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► **To cite this version:**

Sébastien R. David, Sarah Fritsch, Anne Forster, Dris Ihiawakrim, Valérie Geoffroy. Flocking asbestos waste, an iron and magnesium source for *Pseudomonas*. *Science of the Total Environment*, 2020, 709, pp.135936. 10.1016/j.scitotenv.2019.135936 . hal-02981380

HAL Id: hal-02981380

<https://hal.science/hal-02981380>

Submitted on 7 Mar 2022

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1 **Flocking asbestos waste, an iron and magnesium** 2 **source for Pseudomonas**

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17 **Abstract**

18 Iron and magnesium are essential nutrients for most microorganisms. In the environment,
19 the availability of iron is low relative to that of magnesium. Microorganisms have developed
20 various iron acquisition systems, which have been well studied, whereas few studies have
21 examined magnesium acquisition. The production of siderophores is one of the efficient
22 strategies widely used to sustain iron nutritional requirements. Many studies have shown that
23 minerals, such as clays, iron oxides, and silicates, can serve as nutrient sources for bacteria.
24 Asbestos, a natural fibrous silicate present in soil contains iron and/or magnesium, depending on
25 the species of asbestos. Our aim was to study the acquisition of iron and magnesium from
26 flocking asbestos waste by *Pseudomonas aeruginosa* and the involvement of the siderophores,
27 pyoverdine and pyochelin. Flocking asbestos waste promoted growth under iron- and
28 magnesium-limited conditions, together with a decrease in pyoverdine production, correlating
29 with the dissolution of iron from the waste. In long-term experiments, flocking asbestos waste
30 provided these two essential elements for bacterial growth and resulted in a decrease of iron in
31 asbestos fibers. Among the enzymes required for pyochelin and pyoverdine synthesis, PchA and
32 PvdJ were tagged with the fluorescent protein mCherry to analyze the expression patterns of
33 proteins involved in siderophore production. Both enzymes were produced in the presence of
34 flocking asbestos waste, suggesting a role of the pyoverdine and pyochelin pathway in asbestos
35 dissolution. We investigated the involvement of each siderophore in iron and magnesium
36 removal using mutants in one or both siderophore pathways. We observed a significant increase
37 in iron extraction in the presence of siderophores and the absence of one of the two siderophores
38 could be compensated by the other. Flocking asbestos waste represents an iron and magnesium
39 source for *P. aeruginosa*, with iron removal linked to a siderophore-driven mechanism.

40 **Keywords**

41 **asbestos-*Pseudomonas*-siderophores-alteration-iron-magnesium**

42

43 **1. Introduction**

44 Asbestos has been extensively used in many commercial products due to its unique
45 properties. The two major waste products are cement asbestos waste and flocking asbestos waste
46 (FAW) from insulation in buildings. Exposure to asbestos fibers can cause serious health
47 problems, such as asbestosis and cancer, related to their physical and chemical factors. Asbestos
48 toxicity correlates with the presence of iron as a catalyst of the Fenton reaction, leading to the
49 generation of free radicals and reactive oxygen species (ROS) (Toyokuni, 2009). As a
50 consequence of the associated health problems, asbestos was banned in many developed
51 countries. However, following 30 years of intensive use, we are confronted with the generation
52 of a vast amount of asbestos-containing waste. Today, most asbestos waste is typically disposed
53 in controlled landfill sites, which does not remove the potential health risk of waste related to
54 fiber inhalation. Asbestos consists of fibrous silicate minerals present in soil. There are two
55 major families of asbestos i) serpentine, of which the only member is chrysotile, and ii)
56 amphibole, among which the major members used industrially are crocidolite and amosite. The
57 structure of chrysotile consists of a double layer comprised of a silicate sheet and brucite
58 ($\text{Mg}(\text{OH})_2$), in which the Si and Mg can be replaced by Fe^{2+} and Fe^{3+} , respectively (Virta, 2002).
59 The surface of asbestos can also be contaminated by Fe, depending on where the asbestos was
60 mined. Given the presence of Fe and Mg in the structure of asbestos, this mineral can be a
61 nutrient source for microorganisms. Iron (Fe) and magnesium (Mg) are metals required for living
62 organisms. They are present at different concentrations in the cell because of their specific
63 physicochemical properties. Mg is found at high concentrations and is involved in basic cell
64 metabolism, including the maintenance of macromolecules and bacterial walls and as cofactors
65 for various enzymatic reactions (Groisman et al., 2013). Transition metals, such as manganese,

