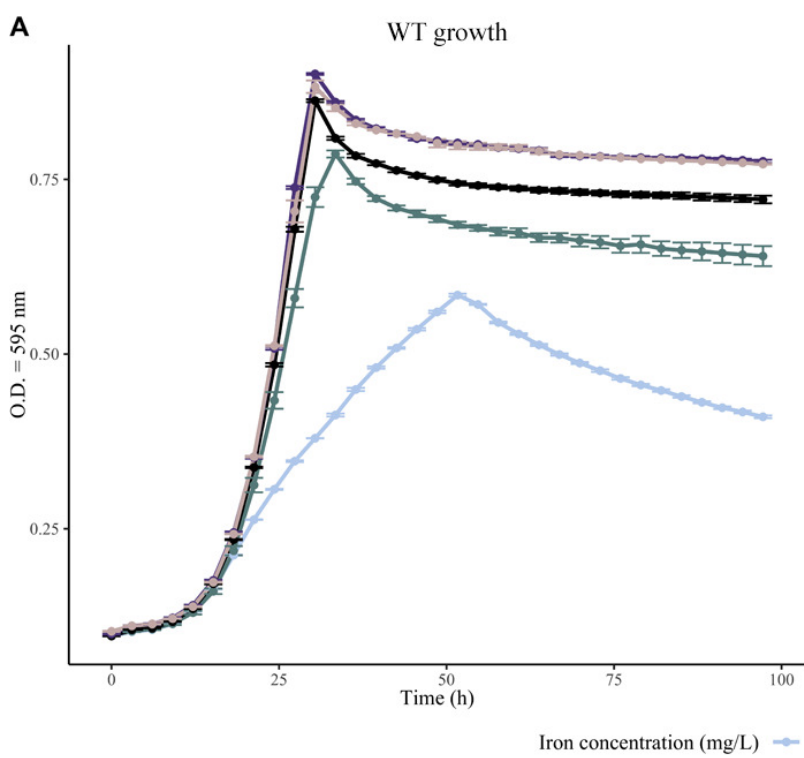
858 **Figure 6. Isotopic pattern of iron complexes.** Comparison between (A) the experimentally  
859 observed isotopic pattern of the putative iron(III) siderophore complex ion ( $m/z=431.0963$ ),  
860 and (B) the theoretical isotopic profile of the complex  $[(C_{15}H_{25}O_8N_3-2H)+Fe^{III}]^+$ ,  $C_{15}H_{25}O_8N_3$   
861 being the raw formula associated with the putative siderophore of  $m/z=378.1868$ .

