The mineral weathering ability of Collimonas pratensis PMB3(1) involves a Malleobactin-mediated iron acquisition system

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2   Malleobactin-mediated iron acquisition system

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24 **SUMMARY**

25 Mineral weathering (MW) by microorganisms is considered to occur through a

26 succession of molecular mechanisms based on acidification and chelation. While the

27 role of acidification is established, the role of siderophores is difficult to disentangle

28 from the effect of the acidification. We took advantage of the ability of strain

29 *Collimonas pratensis* PMB3(1) to weather minerals but not to produce organic acids

30 depending on the corbon source to adress the role of siderophores in MW. We

31 identified a single non-ribosomal peptide synthetase (NRPS) responsible for

32 siderophore biosynthesis in the PMB3(1) genome. By combining iron-chelating

33 assays, targeted mutagenesis and chemical analyses (HPLC and LC-ESI-HRMS),

34 we identified the siderophore produced by strain PMB3(1) as malleobactin X and how

35 its production depends on the concentration of available iron. Comparison with the

36 genome sequences of other collimonads evidenced that malleobactin production

37 seems to be a relatively conserved functional trait, though some collimonads

38 harbored other siderophore synthesis systems. We also revealed by comparing the

39 wild-type strain and its mutant impaired in the production of malleobactin that the

40 ability to produce this siderophore is essential to allow the dissolution of hematite

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41 under non-acidifying conditions. This study represents the first characterization of the

42 siderophore produced by collimonads and its role in mineral weathering.

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44 **Key words:** *Collimonas pratensis* PMB3(1); mineral weathering; siderophore;

45 malleobactine; hematite

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51 **Originality-Significance Statement**

52 Nutrient access is an essential process in nutrient-poor and non-amended

53 ecosystems. To deal with such conditions, plants have developed particular strategies to

54 adapt, noticeably through the selection in their rhizosphere of functional communities

55 effective at mobilizing the nutrients. Among them, bacteria have been shown to be

56 effective at mineral weathering (MW) and at promoting plant growth. Acidification and

57 chelation are considered as the main mechanisms used by MW bacteria. However,

58 our understanding of the molecular mechanisms and genes involved remains limited.

59 While the role of acidification is established, the role of siderophores is difficult to

60 disentangle from the effect of the acidification. In strain PMB3(1) of *Collimonas*

61 *pratensis*, the production of organic acid by the central metabolism is dependent on

62 the carbon source used for growth. Hence, it is one of the few bacterial models in

63 which it is possible to decipher mineral weathering molecular mechanisms that are

64 organic acid-independent. Strain PMB3(1) of *Collimonas pratensis* is a very effective

65 MW bacterium, particularly adapted to oligotrophic environments and effective at

66 promoting plant growth and at protecting plants against fungal pathogens. Mineral

67 weathering has been proposed to be a functional trait of this genus, which molecular

68 mechanisms have not yet been elucidated. The novel results obtained in this study

69 through the combination of bioinformatics, chemical and phylogenetic analyses

70 allowed us to identify and to characterized the siderophore produced by this model

71 strain and to demonstrate the role of this iron acquisition system in mineral

72 weathering. This study represents the first experimental demonstration of the

73 siderophore produced by *Collimonas* (i.e., malleobactin). Our analyses allowed us to

74 evidence the conservation of this iron acquisition system among collimonads and to

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75   improve the siderophore prediction tools, especially to better discriminate ornibactin

76   and malleobactin. Our findings offer relevant informations for different fields of

77   research such as environmental genomics and chemistry, and soil sciences.

78

INTRODUCTION

80   Iron is an essential micronutrient for living organisms. It is involved in key cellular

81   processes such as DNA synthesis, respiration, photosynthesis or metabolism (Rout and Sahoo,

82   2015). Due to its oxidoreduction potential iron is used by several enzymes as energy source

83   (*i.e.* cytochrome subunit of enzymes or cofactors). Although this element is abundant in the

84   Earth crust, its bioavailability is strongly reduced in aerobic soils ranging from neutral to

85   basic pH, while in acidic soils such as in temperate forests, its bioavailability is higher.

86   However, it tends to decrease from the topsoil horizon to the deeper horizons due to the

87   precipitation of iron as iron oxi-hydroxides and its adsorption on different substrates such

88   as organic matter and primary minerals. In soils, the main sources of iron are the primary

89   minerals (*i.e.*, granite and other Fe-carrying minerals and rocks), the secondary

90   minerals such as iron oxi-hydroxides (*i.e.*, hematite, goethite) and the adsorbed

91   forms. However, most of those minerals dissolve at much slower rates than those required to

92   support plant and microbial growth (Lindsay, 1995). Consequently, iron access is subject to

93   important competitions between living organisms (Butaitė *et al.*, 2017).

94   The ability to mobilize iron from the different soil compartments (*i.e.*, minerals, rocks,

95   organic matter) has been reported for several plants, fungi, and bacteria. Noticeably, effective

96   iron mobilizing bacteria have been evidenced in the rhizosphere of various plants and in

97   different soils (Uroz *et al.*, 2009a). The presence of such bacteria and their relative enrichment

98   in the rhizosphere compared to the surrounding bulk soil suggest that these functional

99   communities are recruited in the root environment to make iron bioavailable for plant

100   nutrition. Among the effective iron mobilizing bacteria encountered in nutrient-poor forest

101 soils, bacteria belonging to the genus *Collimonas* have been described for their effectiveness

102 in mobilizing iron (Uroz *et al.*, 2009b, 2007; Leveau *et al.*, 2010). Although, collimonads

103 are considered as members of the rare biosphere in the soil (Leveau *et al.*, 2010),

104 they can be the dominant taxa in specific habitats such as in the mineralosphere

105 (Uroz *et al.*, 2015) or in the mycorrhizosphere (Lepleux *et al.*, 2012; Uroz *et al.*,

106 2012). Representatives of this genus have been isolated from various nutrient-poor

107 environments (*i.e.*, grass land, forests soil; (Uroz *et al.*, 2009b, 2014; Hoppener-

108 Ogawa *et al.*, 2008) and often in the close vicinity of fungi (De Boer, 2004; Uroz *et al.*,

109 2012). Some *Collimonas* species have been used as plant growth promoting agent

110 (*e.g.* tomatoes) to protect them against fungal pathogens (Kamilova *et al.*, 2007;

111 Senechkin *et al.,* 2013; Doan *et al.,* 2020) and to promote the growth of *Pinus*

112 *sylvestris* seedlings (Koele *et al.*, 2009). All these characteristics make this genus an

113 interesting plant growth-promoting (PGP) candidate.

114   The functional screening of *Collimonas* collections revealed that all the collimonads were

115 especially effective at weathering minerals compared to other genera. Among them,

116 *Collimonas pratensis* strain PMB3(1) appeared as the most effective (Uroz *et al.*, 2009b;

117 Picard *et al.*, 2020). This strain is able to solubilize inorganic phosphorous, mobilize iron and

118 dissolve minerals. At the molecular level, this high ability to weather mineral is explained by

119 the ability of the collimonads to produce protons *via* the direct oxidative pathway (Picard *et*

120 *al.*, 2021). Strain PMB3(1) is capable of strongly acidifying its environment when glucose is

121 used as sole carbon source and to produce high concentration of gluconic acid (Uroz *et al.*,

122 2009b; Picard *et al.*, 2021). In that respect, the glucose/methanol/choline (GMC)

123 oxidoreductase was identified as responsible for this acidification process in strain PMB3(1)

124 (Picard *et al.*, 2021). Other molecular mechanisms such as complexolysis may also play a role

125 in the dissolution of minerals (Uroz *et al.*, 2009a; Dong, 2010). Indeed, most of the bacteria

126 recruited in the rhizosphere are capable of producing siderophores. To date, different types of

127 siderophores have been reported and new chelating molecules are continuously discovered

128 (Hernandez *et al.*, 2004; Mathew *et al.*, 2014). Siderophores can impact directly (*i.e.*, surface

129 dissolution) or indirectly (*i.e.*, modification of the solution equilibrium) the mineral

130 weathering process, especially when carbon sources other than glucose are available (*i.e.*,

131 mannitol, trehalose) (Holmén and Casey, 1996; Kraemer, 2004; Parrello *et al.*, 2016; Perez *et*

132 *al.*, 2019).

133   In absence of acidification, the dissolution of minerals may occur through the action of

134 siderophores (Shirvani and Nourbakhsh, 2010), but no experimental study was done on

135 collimonads to functionally characterize the gene clusters involved and the type of

136 siderophore produced. The ability to chelate iron was evidenced for several *Collimonas*

137 strains (Uroz *et al.*, 2009b; Ballhausen *et al.*, 2016). More recently, bioinformatics analyses

138 identified genes encoding a potential Non-Ribosomal Peptide Synthetase (NRPS) in the

139 genomes of collimonads (*i.e.,* Ter6, Ter91, Ter331, Ter10, Ter282 and Ter291) and predicted

140 the production of ornibactin (Song *et al.*, 2015). In this context, the goals of this study were i)

141 to identify and chemically characterize the siderophore produced by the model *Collimonas*

142 *pratensis* strain PMB3(1), ii) to investigate how conserved the gene sequences were among

143 collimonads and related genera and iii) to determine the role of the siderophore in mineral

144 weathering. To do this, we combined bioinformatics, genomics, genetics, geochemistry and

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145 functional bioassays to decipher the genes conferring the chelating and mineral weathering

146 abilities to strain PMB3(1) and other collimonads. The use of high-performance liquid

147 chromatography (HPLC) coupled to high-resolution and tandem mass spectrometry

148 (HRMS and MS/MS) allowed us to identify the siderophore produced as malleobactin X.