66 cobalt, nickel, copper, zinc, and iron, are present at lower concentrations in cells and have key
67 functions in many biological processes, such as energy production, oxido-reduction reactions,
68 and the stabilization of protein structures (Heldal et al., 1985). Iron, the fourth most abundant
69 metal in the Earth's crust, exists in two redox states, ferric (Fe^{3+}) and ferrous (Fe^{2+}), and is
70 involved in enzymatic processes, electron transfer, oxygen metabolism, and DNA and RNA
71 synthesis (Aguado-Santacruz et al., 2012; Andrews et al., 2003). Mg is more soluble than Fe
72 (Schvartz et al., 2005). Its solubility depends on the presence of oxygen and the pH (Cornell and
73 Schwertmann, 2003). Fe is poorly bioavailable in aerobic circumneutral environments and tends
74 to be limited by Fe(III)(hydr)oxide solubility. Microorganisms overcome the poor bioavailability
75 of iron by secreting low molecular-weight organic ligands, called siderophores, with a high
76 affinity for Fe^{3+} (Neilands, 1981). Siderophore production is well known among aerobic
77 microorganisms (Neilands, 1981), including fluorescent *Pseudomonas*, for which a wide variety
78 of siderophores has been described. More than 98% of fluorescent *Pseudomonas* isolated from
79 soil produce detectable siderophores under conditions of iron starvation (Cocoza and Ercolani,
80 1997). The range of siderophore concentrations found in soil is wide, from tens of micromoles to
81 a few millimoles per liter (Hersman et al., 1995). *Pseudomonas aeruginosa* is a ubiquitous
82 fluorescent *Pseudomonas* species found in diverse environments, such as water and the
83 rhizosphere (Goldberg, 2000), that adapts its iron-uptake strategy depending on the
84 environmental or infectious context (Cornelis and Dingemans, 2013). The iron uptake pathways
85 of this bacterium, known to be an opportunistic pathogen, are well characterized. *P. aeruginosa*
86 produces two siderophores, pyochelin (PCH), a low-affinity siderophore and a more energy-
87 demanding high-affinity molecule, pyoverdine (PVD) (Dumas et al., 2013). Pyoverdine consists
88 of three distinct structural entities i) a dihydroxy quinoline chromophore, which confers the color

89 and fluorescence of the molecule, ii) a peptide chain comprised of 6 to 12 amino acids bound to
90 its carboxyl group, and iii) a small dicarboxylic acid (or its monoamide) amidically connected to
91 the NH₂-group (Budzikiewicz, 2004, 1997; Budzikiewicz et al., 2007; Demange et al., 1990;
92 Fuchs and Budzikiewicz, 2001). Pyoverdine binds ferric iron with a high affinity ($K_a = 10^{32}M^{-1}$)
93 in a 1:1 stoichiometric ratio via the catechol group of the chromophore and the two
94 hydroxyornithines of the peptide moiety (Albrecht-Gary et al., 1994). Pyochelin is a derivative of
95 salicylic acid, which chelates Fe³⁺ with an affinity of $10^{28.8}M^{-2}$ (Brandel et al., 2012) in a 2:1
96 (PCH:Fe³⁺) stoichiometry. A tetra-dentate chelator is provided by one molecule of PCH and a bi-
97 dentate chelator by the second PCH to complete the hexacoordinate octahedral geometry
98 necessary for Fe³⁺ chelation (Tseng et al., 2006). Aside from iron, it has been shown that PVD
99 and PCH form stable complexes with other metal cations (Braud et al., 2009; Schalk et al.,
100 2011).