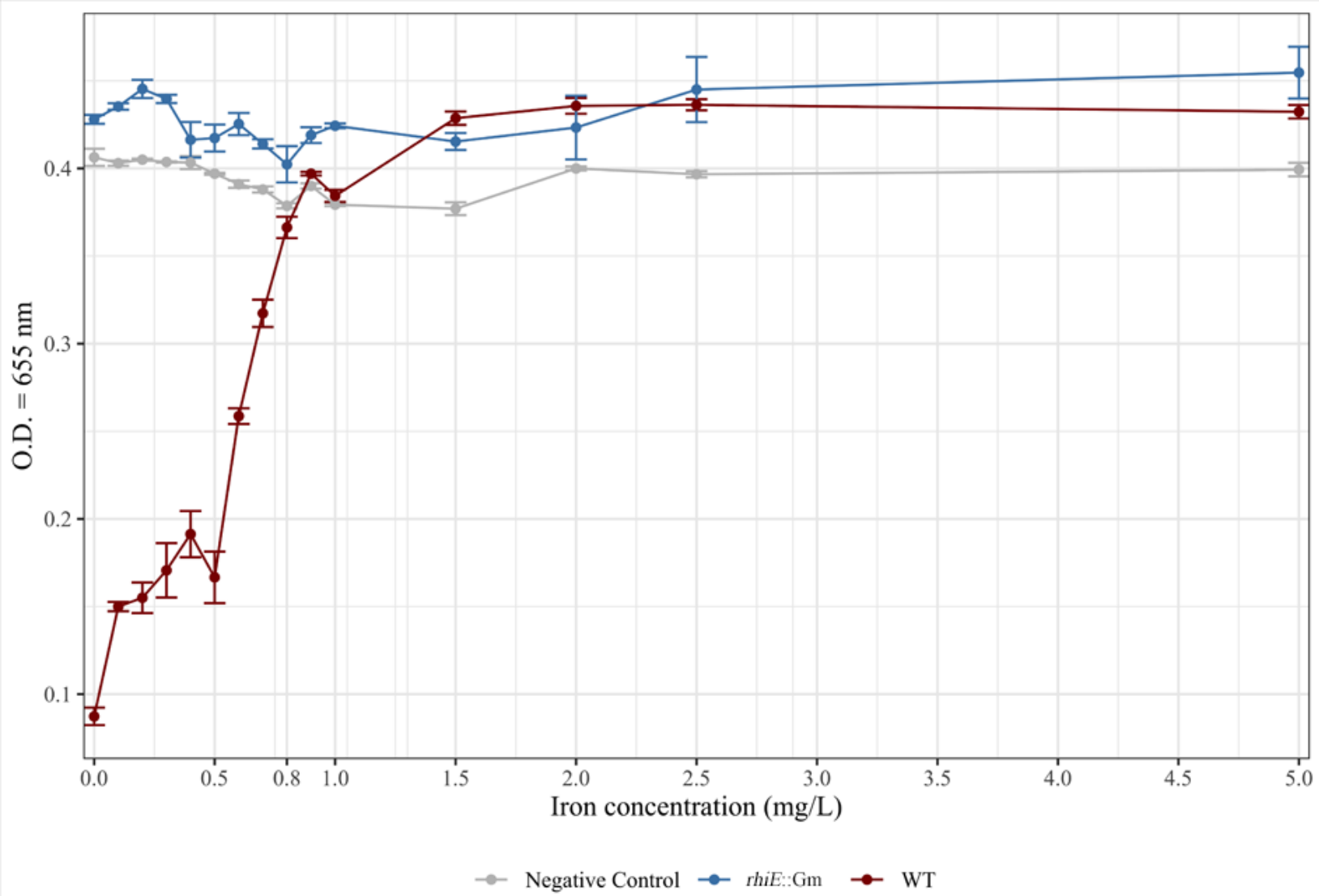
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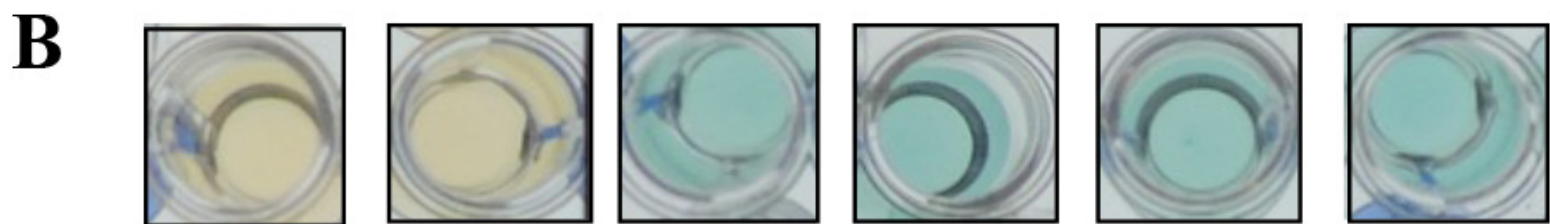
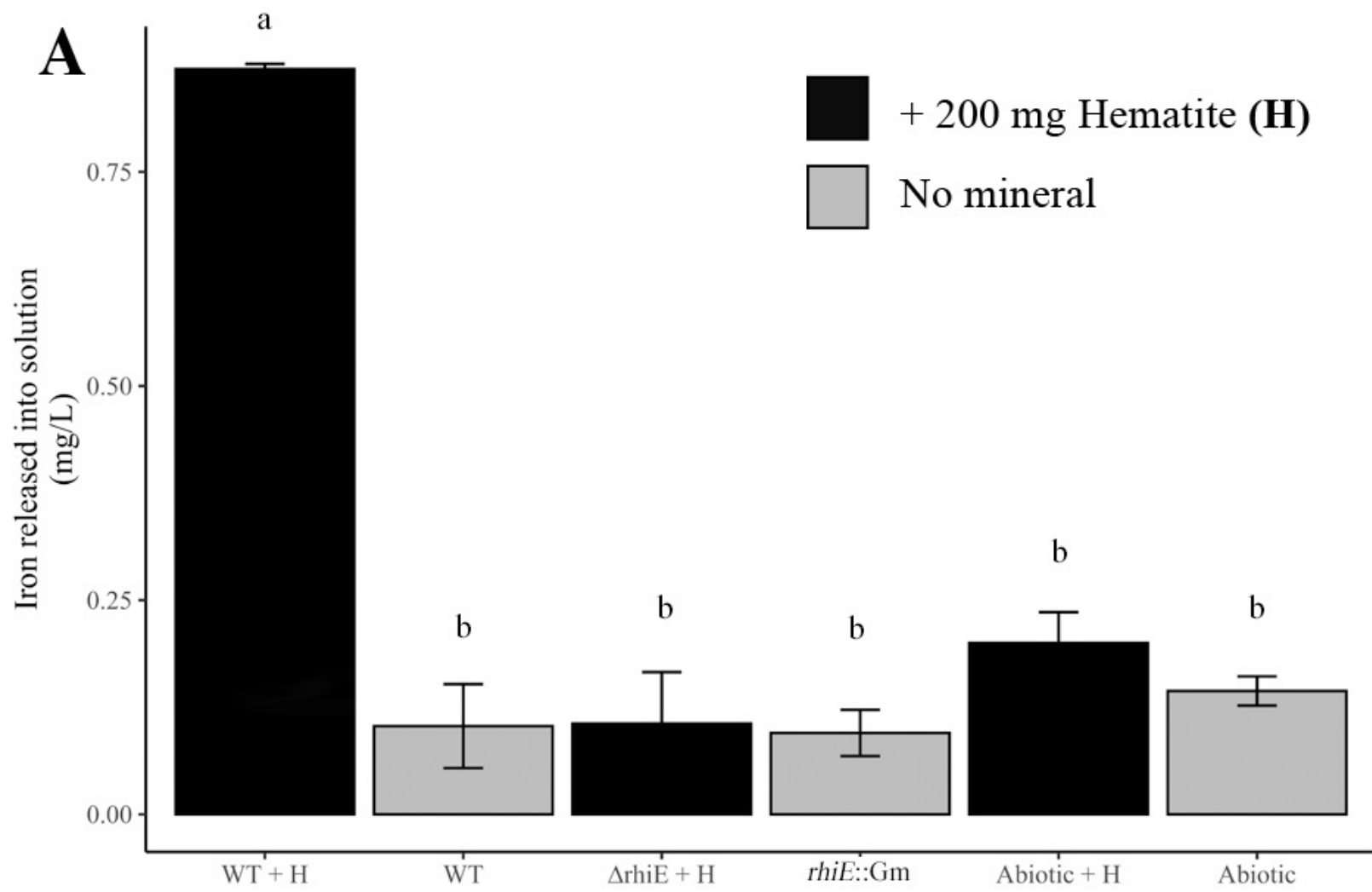
863 **Figure 7. Organization of the cluster of genes involved in rhizobactin biosynthesis in**  
864 **different strains.** Each arrow represents one gene, and its size was scaled according to gene  
865 length. Gene annotation is shown in the right and the complete description is provided in  
866 Table 1. Light grey, dark grey and green genes are involved in siderophore transport. In blue,  
867 red, and yellow, genes are involved in siderophore biosynthesis. In black, genes involved with  
868 siderophore production regulation. In bold, strains for which rhizobactin synthesis was  
869 demonstrated.