149

150 **RESULTS**

151 *Identification of a genomic region involved in siderophore production*

152   The CAS plate assay revealed that the model *Collimonas pratensis* strain

153 PMB3(1) produced chelating molecules as shown by the development of a yellow

154 halo around the colony (Fig. 1A). To identify the genomic region potentially involved

155 in the production of chelators in strain PMB3(1), an antiSMASH search was

156 performed on the genome sequence. Such analysis identified 11 regions encoding

157 putative polyketide synthases (PKS; n=2) or Non-Ribosomal Peptide Synthetases

158 (NRPS; n=9) with prediction or not of the final product (Table S2). Among the

159 potential NRPS, only one was predicted as encoding a siderophore. This region large

160 of 27.7 kb is characterized by 13 genes (Fig. 2; Table 2). Six genes are involved in

161 siderophore biogenesis. Among them the larger genes *mbaA* and *mbaB* encode

162 Non-ribosomal peptide synthetase (NRPS) composed of modules which including

163 domains (Condensation, Adenylation, Thiolation). In addition, the region is

164 characterized by 6 genes involved in siderophore transport and utilization and by one

165 gene encoding a sigma factor.

166

167 *Prediction of the siderophore structure*

168   To predict the structure of the siderophore, the two NRPS encoding genes (*mbaA*

169 NKI69295.1 and *mbaB* NKI69296.1) were submitted to NRPS prediction using three

170 different tools. All predictions identified four biosynthetic modules, three were

171 encoded by the *mbaA* gene and the last one by the *mbaB* gene. Modules are

172 composed of different domains: adenylation (A), thiolation (T), condensation (C) and

173 epimerization (E) following the schema (AT)-(CATE)-(CAT)-(CATC) for modules 1-2-

174 3-4 respectively. The NRPS prediction tools also proposed the amino acid

175 composition of the siderophore based on the Stachelhaus code (Stachelhaus *et al.*,

176 1999). For modules 2 and 3, the three NRPS prediction tools proposed the same

177 amino acids. Module 2 corresponds to a surfactin synthetase B (75 % identity) that

178 recruits an aspartate (Asp). Module 3 has 100 % identity with a pyoverdin synthetase

11

179   that recruits a serine (Ser). No consensus was obtained for modules 1 and 4. Indeed,

180   the signature sequence (DVETLGGISK or DVETLGGI) identified for module 1

181   indicated the recruitment of beta-hydroxy-tyrosine according to 'NRPS predictor 2' or

182   an ornithine according to 'NP.searcher'. For the amino acid recruited by the module

183   4, the signature sequence was DGEYTGGITK or DGEYTGGI. Such sequence is

184   predicted to recruit a leucine according to 'NRPS predictor 2' and 'NP.searcher', or

185   an ornithine according to 'PKS/NRPS analysis'. Altogether, our bioinformatics

186   analyses predicted a siderophore composed of four amino acids, the second being a

187   serine and the third an aspartate (X-Ser-Asp-X).

188

189   *Involvement of the mbaA gene in the production of siderophore*

190   To demonstrate the involvement of this NRPS encoding region in siderophore

191   synthesis, a *mbaA* mutant named ∆NRPS was constructed and tested using different

192   bioassays (*i.e.*, siderophore production, growth assay).

193   Regarding the ability to chelate iron, the WT strain of PMB3(1) was able to

194   mobilize iron in the CAS plate assay after 3 days. The yellow halo formed around the

195   colonies (WT colony diameter: 1.0 ± 0.1 cm) was about 1.5 ± 0.1 cm, while no halo

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196 was observed for the ΔNRPS mutant strain (ΔNRPS colony diameter: 0.6 ± 0.1 cm)

197 (Fig. 1A). The production of chelating agent by the WT strain was also confirmed in

198 liquid CAS assay where the absorbance in absence of iron in the culture medium is

199 significantly decreased (OD at 655 nm = 0.053 ± 0.001) compared to the control.

200 Noticeably, for the WT strain, a siderophore activity was observed for iron

201 concentration ranging from 0 to 0.7 mg/l, while the production of chelator is inhibited

202 for greater values (iron concentration of 0.8 mg/l: OD at 655 nm = 0.372 ± 0.019;

203 P<0.05) (Fig. 1B). Whatever the iron concentration, the ΔNRPS mutant did not

204 present a chelating activity (OD at 655 nm = 0.458 ± 0.005) (Fig. 1B).

205

206 *Impact of the mbaA mutation on growth*

207 To determine how the concentration of iron and the mutation of the *mbaA* gene

208 (ΔNRPS mutant) affect the growth of strain PMB3(1), were performed tests in

209 medium containing increasing concentrations of iron (*i.e.* 0.1; 0.2; 0.3; 0.5; 1.0 mg/l).

210 In this way, we demonstrated that the growth rate increased with increasing

211 concentrations of iron and that strain PMB3(1) and its ΔNRPS mutant presented the

212 same growth rate (P > 0.05) (Fig. S1). With 1 mg/l of iron, the growth rates of the

13

213 ΔNRPS mutant and WT strains without EDTA were ca 0.06 ± 0.005 h^{-1}. However, in

214 presence of EDTA (10 mM), no growth was observed for the ΔNRPS mutant while

215 the growth of the WT strain was maintained, but its growth rate decreased to 0.01 ±

216 0.005 h^{-1} (Fig. 1C).

217

218 *Purification of the siderophore and chemical characterization*

219   To determine the structure of the synthetized siderophore, the supernatants of 3-

220 days old cultures in ABm devoid of iron of the WT strain (containing the siderophore),

221 the ΔNRPS mutant strain and the medium were analysed by HPLC and LC-ESI-MS

222 (or MS/MS).

223   The HPLC chromatograms of the WT, ΔNRPS mutant and medium supernatants

224 are presented in figure 3A. Such analysis evidenced a major peak with a retention

225 time (RT) of 1.2 min only present for the WT strain and absent from the two other

226 conditions. To investigate the potential presence of a siderophore activity associated

227 to this peak, the collected HPLC fractions were tested with the liquid CAS assay.

228 Both HPLC chromatogram and siderophore activity were overlaid and are presented

229 in figure 3B. Noticeably, the CAS assay revealed a strong reduction of the

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230　absorbance (OD_{655nm} = 0.055) for the fractions corresponding to the peak present at

231　RT = 1.2 min, matching with the yellow color observed with the CAS assay. This

232　confirmed the presence of a chelating activity in the first peak. In addition, two other

233　peaks were detected for the WT strain at 2.2 (peak 2) and 3.8 (peak 3) min RT (Fig.

234　3A). The liquid CAS assay revealed a decrease of absorbance (OD_{655nm} = 0.110 and

235　0.103 for peak 2 and 3, respectively; Fig. 3B) after 1h incubation, suggesting the

236　presence of a chelating activity in the two peaks.

237　　A deeper analysis was done by liquid chromatography coupled to positive ion-

238　mode electrospray mass spectrometry (LC-ESI$^+$-HRMS) on the first peak collected to

239　perform the chemical characterization on a fraction which chelating activity was

240　demonstrated. High resolution MS analysis revealed that the siderophore compound

241　(S) has an exact mass of M_S = 622.3069 (monoprotonated ion seen at

242　m/z = 623.3069) (Fig. 4A). This value is strictly similar to the mass of the

243　malleobactin X (Fig. 4E). To confirm the malleobactin X structure, the siderophore

244　was further analyzed by tandem mass spectrometry (MS/MS) and the major product

245　ions (Fig. 4C) compared with theoretical fragments (Fig. 4D). Product ion at m/z =

246　535.4 (Fig. 4C; b4) was attributed to the initial loss of C-terminal putrescine from the

15

247   precursor ion and that at m/z = 517.4 to additional dehydration (H_2O loss from m/z =

248   535.4). Product ion at m/z = 465.3 (also seen in its dehydrated form at m/z = 447.4)

249   (Fig. 4C; y1) was attributed to the initial loss of N-terminal ornithine from the

250   precursor ion. The breaking of the peptide bond between serine and ornithine gave

251   rise to a product ion at m/z = 377.2 (also seen in its dehydrated form at m/z = 359.2)

252   (Fig. 4C; b3) and another product ion at m/z = 247.3 (Fig. 4C; y3). The breaking

253   between aspartate and serine gave rise to a product ion at m/z = 334.3 (Fig. 4C; y2).