101 Microorganisms overcome the iron limitation in soil using siderophores to chelate iron
102 from Fe-bearing minerals. Indeed, siderophores play an important role in the formation of
103 soluble iron complexes for biological iron acquisition (Kraemer, 2004). Microbes then recover
104 ferric iron-siderophore complexes via cell-surface receptors. There is an abundant literature
105 concerning the weathering of minerals by biological processes. Deferoxamine-B, a bacterial
106 siderophore produced by *Streptomyces pilosus*, has been reported to enhance iron solubilization
107 of hornblende (Kalinowski et al., 2000) and kaolinite (Rosenberg and Maurice, 2003). Over the
108 last decades, mineral weathering of iron oxides, iron-rich aluminum silicates, and manganese
109 oxides by siderophore-producing bacteria has been well documented (Duckworth et al., 2009;
110 Maurice et al., 2009). *Pseudomonas* species have shown their ability to mobilize iron from iron
111 oxides, such as hematite or goethite by *Pseudomonas mendocina* (Hersman et al., 2001) or clay,

112 such as smectite by *Pseudomonas aeruginosa* (Ferret et al., 2014). Bacterial weathering of
113 asbestos has also been recently reported. Several studies have investigated the biological
114 alteration of raw asbestos or asbestos cement waste. Indeed, Bhattacharya et al. (2015) isolated
115 well-adapted Gram-positive and Gram-negative bacteria from Indian asbestos mines that
116 decrease the iron content of asbestos rocks, without identifying the species. Wang et al. (2011)
117 and Wang and Cullimore (2010) showed that microorganism colonization induces porous
118 structures in asbestos-cement pipes. Another Gram-positive bacteria, *Bacillus mucilaginosus*,
119 promotes dissolution of serpentine powder by bacterial metabolites, most likely due to the
120 production of organic acids and ligands, as suggested by Yao et al. (2013). Further studies on
121 bacteria isolated from mines (Bhattacharya et al., 2015) showed the production of siderophores
122 that may be involved in the weathering process, but with no characterization of their nature
123 (Bhattacharya et al., 2016). Siderophore production has been shown in native asbestos, leading to
124 iron dissolution from the fibers. Moreover, Rajkumar et al. (2009) described the plant growth-
125 promoting mechanisms of rhizospheric bacteria in a serpentine soil, for which the secretion of
126 siderophores is one of the various strategies used to release elements essential for plant growth.
127 Direct evidence of iron removal by the siderophore deferoxamine from raw asbestos, chrysotile
128 fibers (Mohanty et al., 2018), and amphibole fibers, such as crocidolite and amosite (Chao and
129 Aust, 1994) has been shown. Importantly, the dissolution of iron by siderophores lowers the
130 toxicity of the fibers of the three major commonly used species of asbestos, chrysotile (Poser et
131 al., 2004), amosite (Gold J et al., 1997), and crocidolite (Gold J et al., 1997; Hardy and Aust,
132 1995; Poser et al., 2004).

133 To date, a few studies have focused on asbestos-cement waste, whereas none have
134 focused on FAW. This anthropogenic material, used as insulation in buildings, is composed of

135 chrysotile fibers ($\text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4$, 5 to 90%) and gypsum ($\text{CaSO}_4, 2\text{H}_2\text{O}$, 10 to 95%). The
136 asbestos fibers present in FAW are only weakly bound in the gypsum, resulting in high emission
137 of the fibers into the air. Here, we aimed to determine whether the aerobic *P. aeruginosa* can
138 acquire Fe and Mg from FAW for growth and, at the same time, enhance FAW dissolution. We
139 examined the role of pyoverdine and pyochelin in altering FAW. A better understanding of the
140 bacterial alteration of asbestos waste may allow the development of an efficient bioremediation
141 process, avoiding the continuing disposal of such waste in landfills.

142

143 **2. Materials and Methods**

144 **2.1 Asbestos waste preparation**

145 The flocking asbestos waste, chrysotile-gypsum, used in this work came from the
146 asbestos removal site of Jussieu University of Paris and was kindly provided by the
147 Mediterranean Company of Zeolites (SOMEZ). Asbestos samples (0.2 g) were sterilized by
148 autoclaving (121°C for 20 min) and incubated at 70°C for 14 days before experiments for
149 complete sterilization of the material. The heat removed all water, allowing precise weighing of
150 the samples. Before each experiment, asbestos samples were washed with 20 mL sterile
151 succinate or casamino acids (CAA) without magnesium to remove free Fe and Mg.