870

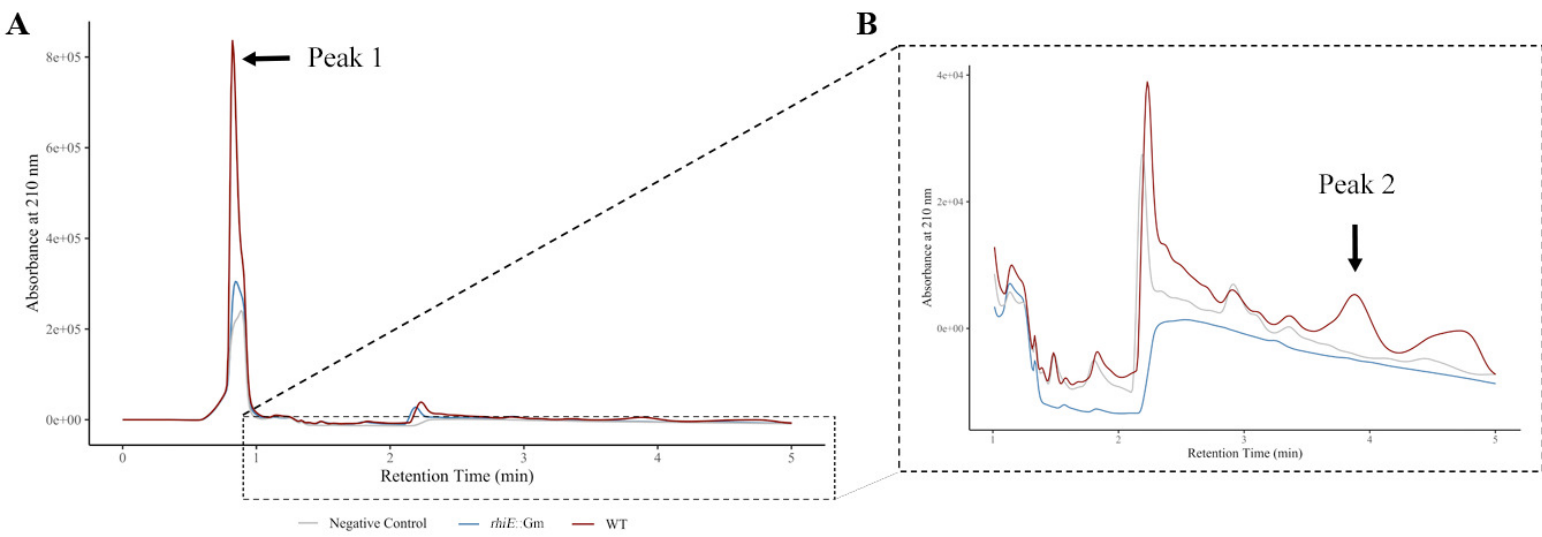
871 **Figure 8. Neighbor-joining phylogenetic analysis of NIS synthetases.** A total of 24  
872 experimentally validated and two proposed NIS-synthetases were aligned using MUSCLE  
873 algorithm and the phylogenetic tree was constructed using SeaView. The code between  
874 brackets corresponds with the NCBI accession number for each protein sequence. NIS-  
875 synthetases are colored depending on their specificity of substrate and thus their group: A  
876 (pink), A' (red), B (green), C (blue), C' (yellow). The enzymes presented in this study (*i.e.*,  
877 RhiE and putative rhizobactin synthetases) are shown in black.