254   An additional LC-ESI[+]-HRMS analysis was performed on the same fraction

255   (collected at RT = 1.2 min), but previously incubated with iron (see details in

256   *Ferrisiderophore preparation* section). As presented on Figure 4B, formation of the

257   expected ferrisiderophore (FS) was confirmed through the clear observation of the

258   ion at m/z = 676.2191 ($M_{FS\ (exp)}$ = 675.2191). This experimental value is in full

259   agreement with the complexation between iron (55.935 u for major isotope [56]Fe) and

260   the triply deprotonated siderophore S (622.3069 – 3 ×1.008 = 619.2829 u), leading to

261   a theoretical mass $M_{FS\ (theo)}$ = 675.2179 (55.935 + 619.2829). The presence of iron in

262   the structure was also validated by the detection of the characteristic isotopic pattern

263   detailed in Figure 4B, showing clearly the contributions of [54]Fe and [56]Fe isotopes

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264 (m/z = 674.2246 and 676.2191, respectively). In addition, the relative intensities of

265 the ions at m/z = 677.2213 (28.8 %) and at m/z = 678.2236 (4.4 %) were found to be

266 in perfect accordance with the predicted isotopic distribution for malleobactin (Manura

267 and Manura, 2015). The MS/MS analysis conducted on the ferrisiderophore FS

268 (fragmentation of the ion at m/z = 676.2) revealed a product ion at m/z =

269 588.3 explained by the loss of C-terminal putrescine (dehydrated form also seen at

270 m/z = 570.2) (Fig. S2). A second intense ion at m/z = 606.2802 was also observed

271 on Figure 4B, in addition to that attributed to the ferrisiderophore FS (m/z =

272 676.2191).

273   The experimental masses found for the siderophore S (without incubation with

274 iron) and for the ferrisiderophore FS (after incubation of the siderophore with iron)

275 agree with the results previously obtained on *Burkholderia xenovorans* strain LB400

276 (Vargas-Straube *et al.*, 2016), allowing us to clearly identify the malleobactin X as the

277 siderophore produced by strain PMB3(1).

278

279 *Comparison with other Collimonas and related genera*

17

280   Our phylogenetic analysis revealed a good differentiation of the NRPS proteins according

281   to the chemical structure of the siderophores (Fig. 5; green, ornibactin; red,

282   malleobactin), the taxonomic belonging and the MbaA protein sequence. Beside the

283   serobactin-related cluster, two clusters can be identified. The cluster 1 is supported

284   by NRPS sequences (*i.e.*, OrbI homologues) assigned to *Burkholderia cepacia, B.*

285   *cenocepacia* or *B. vietnamiensis* and is predicted to produce ornibactin. Noticeably,

286   the production of ornibactin was experimentally demonstrated for three strains (*B.*

287   *contaminans* MS14 (Deng *et al.*, 2017), *B. cenocepacia* K56-2 (Darling *et al.*, 1998), *B.*

288   *cepacia* ATCC25416 (Meyer *et al.*, 1995)) of this cluster. The second cluster is composed

289   of NRPS sequences (*i.e.,* MbaA homologues) assigned to *Burkholderia*

290   *pseudomallei, B. mallei, B. thailandensis* and *Collimonas* and predicted to produce

291   ornibactin or malleobactin. In this cluster, the production of malleobactin was

292   experimentally demonstrated for three strains (*B. pseudmallei* K96243 (Alice *et al.*, 2006), *B.*

293   *thailandensis* (Franke *et al.*, 2013), *B. xenovorans* LB400 (Vargas-Straube *et al.*, 2016)),

294   while the production of ornibactin was only predicted. The strain PMB3(1) represents the

295   fourth strains in this cluster which malleobactin production was experimentally demonstrated

296   and the first collimonads. The strong proximity between the MbaA protein of the

297   collimonads, the ability of strain PMB3(1) to produce malleobactin and the conserved ability

298   to mobilize iron in collimonads suggest that all the collimonads considered synthetize

299   malleobactin and not ornibactin as previously predicted (Fig. 5, green dotted circle; (Song *et*

300   *al.*, 2015). Noticeably, the most distantly related sequence (38.25% of identity) to MbaA of

18

301 strain PMB3(1) is the NRPS protein responsible of the biosynthesis of serobactin in

302 *Herbaspirillum seropedicae* that is however the closest genus of *Collimonas* according to

303 taxonomy (Rosconi *et al.*, 2013).

304

Siderophore involvement in hematite weathering

306   To evaluate the potential involvement of the siderophore produced by *Collimonas*

307 strain PMB3(1) on mineral weathering, a hematite dissolution experiment was

308 performed (Fig. 6). To exclude an acidification effect, the experiment was done with

309 mannitol as sole carbon source, which was previously shown not to lead to the

310 acidification of the medium during growth (Uroz *et al.*, 2009b). After 7-days of

311 incubation, the iron released in solution from hematite as well as the pH were

312 measured, permitting to monitor the mineral weathering potential of the different

313 strains tested. In this experiment, no significant growth difference was observed

314 between the ∆NRPS mutant and the WT strains (OD_{595nm} = 0.8 ± 0.1; P<0.05),

315 though the WT strain released significantly more iron from the mineral (0.99 ± 0.19

316 mg/l; Fig. 6) than the ∆NRPS mutant strain or the non-inoculated controls (*i.e.* 0.01 ±

317 0.01 mg/l; P<0.05). Noticeably, the pH of the medium remained stable at 6.2

318 whatever the treatment (inoculated with the WT strain, inoculated with the ∆NRPS

19

319   mutant strain and non-inoculated controls). In this sense, the CAS assay performed

320   on the supernatant for each condition demonstrated a siderophore activity (yellow

321   colour) only for the WT strain (Fig. 6).

322

323   **DISCUSSION**

324   The high effectiveness of *Collimonas pratensis* strain PMB3(1) to weather mineral

325   led us to investigate in more details the molecular mechanisms involved. In a

326   previous study, we showed that the mineral weathering effectiveness of this strain

327   was related to the carbon substrate used (*i.e.*, glucose, mannitol, trehalose,

328   gluconate; (Uroz *et al.*, 2009b)). In presence of glucose this strain strongly acidified the

329   medium and effectively weathers minerals, through the production of protons and

330   gluconic acids (Picard *et al.*, 2021). Noticeably, the strain PMB3(1) appeared also

331   capable of weathering minerals in presence of mannitol, a sugar highly produced by

332   mycorrhizal fungi and metabolized by this bacterial strain through a moderately-

333   acidifying pathway. The mineral weathering ability observed with mannitol suggests

334   the existence of alternative MW mechanisms such as iron acquisition systems. In this

335   study, we investigated the mechanisms by which this strain was able to mobilize iron

336 from artificial (*i.e.,* CAS) or natural iron complexes (*i.e.,* hematite). The *in silico*

337 analyses revealed that the genome of strain PMB3(1) harbors the *ftrABCD* system

338 (siderophore-independent iron uptake system; (Mathew *et al.*, 2014): [NKI69657.1-

339 NKI69654.1]), the bacterioferritin (iron storage protein; (Andrews, 1998): [NKI72009.1-

340 NKI72013.1]), the fecIR operon ((Braun *et al.*, 2003): [NKI68784.1-NKI68785.1]), and an

341 incomplete hmu operon ((Nienaber *et al.*, 2002); only hmuU [NKI72430.1] and hmuV

342 [NKI72429.1] were identified). In addition, an antiSMASH analysis identified a NRPS

343 cluster, predicted to synthetize a siderophore (*i.e.,* ornibactin). The presence in this

344 strain of all these iron acquisition systems suggests that it is quite adapted to the nutrient-poor

345 conditions occurring in the soil where it was isolated, but may also reflect the competition for

346 iron occurring in the mycorrhizosphere with other bacteria and fungi.

347

348   Although, strain PMB3(1) possesses all these putative iron acquisition systems, the knock-

349 out of a single gene of the NRPS cluster fully abolished its ability to mobilize iron as seen

350 using the CAS assay. These results suggest that the other iron acquisition systems play a

351 minor role in comparison to the production of siderophores or that they are important in other

352 processes such as the mobilization of intracellular iron reserves. The NRPS region detected

353 in strain PMB3(1) presents the classical structure found in other bacterial species

354 producing NRPS-dependent siderophores with the identification of genes involved in

355 siderophore biogenesis (including the 2 NRPS genes), in siderophore transport and

21

356   utilization (Fig. 2; (Esmaeel *et al.*, 2016, 2018; Butt and Thomas, 2017)). Noticeably, the

357   *mbaA* and *mbaB* genes coding for siderophore production presented 36 % identity

358   with genes *mbaA* and *mbaB* of *B. xenovorans* LB400, a strain capable of chelating

359   iron and producing malleobactin (Vargas-Straube *et al.*, 2016). Different homologues of

360   enzymes involved in N- and C-terminal modifications during siderophore production

361   were also detected. These included *mbaH* which encodes a taurine dioxygenase

362   TauD/F involved in the modifications on the N-terminal ornithine (Franke *et al.*, 2013),

363   *mbaC* which encodes a L-ornithine 5-monooxygenase involved in the formation of a

364   nitrogroup (Franke *et al.*, 2013) and *mbaE* encoding a N(5)-hydroxyornithine

365   transformylase PvdF-type involved in modifications of the C-terminal ornithine

366   (McMorran *et al.*, 2001; Vargas-Straube *et al.*, 2016). The *mbaI, J, L, N* and D genes

367   involved in the ferrisiderophore complex recognition and transport were also

368   identified (Butt and Thomas, 2017). In addition, the enzyme in charge of the

369   reduction of the ferrisiderophore and of the release of Fe^{2+} and siderophore in the

370   cytoplasm was identified as the *mbaK* gene encoding a siderophore-iron reductase

371   FhuF (Matzanke *et al.*, 2004). The mbaF gene responsible of the siderophore

372   expression and regulation was also identified as well as *mbaF*-dependent promoters

22

373 with specific -35 and -10 regions (TAAA/n(17)/CGTC) (Agnoli *et al.*, 2006; Thomas,

374 2007). Last, two genes (*iorA*, *iorB*) were identified at the end of the siderophore

375 biosynthesis region. They are predicted to encode two subunits of an aldehyde

376 dehydrogenase responsible of the formation of a carboxyl group. This function may

377 be responsible of modifications occurring after the synthesis of the siderophore.