152 **2.2 Bacterial strains, plasmids and growth conditions**

153 The bacterial strains and plasmids used in this study are listed in Table 1. Stocks of
154 bacterial strains were stored in LB-20% glycerol at -80°C. *Escherichia coli* was routinely grown
155 in Luria Bertani medium (LB) at 37°C with shaking (220 rpm). *Pseudomonas* was routinely
156 grown overnight at 30°C in Luria Bertani medium (LB) with shaking (220 rpm). Succinate
157 (composition in g.L^{-1} : $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 6.0; KH_2PO_4 , 3.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2;

158 sodium succinate, 4.0; pH adjusted to 7.0 by addition of NaOH) or CAA (composition in g.L⁻¹:
159 low-iron-casamino acids, 5.0; K₂HPO₄·3H₂O, 1.46; MgSO₄·7H₂O, 0.25) media were used as iron-
160 restricted media and sterilized using 0.2 µm polyethersulfone membrane filter units (Stericup
161 Merck). For iron and magnesium-depleted cultures, cells were harvested from a LB pre-culture
162 (24 h incubation), washed once with sterile succinate or CAA media not supplemented with
163 magnesium, and used to inoculate fresh succinate or CAA media without magnesium. The
164 cultures were incubated for 24 h at 25°C with shaking (200 rpm). For bacterial suspension, cells
165 were washed once with sterile iron- and magnesium-restricted medium and cell density adjusted
166 to an OD₆₀₀ of 0.1, corresponding to 1-2.10⁸ CFU.ml⁻¹.

167 **2.3 Mutant construction**

168 All enzymes for DNA manipulation were purchased from Thermo Fisher Scientific and
169 used in accordance with the manufacturer's instructions. *E. coli* strain SM10 (Herrero et al.,
170 1990) was used as a host strain for the plasmid. DNA fragments from *P. aeruginosa* used for
171 cloning were amplified from the plasmid pLG45 *pvdJmcherry* using Phusion High-Fidelity DNA
172 polymerase (Thermo Fisher Scientific). The primers used are listed in Supplementary Table 1.
173 The procedure for the construction of the pLG45 *pvdJmcherry* plasmid involved amplification of
174 DNA fragments from *P. aeruginosa* and insertion of the *mcherry* gene flanked by upstream and
175 downstream regions of 700 bp, corresponding to the insertion site, into the pLG45 vector, as
176 previously described (Gasser et al., 2015).

177 The construction of the pEXG2 *pvdJmcherry* plasmid was obtained by amplification of
178 DNA fragments from pLG45 *pvdJmcherry* and their insertion into the pEXG2 vector (Rietsch et
179 al., 2005). Mutations in the chromosomal genome of *P. aeruginosa* were generated by

180 homologous recombination between the plasmid pEXG2 and the chromosome, with selection for
181 gentamicin resistance. Gene-replacement mutants were verified by PCR.

182 **2.4 Magnesium growth requirements for *Pseudomonas aeruginosa***

183 The *Pseudomonas aeruginosa* strain PAO1 was cultured overnight at 30°C in LB medium
184 with shaking (220 rpm). The culture was centrifuged (10 min at 9,871 g) and washed twice with
185 succinate medium without magnesium. Bacterial cell density was adjusted to an OD₆₀₀ of 0.01.
186 We dispensed 200 µl of the suspension into individual wells of a 96-well plate (Greiner, PS U-
187 bottomed microplate) with 0, 0.005, 0.05, 0.5, 1, 2, 5, and 20 mg.L⁻¹ MgSO₄7H₂O,
188 corresponding to 0, 0.0005, 0.005, 0.05, 0.1, 0.2, 0.5, and 2 mg.L⁻¹ Mg. Plates were incubated at
189 30°C, with shaking, in a Tecan microplate reader (Infinite M200, Tecan) for measurements of
190 the OD₆₀₀ at 30-min intervals, for 37.5 h. Assays were performed in triplicate.