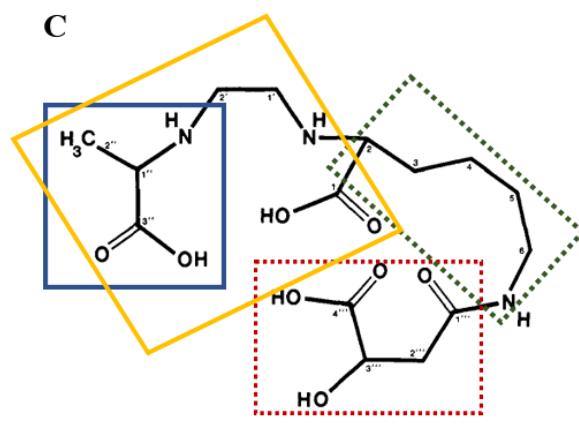
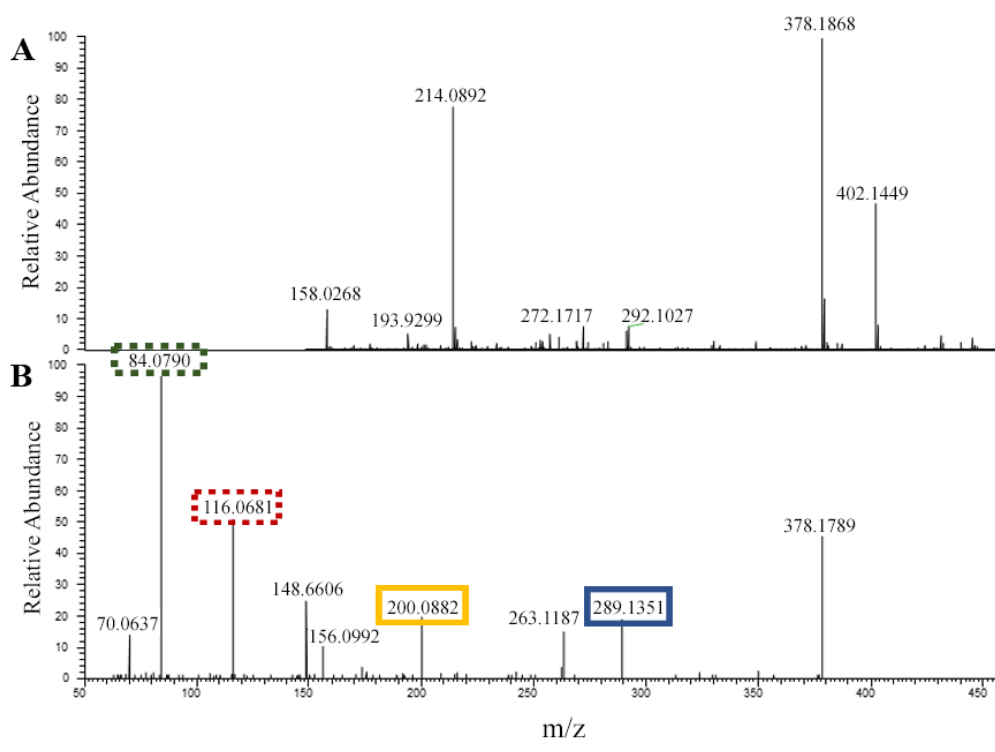