378 However, a ∆IorB mutant presented no differences in siderophore mass (M_S =

379 622.3069) or in its chelating activity compared to the WT strain (data not shown). A

380 comparison with all the other available collimonads genomes revealed a good

381 conservation of the NRPS regions as well as of the *iorAB* genes. Indeed, the

382 complete NRPS region was found in nine of the 14 genomes and was absent from

383 the other five (*Collimonas sp.* OK242; *Collimonas sp.* OK307; *Collimonas sp.* OK607;

384 *Collimonas sp.* PA-H2 and *Collimonas arenae* Cal35). A more in-depth analysis of

385 the genomes of these collimonads revealed that some of them possessed genes

386 involved in the synthesis of other siderophores predicted to be myxochelin (41% of

387 homology for strains Cal35 and PA-H2) or staphylobactin (18% of homology for

388 strains OK242 and OK307). No siderophore region was detected for strain OK607.

389 These results suggest that malleobactin may not be the sole siderophore produced

390 by collimonads.

391

392   The combination of HPLC, fraction collect and mass spectrometry allowed us to

393 identify the siderophore produced by *Collimonas pratensis* strain PMB3(1) as

394 malleobactin. This hydroxamate type siderophore was reported for the first time to be

395 produced by *Pseudomonas pseudomallei* (reclassified as *Burkholderia pseudomallei*)

396 hence its name (Yang *et al.*, 1991). To date, different forms of malleobactin have been

397 discovered in different *Burkholderia* and *Paraburkholderia* species, be they members

398 or not of the *Burkholderia cepacia* complex (Bcc) (*i.e.* most pathogenic *Burkholderia*)

399 (Esmaeel *et al.*, 2016). In our study, the ESI-MS analysis revealed that the major

400 metabolite in the purified extract (*i.e.*, peak 1 with RT=1.2 min) had an exact mass of

401 M_S = 622.3069 and m/z = 623.3069 without iron and of M_{FS} = 675.2191 with iron (Fig.

402 4B). This compound effectively chelated iron as stated with the CAS assay. This

403 mass value obtained with iron corresponds perfectly with the mass of the

404 malleobactin previously identified in the strains *Burkholderia xenovorans* LB400

405 (Vargas-Straube *et al.*, 2016) and *Burkholderia thailandensis* (Franke *et al.*, 2013). In both

24

406   studies, the molecule detected was named malleobactin, but Vargas-Straube et al.

407   (2016) proposed the name malleobactin X and Franke et al. (2013) proposed

408   malleobactin B. The single difference between malleobactins B and X is the position

409   of an aldehyde group on the N-terminal ornithine. Such position was predicted by

410   Vargas-Straube et al., (2016) to be on the second nitrogen of the N-ter ornithine,

411   while Franke et al., (2013) determined it by NMR to be on the first nitrogen of the N-

412   ter ornithine. As the two molecules present the same properties (*i.e.*, mass,

413   fragmentation peaks, chelation ability) and the genome sequences are quite similar,

414   malleobactin B and X may correspond to the same molecule. For clarity in the text

415   we used the term malleobactin X. Finally, Vargas-Straube et al. (2016) also identified

416   another compound in the MS spectrum of the malleobactin produced by *B.*

417   *xenovorans* LB400 presenting a peak at m/z = 606.4 that they hypothesized to be a

418   fragment of the ferrisiderophore produced by a McLafferty rearrangement

419   (elimination of $CH_2CHCH_2CH_2NH_2$ corresponding to $[M_{FS}-70]^+$), but without the

420   demonstration of the presence of iron in this compound. For our strain PMB3(1) of

421   *Collimonas*, we detected a similar compound (m/z = 606.2802) in the MS spectrum of

422   the ferrisiderophore (*i.e.*, malleobactin + Fe). However, the isotopic profile of this ion

25

423 (m/z = 606.2802) did not present the characteristic iron signature, *i.e.* the clear

424 contributions of the two major iron isotopes [54]Fe and [56]Fe (Fig.S3). The absence of

425 iron signal indicates that the m/z = 606.2802 molecule is consequently not the result

426 of a Mc Lafferty rearrangement of the ferrisiderophore.

427

428   The chemical analyses granted us the possibility to authenticate two other minor

429 peaks (2 and 3; Fig. 3) in the purified extract, far less intense than the peak 1

430 corresponding to malleobactin (RT = 1.2 min). The absence of these two peaks in the

431 HPLC chromatogram of the extract of the ∆NRPS mutant (Fig. 3A) demonstrated that

432 the NRPS region we identified was responsible for the synthesis of (at least) the

433 three molecules (corresponding to the main peak 1 and the minor peaks 2 and 3).

434 Interestingly, the production of three different malleobactins by a single NRPS region

435 was also proposed for *Burkholderia pseudomallei* strain K96243, but the exact

436 structure and identity of these malleobactins were not reported (Alice *et al.*, 2006). In

437 the current work, we show that these two other molecules presented a lower

438 chelating activity than the main molecule we identified as malleobactin X. This is not

439 incongruent with these molecules being malleobactins. In fact, among the wide range

26

440 of malleobactins described (malleobactin A-H and X; (Franke *et al.*, 2015; Vargas-

441 Straube *et al.*, 2016), the affinity for iron strongly varies. Some of them (*i.e.*

442 malleobactin B, F and H) present such a low chelating activity compared to

443 malleobactin E that their real role in cell has been questioned (Franke *et al.*, 2015).

444 Thus diversity of malleobactin forms was proposed to result from the low specificity of

445 the adenylation domain A1 and A4 of the NRPS modules (Misiek *et al.*, 2011; Franke *et*

446 *al.*, 2015), but also from a spontaneous structural change (Franke *et al.*, 2013).

447 Noticeably, malleobactin B (similar to malleobactin X) was shown to undergo such a

448 structural change to other malleobactin forms (A, C or D) without any enzyme activity

449 (Franke *et al.*, 2013). Comparing the affinity for iron of these different forms revealed

450 that malleobactin A has a very low affinity. Based on the literature, our observations

451 suggest that the two minor peaks (2 and 3) represent alternate structures of the main

452 malleobactin detected in peak 1. However, due to their low concentration and low

453 affinity for iron, we were not able to determine their mass.

454

455 While the chemical characterization of the siderophore produced by strain

456 PMB3(1) allowed the identification of a malleobactin, the bioinformatic analyses done

27

457   on the NRPS genomic sequence (antiSMASH) predicted the production of ornibactin

458   (Stephan *et al.*, 1993). This discrepancy is mostly explained by the antiSMASH

459   database that does not contain malleobactin standard regions as few malleobactins

460   have been characterized at both chemical and genomic levels. The comparison of

461   the chemical structures of ornibactin and malleobactin reveals that they are strongly

462   similar (Alice *et al.*, 2006; Deng *et al.*, 2017). Indeed, the two siderophores differ only on

463   the N-terminal ornithine residue, where an aliphatic chain is present only on

464   ornibactin (Franke *et al.*, 2013). Such similarities suggest that the two siderophores are

465   encoded by very similar NRPS systems and that many predicted ornibactin-

466   producing bacteria may be malleobactin producers. To solve this issue of

467   identification, Esmaeel *et al.* (2016) proposed to add new criteria to differentiate the

468   NRPS-encoding ornibactin and malleobactin based on particular genes present or

469   absent in the NRPS clusters (Esmaeel *et al.*, 2016). They suggested that *orbK*-like and

470   *orbL*-like genes (encoding acetylases) are only present in ornibactin clusters,

471   whereas the presence of *mbaM* gene (encoding a protein of unknown function) was

472   only present in malleobactin clusters (Esmaeel *et al.*, 2016). However, the presence of

473   the *mbaM* gene was only highlighted for Burkholderiales (Butt and Thomas, 2017). In

28

474   our study, we do not detect a *mbaM* homologue in our model strain nor in the other

475   *Collimonas* strains or in *Burkholderia xenovorans* strain LB400, indicating that this

476   protein is not necessary for malleobactin production and is not a good marker of the

477   malleobactin cluster. The absence of the *orbK*-like and *orbL*-like genes are therefore

478   the most relevant for the identification of the malleobactin cluster.