191 **2.5 Expression of the pyoverdine and pyochelin pathway in the presence of FAW**

192 We analyzed the expression of the biosynthetic enzymes for siderophore production
193 using *Pseudomonas aeruginosa* PAO1 strains with the biosynthetic enzymes for pyochelin and
194 pyoverdine, PvdJ (this study) and PchA (Cunrath et al., 2015), tagged with the fluorescent
195 protein mCherry. Bacterial suspensions (20 mL) prepared in CAA medium without magnesium,
196 as described in paragraph 2.2, were incubated with chrysotile-gypsum samples. After 24 h of
197 incubation at 25°C with shaking (220 rpm), the number of bacteria was determined by serial
198 dilution, plating on LB-agar plates, and incubation at 30°C for 24 h. For the expression analysis,
199 the samples were centrifuged for 5 min at 67 g to separate the bacteria from the asbestos fibers.
200 Bacterial cells (100 µl) were dispensed into individual wells of a 96-well plate (Greiner, PS flat-
201 bottomed microplate). mCherry fluorescence (excitation/emission wavelengths: 570 nm/610 nm)

202 was measured in a Tecan microplate reader (Infinite M200, Tecan). Assays were performed
203 using three to five replicates.

204 **2.6 Asbestos dissolution by *Pseudomonas***

205 Microbial growth in the presence of flocking asbestos waste was assessed at 25°C with
206 shaking (220 rpm) for 24 h. *Pseudomonas aeruginosa* PAO1 suspensions (20 mL), prepared in
207 succinate medium without magnesium, as described in paragraph 2.2, were incubated with
208 chrysotile-gypsum samples pre-treated by eight 24-h cycles with shaking (220 rpm) in succinate
209 medium not supplemented with magnesium to remove free Fe and Mg. Controls without
210 asbestos, supplemented or not with magnesium, were included. The number of bacteria was
211 determined after 0, 4, 8, 12, and 24 h of incubation. Samples were serially diluted, plated on LB-
212 agar plates, and incubated at 30°C for 24 h. The kinetics of iron and magnesium release from
213 chrysotile-gypsum, with or without bacteria, were followed for 24 h by regular sampling at 0, 1,
214 2, 4, 6, 8, 12, and 24 h. Material was eliminated from supernatants by filtration (0.22 µm
215 porosity). At the end of the experiment, cultures were gently centrifuged for 5 min at 67 g to
216 separate the bacterial cells from asbestos fibers. Bacterial cells (10 mL) were centrifuged 10 min
217 at 9,871 g, the pellets mineralized, and the supernatants filtered (0.22 µm porosity). The iron and
218 magnesium content as well as the pH were measured and the amount of pyoverdine in solution in
219 the supernatant estimated by monitoring the absorbance at 400 nm (Folschweiller et al., 2002).
220 Assays were performed in three to five replicates.

221 Long-term alteration of chrysotile gypsum by *Pseudomonas aeruginosa* was performed
222 by renewal cycles at 25°C with shaking (220 rpm). Controls without bacterial cells were
223 included. Sixteen 8 h cycles were performed in the presence of 20 mL of bacterial cells prepared
224 in succinate medium without magnesium, as described in paragraph 2.2, and reinoculated for

225 each renewal. For every cycle, bacterial enumeration was performed by serial dilution, plating on
226 LB-agar plates, and incubation at 30°C for 24 h. Assays were performed in triplicate. Chrysotile
227 fibers, with or without bacteria, were observed by transmission electron microscopy at the end of
228 the experimental cycles.

229 The role of each siderophore in iron and magnesium dissolution from chrysotile-gypsum
230 was evaluated using the wildtype *Pseudomonas aeruginosa* PAO1 strain (Stover et al., 2000) and
231 three siderophore mutants: i) a pyoverdine-deficient strain ($\Delta pvdF$) (Hoegy et al., 2009), ii) a
232 pyochelin-deficient strain ($\Delta pchA$) (Cunrath et al., 2015), and iii) a pyoverdine- and pyochelin-
233 deficient strain ($\Delta pvdF\Delta pchA$) (Gasser et al., 2016). Bacterial suspensions (20 mL) prepared in
234 CAA medium without magnesium, as described in paragraph 2.2, were added to chrysotile-
235 gypsum samples. Controls without bacterial cells were included. After 18 h of incubation at
236 25°C with shaking (220 rpm), the number of bacteria was determined by serial dilution, plating
237 on LB-agar plates, and incubating at 30°C for 24 h. The assays were gently centrifuged for 5 min
238 at 67 g to separate the bacterial cells from asbestos fibers. Bacterial cells (10 mL) were
239 centrifuged 10 min at 9,871 g, the pellets mineralized, and the supernatants filtered (0.22 μm
240 porosity). The iron and magnesium content as well as the pH were measured. Assays were
241 performed in triplicate.