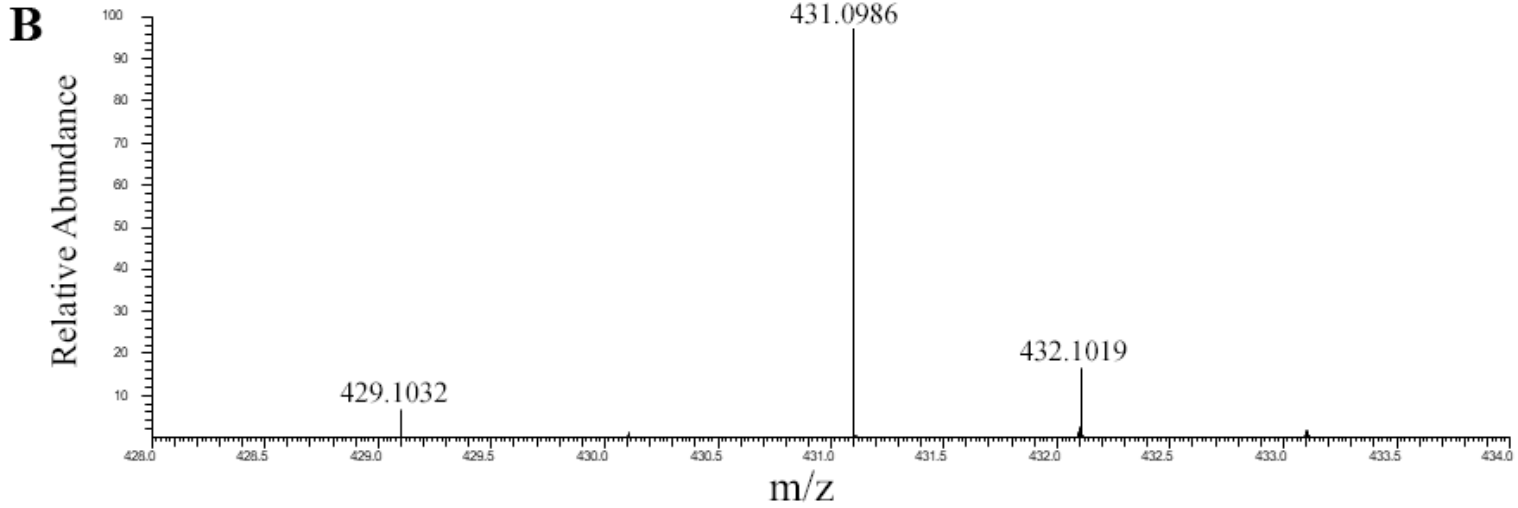
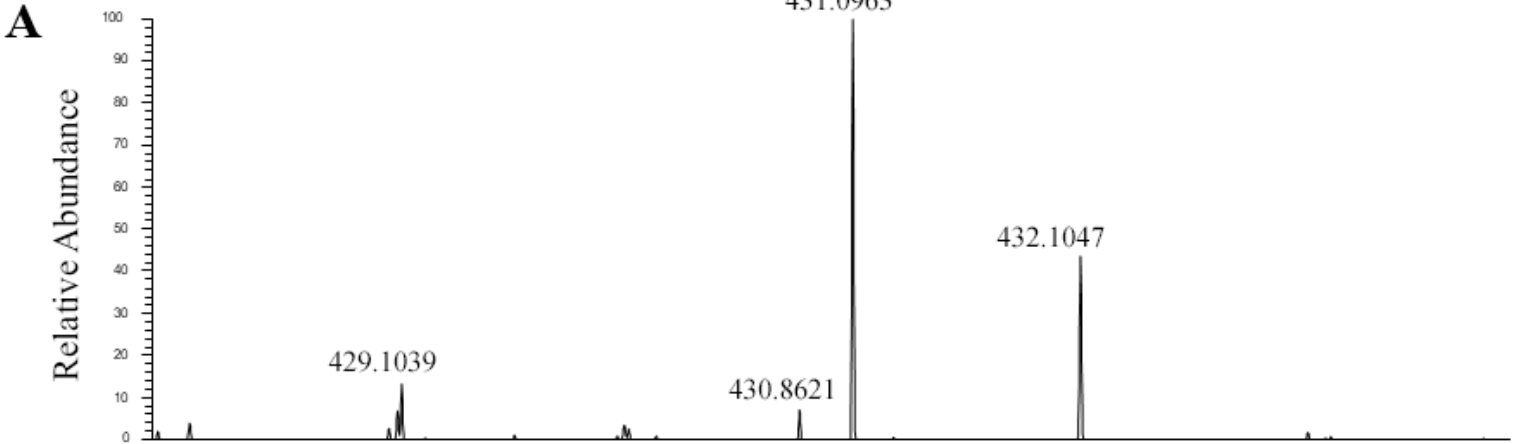












*Caballeronia mineralivorans* PML1 (12)