479

480   Siderophores represent one of the main class of molecules reported to play an

481   important role in mineral weathering which are frequently detected in soil (Holmström

482   *et al.*, 2004). They are suspected to act directly on the mineral surfaces where they

483   form iron-siderophore complexes that are then transferred into the soil solution

484   (Kalinowski *et al.*, 2000; Kraemer, 2004). They may also indirectly act in MW by

485   chelating the iron released in solution, a phenomenon that modifies the equilibrium of

486   the surrounding solution and thus enhances mineral dissolution. While the effect of

487   pure molecules was clearly evidenced, the experiments done with bacteria producing

488   such molecules were less clear. Indeed, concomitantly to the production of

489   siderophores, many bacteria acidify their environment, making it difficult to

490   disentangle the relative effects of the acidification and the chelating molecules in the

491 mineral weathering process (Hersman *et al.*, 1995; Holmén and Casey, 1996; Maurice,

492 2000). Furthermore, several studies have shown a synergistic effect of siderophores

493 and acidifying molecules on mineral weathering (Reichard *et al.*, 2007; Dehner *et al.*,

494 2010; Lin *et al.*, 2018). Controlling the pH of the solution is therefore essential at

495 chemical, mineralogical and microbiological levels. First, it conditions the activity of

496 the siderophores. Deferrioxamine B, protochelin or rhizoferrin clearly showed

497 contrasted chelating activity with pH (Akafia *et al.*, 2014). Second, acidic conditions

498 allow a spontaneous dissolution of minerals (Brantley, 2008), which may release

499 significant quantities of iron in solution and consequently inhibit siderophore

500 production by bacteria. Indeed, the production of siderophore was shown to be

501 inhibited by iron concentrations ranging from 0.2 to 5 mg/l (Dave and Dubc, 2000; Deng

502 *et al.*, 2017) or in presence of Fe-bearing glasses (Perez *et al.*, 2019). In our study,

503 the production of malleobactin by strain PMB3(1) appeared inhibited from 0.8 mg/l of

504 iron in the solution, while the amount of iron released passively in solution from

505 hematite in our experimental conditions was below 0.1 mg/l. To exclude a potential

506 effect of acidification, our mineral weathering experiments were performed in a

507 buffered medium (constant pH = 6.2), devoid of iron and containing a carbon source

30

508   (*i.e.* mannitol), which does not permit an acidifying metabolism. Using this approach,

509   we were able to demonstrate for the first time that malleobactin production by

510   *Collimonas pratensis* strain PMB3(1) contribute to a significant increase of the

511   hematite dissolution as showed by comparing the WT and ΔNRPS mutant strains.

512   Together, our results suggest that depending on the availability of carbon substrates

513   (*i.e.*, glucose vs mannitol) and the nutrients (*i.e.,* quantity of iron in solution), strain

514   PMB3(1) may use high levels of organic acid such as gluconate and 2-keto-

515   gluconate or malleobactin to weather minerals and to scavenge iron from its

516   surrounding environment, two mechanisms which can act independently or in a

517   synergistic way (Picard *et al.*, 2021).

518

519   **CONCLUSION**

520   The combination of molecular and chemical methods allowed us to identify the

521   siderophore produced by *Collimonas pratensis* strain PMB3(1) as malleobactin X.

522   This is the first chemical and molecular characterization of a siderophore produced

523   by a collimonads as previous studies were only based on *in silico* analyses which

524   predicted that the siderophore produced by *Collimonas* was ornibactin (Song *et al.*,

     31

525   2015). Malleobactin is a hydroxamate type siderophore characterized by a large

526   diversity of structures that was previously reported in Burkholderiales and for several

527   strains of *Burkholderia*. The conservation of the NRPS region among *Collimonas*

528   species and the ability of the related strains to mobilize iron indicate that the

529   production of malleobactin is a common trait among collimonads. It would be

530   interesting to extend such analysis to a broader number of collimonads and

531   neighboring genera. This study is also the first one demonstrating that malleobactin

532   production is involved in mineral weathering. The use of a non-acidifying C source

533   and of buffered conditions clearly proved the impact of malleobactin on the

534   dissolution of hematite. Although malleobactin impacted hematite, a highly

535   weatherable iron oxide, its impact on primary minerals present in soils such as

536   phyllosilicate remains to be tested.

537

538   **EXPERIMENTAL PROCEDURES**

539

540   **Bacterial strains and growth media**

32

541   Bacterial strains, plasmids and primers are listed in Table 1. The model *Collimonas*

542   *pratensis* strain PMB3(1) studied here was isolated from oak (*Quercus petraea*)–

543   *Scleroderma citrinum* ectomycorrhizae sampled in the organo-mineral soil horizon

544   from the long term experimental forest site of Breuil-Chenue (Morvan region, France;

545   for sampling details see (Calvaruso *et al.*, 2007). This model strain was chosen for its

546   high mineral weathering effectiveness (Uroz *et al.*, 2009b, 2007). All strains were grown

547   at 25°C, except the strains of *Escherichia coli*, which were grown at 37°C. The media

548   used were Luria-Bertani (LB) and AB medium supplemented with mannitol (2 g/l final

549   concentration) as carbon source (ABm; (Chilton *et al.*, 1974). Gentamycin was added

550   to the media at 20 µg/ml (final concentration) when required.

551

552   *Preparation of bacterial inocula*

553   For each growth or functional assay presented below, the wild type (WT) strain

554   PMB3(1) and the mutant strain affected in its ability to mobilize iron (∆NRPS) were

555   recovered from glycerol stock (-80 °C) and grown on ABm solid medium, amended

556   with gentamycin for the ∆NRPS mutant. After a 2-day incubation at 25 °C, one colony

557   of each strain was inoculated in liquid ABm medium (10 ml) and incubated for 2 days

        33

558   at 25 °C under 200 rpm agitation. The cultures were then centrifuged at 8,000 g for

559   15 min at 10 °C and the pellet was washed twice with 5 ml of sterile Milli-Q water to

560   eliminate all culture medium and antibiotic traces. The pellet was recovered in 5 ml of

561   sterile Milli-Q water. To control the quantity of cells inoculated, the optical density

562   (OD) was measured at 590 nm and cell suspensions were adjusted at 0.90 · 0.03.

563

564   **Siderophore activity**

565   To determine the siderophore activity, the Chrome Azurol S (CAS) assay was

566   performed according to (Schwyn and Neilands, 1987) using the solid and liquid

567   versions. Both assays are based on the competition for iron between the CAS-Fe(III)

568   complex and siderophores. In absence of chelating agent, iron is associated to the

569   Chrome Azurol S and forms a blue complex. When a siderophore with a higher

570   affinity for iron is present, iron is released from the CAS-Fe(III) complex and the

571   colour of the CAS solution turns to yellow.

572   For the solid CAS assay, a volume of 5 µl of the bacterial inoculum of the WT or

573   the mutant strains were inoculated on CAS agar plates and incubated at 25 °C for 3

34

574 days. The presence of yellow halo was scored and its diameter as well as that of the

575 colony diameter was measured, to determine siderophore production and the growth.

576   For the liquid CAS assay, a volume of 100 µl of the bacterial inoculum of the WT

577 or the mutant strains were inoculated in liquid ABm medium devoid of iron (10 ml)

578 and amended with gentamycin for the mutant strain. After 2-days incubation at 25 °C,

579 the cultures were centrifugated at 8,000 g for 15 min at 10 °C to recover the culture

580 supernatant. The supernatant was then filtered at 0.22 µm (GHP Acrodisc 25 mm

581 syringe filter; PALL) and stored at -20 °C for further analyses. To evaluate the

582 presence of siderophore activity, 100 µl of the filtered supernatant was added to

583 100 µl of liquid CAS medium devoid of carbon source in a 96-well microplate. The

584 supernatant of non-inoculated medium was used as a negative control. After 1 hour

585 of incubation at room temperature in the dark, the absorbance was measured at OD

586 = 655 nm with a microplate reader (Bio-Rad, model iMark). The decrease of

587 absorbance corresponds to a change from blue color of the CAS complex to a yellow

588 color when a siderophore is present.

589

590 **ΔNRPS mutant construction**

35

591   Total DNA was extracted from the WT strain using the protocol of (Pospiech and

592   Neumann, 1995). To construct a targeted mutant of the gene *mbaA*, a portion of this

593   gene was amplified using the primers For-NRPS and Rev-NRPS (Table 1). These

594   primers amplified a region of ca. 2 kb of the *mbaA* gene characterized by the

595   presence of a natural *Sma*I site in its center. The purified PCR product was ligated

596   into the pGEM-T Easy plasmid (Promega) resulting in the plasmid pGEM-NRPS

597   (Table 1). The plasmid pGEM-NRPS was then digested with *Sma*I to introduce the

598   SmaI-digested gentamycin (Gm) resistance cassette from plasmid pUC1318,

599   resulting in the plasmid pGEM-NRPS::Gm. A fragment containing the *mbaA*::Gm

600   cassette was then obtained by *Eco*RI restriction and cloned in the plasmid pK19mob,

601   resulting in the pK19mob-NRPS::Gm plasmid. This construction was transferred to *E.*

602   *coli* S17.1λpir and then to strain PMB3(1) by bi-parental conjugation. ⊿NRPS mutants

603   were recovered after 5 days of incubation at 25 °C on gentamycin-containing ABm

604   plates and organized in 96-microtiter plates. The transconjugants obtained were

605   verified by PCR with For-NRPS and Rev-NRPS primers, to differentiate the single

606   and double crossing-over events. Several single and double crossing-over mutants

36

607   were conserved. The experiments described in our study were done using a double

608   crossing-over mutant named ΔNRPS.