*Paraburkholderia* sp. BL2311N1



*Paraburkholderia haematera*



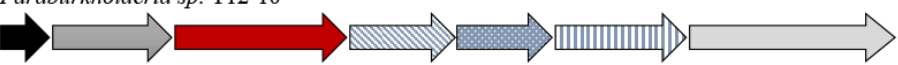
*Paraburkholderia* sp. NMBU\_R16



*Paraburkholderia* sp. Ac-20340



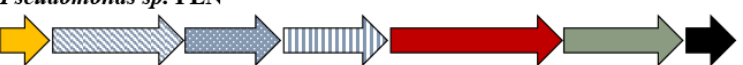
*Paraburkholderia* sp. T12-10



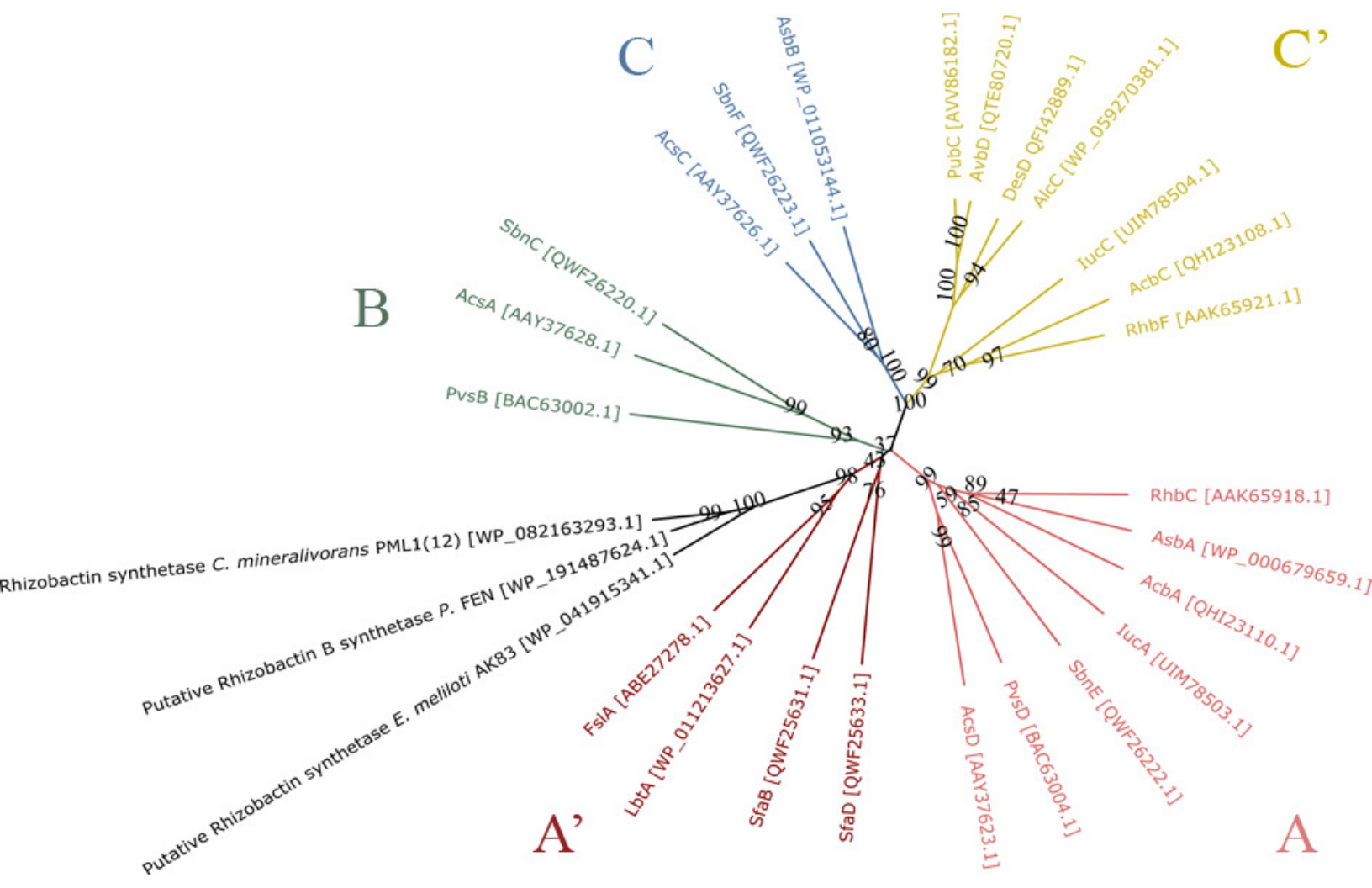
*Ensifer meliloti* AK83



*Pseudomonas* sp. FEN



- TonB-dependent siderophore receptor
- Y4yA family PLP-dependent enzyme
- Cysteine synthase family protein
- NAD/NADP octopine/nopaline dehydrogenase family protein
- iucA/iucC* family siderophore biosynthesis protein
- MFS transporter
- ABC transporters
- Transcriptional regulator/GNAT family N-acetyltransferase
- Ester-coA lyase



**Table 1. Description of the NRPS-independent gene region involved in siderophore production by *Caballeronia mineralivorans* strain PML1(12).** Each gene product of the cluster is presented by its NCBI accession number. The annotation is based on BLASTp homology. NIS synthetases showing the highest homology with RhiE of strain PML1(12) are shown.

Putative function	Accession number (NCBI)	Length	Gene	Annotation (based in BLASTp Homology)	Homology
Transport	KLU26370.1	745	<i>rhiA</i>	TonB-dependent siderophore receptor	88.19% <i>Paraburkholderia</i> sp. BL23IN1 (WP_120293907.1)
Synthesis	KLU26369.1	480	<i>rhiB</i>	Y4yA family PLP-dependent enzyme	80.62% <i>Paraburkholderia haematera</i> (CAE6738131.1)
Synthesis	KLU26368.1	346	<i>rhiC</i>	Cysteine synthase family protein	93.64% <i>Paraburkholderia</i> sp. BL23IN1 (WP_120293908.1)
Synthesis	KLU26367.1	388	<i>rhiD</i>	NAD/NADP octopine/nopaline dehydrogenase family protein	87.37% <i>Paraburkholderia haematera</i> (WP_236066808.1)
Synthesis	KLU26366.1	635	<i>rhiE</i>	<i>iucA/iucC</i> family siderophore biosynthesis protein	85.83% <i>Paraburkholderia</i> sp. BL23IN1 (WP_120293909.1)
Transport	KLU26365.1	421	<i>rhiF</i>	MFS transporter	85.34% <i>Paraburkholderia haematera</i> (WP_211611243.1) <i>Paraburkholderia</i> sp. BL23IN1 (WP_120293910.1)
Transport	KLU26364.1	398	<i>rhiG</i>	ABC transporter substrate-binding protein	88.69% <i>Paraburkholderia haematera</i> WP_211611244.1
Transport	KLU26363.1	353	<i>rhiH</i>	Iron ABC transporter permease	92.63% <i>Paraburkholderia</i> sp. BL23IN1 WP_120293912.1
Transport	KLU26362.1	265	<i>rhiI</i>	ABC transporter ATP-binding protein	92.83% <i>Paraburkholderia</i> sp. BL23IN1

					WP_120293913.1
<b>Regulation</b>	KLU26361.1	138	<i>rhiJ</i>	Transcriptional regulator/GNAT family N- acetyltransferase	87.71% <i>Caballeronia sordidicola</i> WP_031357714

**Table 2. List of bacterial strains, constructions and primers used in this study.**

Strains, plasmids, or primers	Characteristics	Reference
<b>Strains</b>		
<i>Caballeronia mineralivorans</i>		
Strain PML1(12)	Wild type strain	[18]
<i>rhiE</i> ::Gm (PML1(12)( <i>rhiE</i> ::Gm))	Wild type strain, with Gm <sup>R</sup> cassette inserted in double crossing over	This study
<i>Escherichia coli</i>		
DH5α	<i>supE44, ΔlacU169, (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1</i>	Lab collection
S17.1λpir	Tpr Sm <sup>R</sup> <i>recA, thi, pro, hsdRM<sup>+</sup></i> , RP4::2-Tc::Mu::Km Tn7 λpir	Lab collection
<b>Plasmids</b>		
pUC1318	Amp <sup>R</sup> , Gm <sup>R</sup>	Lab collection
pGEM-T Easy	Amp <sup>R</sup>	Kit Promega
pGEM- <i>rhiE</i> _SmaI	pGEM-T Easy carrying a <i>rhiE</i> gene	This study
pGEM- <i>rhiE</i> ::Gm	pGEM- <i>rhiE</i> ::Gm with insertion in <i>SmaI</i> of the Gm <sup>R</sup> cassette	This study
pK19mob	Km <sup>R</sup>	Lab collection
pK19mob- <i>rhiE</i> ::Gm	pK19mob containing a <i>EcoRI</i> fragment obtained from pGEM- <i>rhiE</i> ::Gm	This study
<b>Primers (Tm = 54 °C)</b>		
For_ <i>rhiE</i>	5'- ATGAAGACCACTCCTTCGCTATT - 3'	This study
Rev_ <i>rhiE</i> _SmaI	5'- GCACCGGTCCCGGGAGCCGCGGCAT - 3'	This study
For_ <i>rhiE</i> _SmaI	5'- ATGCCGCGGCTCCCGGGACCGGTGC - 3'	This study
Rev_ <i>rhiE</i>	5'- TCAACCCTCGTTGTGCGCCGCCAGC - 3'	This study

Gm<sup>R</sup>, Km<sup>R</sup>, Sm<sup>R</sup>, and Amp<sup>R</sup>, indicate gentamicin, kanamycin, spectinomycin and ampicillin resistance cassettes, respectively.