609

610   **Minimal concentration of iron inhibiting siderophore production**

611   To evaluate the concentration of iron inhibiting the siderophore production, the WT

612   strain and its ΔNRPS mutant were incubated with different concentrations of iron.

613   Briefly, in a 96-wells microplate, 10 µl of calibrated suspension (described above) of

614   WT and ΔNRPS were inoculated in 180 µl of ABm medium devoid of iron and 10 µl of

615   a $FeCl_3$ solution. Different $FeCl_3$ solutions were prepared in Milli-Q water to obtain

616   final concentrations of iron ranging from 0 to 5 mg/l (0; 0.1; 0.2; 0.3; 0.5; 0.6; 0.7; 0.8;

617   0.9; 1.0; 1.5; 2.0; 2.5; 5.0 mg/l). A non-inoculated condition was performed as control.

618   After 3 days of incubation, the 96-wells microplate was centrifuged (1,100 g; 15 min)

619   and 100 µl of supernatant were transferred to a new 96-wells microplate and mixed

620   with 100 µl of CAS liquid solution (as described above). This solution was incubated

621   1 h in the dark before measuring the absorbance at 655 nm.

622

37

623   **Impact of iron concentration and iron competition on the growth of strain PMB3(1)**

624   **and its ΔNRPS mutant**

625   To determine how iron concentration affects the growth of the WT strain and its

626   ΔNRPS mutant, cultures were done in ABm medium devoid of iron and

627   supplemented or not with $FeCl_3$ (final concentration of iron: 0; 0.1; 0.2; 0.3; 0.5; 1.0

628   mg/l). A volume of 10 µl each of bacterial inoculum (*i.e.*, WT and mutant) was added

629   to 190 µl each of ABm medium described above. In addition, a competition assay

630   was done to determine how iron availability may affect the growth of strain PMB3(1)

631   and its ΔNRPS mutant. To do it, a strong chelator agent (*i.e.*, 10 mM of EDTA) was

632   added or not to the culture medium supplemented with 1 mg/l of iron. All samples

633   were tested in triplicate. The 96-well microplate was incubated at 25 °C on orbital

634   shaking during 3 days in a microplate reader (Tecan infinite M200 pro). The

635   absorbance was measured at OD = 600 nm every 3 hours. To evaluate the growth of

636   the strains, the slope of the exponential phase (*i.e.* growth rate) was calculated for

637   each condition and replicate.

638

639   **Chemical characterization of the siderophore**

38

Siderophore production and purification

641   To enable the chemical characterization of the siderophore(s) produced by strain

642   PMB3(1), cultures of 50 ml were done in iron-depleted ABm culture medium. The WT

643   strain and its ΔNRPS mutant were incubated 3 days at 25 °C under shaking (200

644   rpm). A non-inoculated condition was used as negative control. After this incubation

645   period, the cultures were centrifugated at 8,000 g for 15 min and the supernatant was

646   filtered at 0.22 µm and stored at -20 °C for further analyses. The siderophore was

647   concentrated and purified using a Sep pack Vac 6cc tC18 Cartridges (Waters). The

648   column was first washed with 10 ml of 100 % methanol, then it was equilibrated with

649   10 ml of 0.01% trifluoroacetic acid (TFA) to acidify the column. Next, 25 ml of

650   supernatant was applied on the column. The siderophore was eluted with 5 ml of 10

651   % methanol solution (*i.e.* methanol / MQ water). The eluent was collected into 2 ml

652   eppendorf tubes and evaporated in a speedvacuum system for 5h. The dried samples

653   were dissolved in 100 µl of MQ water. The siderophore activity was tested using the

654   liquid CAS assay on the culture filtrate (0.22 µm) as well as on the Sep pack purified

655   solution.

656

39

657   *Ferrisiderophore (FS) preparation*

658   A siderophore-containing extract was analyzed in the presence and in the absence

659   of an iron(III) acetate solution. Iron(III) solution was prepared with a 4-fold excess of

660   acetate relative to iron (25 mM) / acetate (100 mM). Iron(III) chloride ($FeCl_3$) was

661   initially dissolved in glacial acetic acid before dilution with water to desired volume

662   (pH=1.90) and finally double filtrated.

663   The formation of ferrisiderophore was carried out under non-inert atmosphere as

664   follows. Extract was first diluted 10 times (pH = 4.01) and pH was adjusted at pH 2.79

665   with 0.1 M HCl. Then, iron(III) acetate solution (25 mM, 100 µl) was added to 400 µl

666   of diluted extract to get the final reaction mixture (500 µl), at pH 2.46.

667   The control test was simply carried out by mixing 400 µl of 10-times diluted extract

668   with 100 µl of pure water, pH being adjusted under pH 3 with 0.1 M HCl.

669

670   *High Performance Liquid Chromatography (HPLC) analyses and fractionation*

671   The analysis was done on Sep pack treated supernatants coming from inoculated

672   (WT and ΔNRPS strains) or non-inoculated (control medium) media. Fifty microliters

673   of each supernatant were injected onto a Gemini C18 column (150 x 3.0 mm internal

40

674 diameter, 5 µm particle size, Phenomenex) equilibrated with Milli-Q water with 0.01%

675 formic acid at 25 °C and connected to a Shimadzu Prominence HPLC system. A

676 gradient elution of Milli-Q water with 0.01% formic acid (buffer A) and acetonitrile with

677 0.01% formic acid (buffer B) for 40 min was used at a flow-rate of 1 ml/min and

678 detection was monitored with diode array UV–vis detector at 210 nm. The potential

679 peaks of interest were identified by comparing HPLC spectra and retention times

680 from the WT and ∆NRPS strains and the control medium samples.

681   To determine the siderophore activity of the peaks of interest, a fraction collect

682 was performed in 96-wells microplate using fraction collector system (FRC-10A

683 fraction collector system, Shimadzu). For each sample, fractions of 200 µl were

684 recovered each 12 s using the same HPLC conditions described above. Fractions

685 were then evaporated in a speedvacum system. Samples were suspended in 25 µl of

686 CAS liquid assay diluted with 25 µl of Milli-Q water and transferred in a new

687 microplate for absorbance measure at 655 nm.

688   To perform further mass spectrum analyses, the major peaks identified were

689 collected and the fractions combined to concentrate the metabolite of interest.

690

41

691 *Liquid chromatography coupled to high-resolution electrospray mass spectrometry*

692 *(LC-ESI-HRMS)*

693 Qualitative and semi-quantitative analysis of siderophores (S) and

694 ferrisiderophores (FS) was realized using a LC-ESI-HRMS method previously

695 described by (Paris *et al.*, 2021). The UHPLC-MS system (ThermoFisher Scientific,

696 San Jose, CA, USA) consists in a quaternary UltiMate 3000™ solvent delivery pump

697 connected to a photodiode array detector (PDA) and a LTQ-Orbitrap™ hybrid mass

698 spectrometer. Ten microliters of pre-purified HPLC extract were separated on a C18

699 Alltima reverse phase column (150 x 2.1 mm, 5 µm - Grace/Alltech, Darmstadt,

700 Germany) equipped with a C18 Alltima pre-column (7.5 x 2.1 mm, 5 µm) at 25 °C.

701 The flow rate was set at 0.2 ml/min and mobile phases consisted in water modified

702 with nonafluoropentanoic acid (NFPA) (5 mM) for A and pure methanol for B.

703 Compounds of interest were separated thanks to a program of 36 min, using a linear

704 gradient from 5 % to 98 % of B for 25 min.

705 Mass analysis was carried out in ESI positive ion mode (ESI$^+$) and mass

706 spectrometry conditions were as follows: spray voltage was set at 4.5 kV; source

707 gases were set (in arbitrary units/min) for sheath gas, auxiliary gas and sweep gas at

708 40, 5 and 5, respectively; capillary temperature was set at 300°C; capillary voltage at

709 46 V; tube lens, split lens and front lens voltages at 112 V, - 70 V and - 6.25 V,

710 respectively.

711   Full scan MS spectra were performed at high resolution (R=60000 at *m/z* 400) on

712 the Orbitrap™ analyzer from 120 to 2000 m/z to obtain exact masses of siderophores

713 and ferrisiderophores on the HPLC purified peaks as well as on the total extract. MS^2

714 full scan spectra were additionally realized for structural elucidation thanks to LTQ™

715 analyzer (Linear Trap Quadrupole). Raw data were processed using the

716 XCALIBUR™ software program (version 2.1, http://www.thermoscientific. com).

717

718 **Hematite weathering assay**

719 *Mineral description*

720   Hematite used as mineral in our study comes from a batch isolated in Brazil.

721 Hematite is an iron-oxide mineral of the oxides and hydroxides group. Iron oxides

722 (*i.e.*, hematite, goethite) occur, at least in small amounts, in nearly all soils (Dixon *et*

723 *al.*, 1989). Hematite is a widespread mineral occurring in a great variety of soils as a

724 result of the weathering of Fe-containing minerals (Dixon *et al.*, 1989). Igneous rocks

    43

725 (*i.e.*, granite, trachyte, and rhyolite) can contain primary accessory grains of hematite.

726 This mineral was chosen for its high weatherability, its high content of iron and the

727 presence of hematite as secondary minerals in the experimental site where was

728 isolated the strain PMB3(1) (Calvaruso *et al.*, 2009). In this study, pure hematite

729 crystals were used (Fe_2O_3 pure at 99.3%) and containing in small amounts: 0.40%

730 TiO_2, 0.18% SiO_2, 0.16% Al_2O_3, 0.10% MgO, 0.037% MnO.

731

732 *Mineral weathering assay*

733   The assay was performed in glass tubes containing 2 g/l of sterile hematite and

734 10 ml of ABm medium devoid of iron. Before introduction of hematite, the glass tubes

735 were rinsed with hydrochloric acid (HCl, 3.6 %) and MilliQ water. The tubes

736 containing the culture medium and hematite were then sterilised by autoclaving at

737 121°C. For the weathering assay, 100 µl of each bacterial inoculum (described

738 above; WT and ΔNRPS mutant strains) were used to inoculate the sterile glass

739 tubes. Non-inoculated media with and without hematite were used as controls. After

740 seven days of incubation at 25 °C (200 rpm), 200 µl of supernatant were sampled

741 and the OD at 595 nm was measured to determine bacterial growth. Then, 1 ml of

44

742 supernatant was centrifuged at 11,000 g for 15 min to remove bacterial cells.

743 Quantification of the iron released from hematite in the culture supernatant as well as

744 the pH were determined by adding 180 µl of supernatant of each culture with 20 µl of

745 ferrospectral or bromocresol green (1 g/l), respectively (Uroz *et al.*, 2007). The

746 absorbance of these suspensions was measured at 595 nm. The same culture

747 supernatant was also used to measure siderophore production with the liquid CAS

748 assay. The average values for three independent replicates for growth, iron

749 quantification, pH and siderophore production were used to determine the weathering

750 potential of the ΔNRPS mutant and the wild-type strains.

751

752 **Bioinformatic analyses**

753 *Genome properties gene localization and homology*

754   Genome analysis was performed using NCBI and MaGe (Vallenet, 2006). Homology

755 search analysis and alignment were performed with BLAST (BlastN and BlastP) (Altschul *et*

756 *al.*, 1990).

757 *In silico* search and identification of NRPS genes.

758   An *in silico* survey was done on the genome sequence of strain PMB3(1) to

759 identify the conserved domains of NRPS involved in siderophore production using

760   antiSMASH (Blin *et al.*, 2019). A combination of 3 tools based on the Stachelhaus

761   code of the adenylation domain was used to predict the amino acids recruited and the

762   potential structure of the siderophore produced (NRPS predictor (Rausch, 2005);

763   PKS/NRPS Analysis (Ansari *et al.*, 2004); NP.searcher (Li *et al.*, 2009)).

764

765   *Phylogenetic analysis on Collimonas genus*

766   As the MbaA protein is involved in the synthesis of the most variable part (*i.e.* N-

767   terminal part) of the siderophore, a phylogenetic analysis was performed on it (Franke

768   *et al.*, 2015). A total of 52 NRPS protein sequences were used to build a phylogenetic

769   PhyML tree with Seaview (version 4.5.4; (Gouy *et al.*, 2010)). These sequences were

770   chosen for their similarity to OrbI or MbaA proteins that are NRPS responsible of

771   ornibactin or malleobactin biosynthesis, respectively. Our analysis includes

772   sequences from *Collimonas* genera available online and other *Collimonas* related

773   genera (*i.e. Paraburkholderia, Burkholderia, Caballeronia, Herbaspirillum*) for which

774   the siderophores produced were predicted or experimentally demonstrated (Table

775   S1). As *Collimonas* is strongly related to *Herbaspirillum*, the NRPS protein involved in

46

776 serobactin production by *Herbaspirillum seropedicae* was included (Rosconi *et al.*,

777 2013).

778

779 **Statistical analyses.**

780   Statistical analyses were performed in R software. Data shown were means of at

781 least triplicates. Differences between sample's means were analysed by ANOVA and

782 TukeyHSD tests.

783

784 **Nucleotide sequence accession number.**

785 The whole-genome is available on NCBI under the accession no. WXXL01000000.

786

47

793

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1032

1034 Table 1: List of bacterial strains, constructions and primers used in this study

Strains, plasmids or primers	Characteristics	Reference

Strains		
Collimonas pratensis		
Strain PMB3(1)	Wild type strain	U
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ΔNRPS (PMB3(1)(NRPS::Gm))   Wild type strain, with Gm^R cassette inserted in double crossing over   This study

Escherichia coli

		y
DH5α	sup E44, ΔlacU169, (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1	L a b c o l l

68

| S17.1λpir | TpR Sm^R recA, thi, pro, hsdRM+, RP4: 2-Tc:Mu: Km Tn7 λpir | Lab |

collection

70

Plasmids		
pUC1318	Amp^R, Gm^R	Lab collee

71

pGEM-T Easy   Amp^R

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P

72

pGEM-NRPS   pGEM-T Easy carrying a NRPS fragment   This transgenomer

73

pGEM-NRPS::Gm   pGEM-NRPS with insertion in SmaI of the Gm^R cassette   This study

pK19mob   Km^R

study Lab

75

collection

76

pK19mob-NRPS::Gm   pK19mob containing a EcoRI fragment obtained from pGEM-NRPS::Gm   This study

Primers		Tm (°C)	
For-NRPS	5'-GCCCATCCAATACGCCGATTAC-3'	54	This study
Rev-NRPS	5'-GGTCGGACCGTAGTGATTGATG-3'		This study

1035

1036

1037 **Table 2: Description of the NRPS region involved in siderophore production by *Collimonas pratensis* strain PMB3(1)**

1038 Each gene of the region of interest is presented with its NCBI accession number. Both MaGe and NCBI annotations are displayed.

1039 The putative function of each gene is indicated by a color code (regulation (purple); siderophore biogenesis (green) including NRPS

1040 (red); transport and utilization of the siderophore (blue); unknown function (grey)).

1041

Putative function	Accession number	Length (pb)	Gene	MaGe Annotation	NCBI analyses Annotation	% identity *	
●	NKI69287.1	651	*mbaF*	DNA-directed RNA polymerase specialized sigma subunit,	DNA-directed RNA polymerase specialized sigma subunit, sigma24-like protein	77%	*Collimonas fungivorans* Ter331

79

	Accession	Length	Gene	Annotation	Function	Identity	Organism
🟢	NKI69288.1	309	*mbaG*	sigma24-like protein putative MbtH-like domain	putative MbtH-like domain	81%	*Collimonas sp.* MPS11E8
🟢	NKI69289.1	1023	*mbaH*	putative taurine catabolism dioxygenase TauD/F	putative taurine catabolism dioxygenase TauD/F	92%	*Collimonas sp.* MPS11E8
🔵	NKI69290.1	876	*mbaI*	putative siderophore transport system ATP-binding protein YusV	ABC cobalamin/Fe3+-siderophores transporter, ATPase subunit	81%	*Collimonas fungivorans* Ter331

	NKI69291.1	2094	*mbaJ*	putative iron-hydroxamate transporter permease subunit	Fe(3+)-hydroxamate ABC transporter permease FhuB	79%	*Burkholderia sp.* K24
	NKI69292.1	813	*mbaK*	Ferric reductase	siderophore-iron reductase FhuF	71%	*Collimonas sp.* OK412
	NKI69293.1	1014	*mbaL*	putative iron compound ABC transporter	putative iron compound ABC transporter	86%	*Collimonas sp.* MPS11E8
	NKI69294.1	1746	*mbaN*	ABC-type siderophore export	cyclic peptide export ABC transporter	93%	*Collimonas sp.* OK412

				system, fused ATPase and permease components			
🔴	NKI69295.1	9666	*mbaA*	Non-ribosomal peptide synthetase module	non-ribosomal peptide synthase domain TIGR01720/amino acid adenylation domain-containing protein	79%	*Collimonas sp.* OK412
🔴	NKI69296.1	5076	*mbaB*	putative NRPS amino acid adenylation domain	putative NRPS amino acid adenylation domain	84%	*Collimonas sp.* MPS11E8

🟢	NKI69297.1	1374	*mbaC*	L-ornithine 5-monooxygenase	putative L-ornithin-5-monooxygenase	96%	*Collimonas sp.* MPS11E8
🔵	NKI69298.1	2220	*mbaD*	putative TonB-dependent siderophore receptor	putative TonB-dependent siderophore receptor, partial	96%	*Collimonas sp.* MPS11E8
🟢	NKI69299.1	822	*mbaE*	N(5)-hydroxyornithine formyltransferase, PvdF-type	N(5)-hydroxyornithine transformylase, PvdF-type	89%	*Collimonas sp.* OK412
⚪	NKI69300.1	2358	*iorB*	Isoquinoline 1-oxidoreductase	xanthine dehydrogenase family protein molybdopterin-binding	95%	*Collimonas sp.* PA-H2

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⬤	NKI69301.1	465	*iorA*	beta subunit Isoquino line 1-oxidored uctase subunit alpha	subunit (2Fe-2S)-binding protein	99%	*Collimonas sp.* PA-H2

1042   **Figure 1: Impact of increasing concentrations of iron on chelating and growth abilities**

1043   **Siderophore activity**. (**A**) The *Collimonas pratensis* strain PMB3(1) WT (top) and its

1044   ΔNRPS mutant (bottom) were incubated for 3 days at 25 °C on CAS solid medium.

1045   The presence of a siderophore activity is determined by the production of yellow halo

1046   around the colony. (**B**) To determine the concentration of iron inhibiting siderophore

1047   production, liquid cultures of WT (red) and ΔNRPS (blue) were performed with

1048   different concentrations of iron (*i.e.* 0.5; 0.6; 0.7; 0.8; 0.9; 1.0 mg/l). Pure water

1049   (black) was used as a negative control. After 3 days, the absorbance at 655 nm was

1050   measured. The absorbance measured at 655 nm detects blue colour, yellow colour

1051   (corresponding to siderophore activity) will result of a decrease of the absorbance

1052   measured.

1053   **Growth assay. (C)** The growth of the WT strain (red) and ΔNRPS mutant (blue) was

1054   monitored in presence (full circle) or absence (empty circle) of EDTA (10 mM) and

1055   with iron (1 mg/l) in AB medium (2 g/l mannitol). The growth was performed under

1056   orbital shaking at 25°C for 90 h. The absorbance was measured at 600 nm every 3

1057   hours. Each dot is the mean of independent triplicates.

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1059   **Figure 2: Organization of the genomic region involved in the biosynthesis of**

1060   **siderophore in *Collimonas pratensis* strain PMB3(1)**

1061   Each wide arrow represents one gene and the size is according to the gene length.

1062   Gene annotation is shown under each gene and the complete description is provided

1063   in Table 2. Fine grey arrows represent promoters. Thin black arrows represent *mbaF*-

1064   dependent promoters with specific -35 and -10 regions (TAAA/n(17)/CGTC)

1065   (Thomas, 2007). The purple gene encodes a putative sigma regulation factor. Green

1066   genes are responsible for the siderophore biosynthesis and the two NRPS genes are

1067   specifically represented in red. The blue genes are involved in the siderophore

1068   utilisation and transport. The two white genes have unknown function in siderophore

1069   biosynthesis or utilisation, and represent potential accessory genes involved in

1070   siderophore production. The little red arrow in the *mbaA* gene indicates the *Sma*I

1071   restriction site used for the insertion of the gentamycin resistance cassette to create

1072   the ΔNRPS mutant.

1073

1074   **Figure 3: HPLC analysis and determination of the siderophore activity in the fractions**

1075   **collected**

1076 **(A) HPLC analysis and UV detection.** Products analysis of the pre-purified

1077 supernatant of the WT strain (red), ΔNRPS mutant (blue) and non-inoculated medium

1078 (black) were monitored at 210 nm after HPLC separation using a Milli-Q water /

1079 acetonitrile gradient for 40 min at 1 ml/min. A major peak at 1.2 min retention time

1080 and two smaller peaks at 2.2 and 3.8 min retention time were detected in the

1081 supernatant of the WT strain. These three peaks are highlighted by red arrows. **(B)**

1082 **Overlay of the HPLC and CAS analyses.** To determine the presence of a siderophore

1083 activity, fractions were collected during the HPLC run and tested using the liquid CAS

1084 assay (activity level on the right axis). The HPLC spectra of the WT strain (red line;

1085 left axis), the mutant ΔNRPS (blue line; left axis) and the medium (black line; left

1086 axis) were overlaid with the siderophore activity measured by the CAS test on the

1087 fractions for the WT strain (red dotted line; right axis), the mutant ΔNRPS (blue

1088 dotted line; right axis) and the medium (black dotted line; right axis).

1089

1090 **Figure 4: Chemical characterization of siderophore and ferrisiderophore**

1091 ESI$^+$-HRMS analysis of the fraction collected at 1.2 min (after HPLC purification) from

1092 the crude extract of the WT strain was performed. **(A) Without prior incubation with**

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1093   **iron**, the spectrum shows a major signal at m/z = 623.3069 (mono-protonated ion

1094   [M+H]$^+$) which indicates a siderophore exact mass of M_S = 622.3069. **(B) After prior**

1095   **incubation with iron**, the analysis reveals a major signal at m/z = 676.2191 (mono-

1096   protonated ion [M+H]$^+$) which indicates a ferrosiderophore exact mass of M_{FS} =

1097   675.2191. The isotopic pattern detailed in the dashed inset shows the obvious

1098   presence of an iron atom in the structure, thanks to the clear contributions of ^{54}Fe

1099   and ^{56}Fe (*i.e.,* m/z = 674.2246 with 5.7 % ratio and m/z = 676.2191 respectively). **(C)**

1100   **MS/MS spectrum.** The previously highlighted siderophore compound (MS =

1101   622.3069) was further analyzed by ESI-MS/MS to validate its structure. M/z values

1102   framed in red correspond to theoretical fragments consecutive to breaking of the

1103   peptide bonds of the expected siderophore (m/z values framed in orange correspond

1104   to the dehydrated forms). **(D) Theoretical structure and fragmentation of malleobactin**

1105   **X.** Each letter represents the amino acid letter code forming the malleobactin X. The

1106   cleavage of the different peptide bonds (blue dashed lines) results in the formation of

1107   different ions containing the N-terminus of the peptide (b) and to ions containing the

1108   C-terminus of the peptide (y). Depending on the peptide bond cleaved, different

1109   fragments can be formed and their mass is presented as b(1,2,3,4) and y(1,2,3,4).

1110 The fragments identified experimentally (see panel C), which mass corresponds to

1111 the theoretical masses, are presented in red, the other are presented in blue. (**E**)

1112 **Structure and composition of the malleobactin X**. The molecule represented

1113 corresponds to the reference malleobactin X predicted by Vargas-Straube et al.

1114 2016. The name of each amino acid is written in full under the molecule as well as its

1115 one letter abbreviation.

1116

1117 **Figure 5: Phylogenetic tree based on the NRPS MbaA protein**

1118 The conservation of the protein MbaA among *Collimonas* and other related genera

1119 (*Paraburkholderia, Burkholderia, Caballeronia, Herbaspirillum*) is presented on this

1120 phylogenetic tree. A set of 52 NRPS protein sequences was analyzed and is detailed

1121 in the Table S1. Our model strain *Collimonas pratensis* PMB3(1) is in bold. The

1122 distribution and relatedness to known NRPS types is indicated by a color code:

1123 ornibactin (*i.e.* OrbI-like protein: green circles), malleobactin (*i.e.* MbaA-like protein;

1124 red circles) or serobactin (purple circles). The MbaA homologues for which

1125 production of such siderophore was experimentally demonstrated or only predicted

1126 are presented with full circles and empty circles, respectively. The production of

1127 ornibactin have been experimentally demonstrated for 3 strains included in the

1128 analysis (full green circle; (Meyer *et al.*, 1995; Darling *et al.*, 1998; Deng *et al.*, 2017).

1129 Malleobactin was identified in three strains included in the analysis (full red circle;

1130 (Alice *et al.*, 2006; Franke *et al.*, 2013; Vargas-Straube *et al.*, 2016). The other proteins

1131 considered in this analysis had only predicted function (empty circle; (Holden *et al.*,

1132 2009; Schwager *et al.*, 2013; Song *et al.*, 2015; Esmaeel *et al.*, 2016, 2018). The

1133 siderophores predicted as ornibactin and discussed in this study are represented by

1134 green dotted circles. Letters inside circles correspond to the cited reference (in the

1135 figure) in which the prediction or demonstration was/were done.

1136

1137 **Figure 6: Determination of the weathering ability of the WT *Collimonas pratensis***

1138 **strain PMB3(1) and its ΔNRPS mutant**

1139 The hematite weathering potential of strain PMB3(1) and its ΔNRPS mutant was

1140 evaluated by the measure of iron released from hematite in ABm medium devoid of

1141 iron, after 7 days of incubation at 25°C under agitation (200 rpm). Non-inoculated

1142 media with and without hematite (termed Hematite and Medium respectively) were

1143 used as control. **(A) Quantity of iron released in solution**. The iron released from

1144   hematite was measured by ferrospectral determination (optical density measured at

1145   595nm). A standard calibration of iron ($FeCl_3$) was used to calculate the iron

1146   concentration of the different samples tested (WT, ΔNRPS, hematite alone or

1147   Medium alone). Samples with the same letter indicate no significant difference

1148   ($P<0.05$). **(B) pH of the supernatant**. The acidification of the medium was measured

1149   by absorbance at 595nm using the bromocresol green method. **(C) Siderophore**

1150   **activity**. The siderophore activity of the different samples was determined using the

1151   CAS method. The yellow colour indicates a siderophore activity. For each measure,

1152   data are presented under the corresponding pictures and are the mean of

1153   independent triplicates, the standard deviation is indicated in italic.