242 Bacterial pellets were mineralized by washing them once with ultra-pure water and
243 drying at 50°C for 48 h. Cells were mineralized by incubation in 77 μL 65% (v/v) HNO_3 for 48 h
244 at room temperature. The volume was brought up to 5 ml with ultra-pure water to obtain 1%
245 HNO_3 and the samples filtered through a membrane with a 0.22 μm filter unit.

246 **2.7 Determination of Fe and Mg content**

247 The iron content was determined using the bathophenanthroline method, which is a
248 sensitive colorimetric method for determining ferrous iron content. Total iron was determined by
249 converting ferric iron to ferrous iron using thioglycolic acid. Each sample was analyzed in
250 triplicate. Samples (20 μL) were dispensed into each well of a 96-well plate (Greiner, PS flat-
251 bottomed microplate) and 40 μL saturated sodium acetate (5.5 M) (Sigma-Aldrich), 80 μL cold
252 distilled water, 10 μL thioglycolic acid solution diluted 10 times in water (Sigma-Aldrich), and
253 10 μL bathophenanthroline (5 $\text{mg}\cdot\text{mL}^{-1}$) (Sigma-Aldrich) added per well. After shaking,
254 microplates were stored overnight at 4°C. Absorbance was measured at 535 nm in a Tecan
255 microplate reader (Infinite M200, Tecan) and iron concentration ($\text{mg}\cdot\text{L}^{-1}$) determined by
256 comparing the values to those of a standard reference curve.

257 For magnesium content, the calmagite method was used. Magnesium forms a colored
258 complex with calmagite. Ethylene glycol tetra acetic acid (EGTA) was used to eliminate any
259 calcium interference. Each sample was analyzed in triplicate. The reactive solution was prepared
260 by mixing one volume of reagent 1 (1 $\text{mol}\cdot\text{L}^{-1}$ 2-Methyl-2-Amino-1-Propanol and 215 $\mu\text{mol}\cdot\text{L}^{-1}$
261 EGTA) with one volume of reagent 2 (300 $\mu\text{mol}\cdot\text{L}^{-1}$ calmagite). Samples (3 μL) were dispensed
262 into each well of a 96-well plate (Greiner, PS flat-bottomed microplate) and 300 μL reactive
263 solution mix added. After shaking and 30 s of incubation, absorbance was measured at 500 nm in
264 a Tecan microplate reader (Infinite M200, Tecan) and the magnesium concentration ($\text{mg}\cdot\text{L}^{-1}$)
265 determined by comparing the values to those of a standard reference curve.

266 **2.8 STEM-EDX of asbestos fibers**

267 Transmission electron microscopy (TEM) images were recorded with a JEOL 2100
268 microscope using a 200-kV potential applied to a LaB6 filament as an electron source.
269 Resolution of the TEM was 0.21 nm. TEM mapping was performed in scanning transmission

270 electron microscopy (STEM) mode (resolution 2 nm) using a silicon drift detector-energy
271 dispersive X-ray (SSD-EDX) spectrometer to determine the chemical composition.

272 **2.9 Statistical analysis**

273 Results presented in all figures correspond to the mean values of three to five replicates.
274 The Kruskal-Wallis test (R version 1.0.153) followed by a Conover post-hoc analysis was used
275 to determine differences among values of pyoverdine production after bacterial growth, with or
276 without asbestos fibers, and magnesium extraction from chrysotile-gypsum after incubation in
277 the presence of WT or mutant *P. aeruginosa* PAO1. Differences in the iron dissolution from
278 chrysotile-gypsum after 24 h of bacterial growth were determined using the Wilcoxon signed
279 rank test (R version 1.0.153). One-way ANOVA (R version 1.0.153), followed by Tukey's post-
280 hoc analysis was used to determine differences in mCherry fluorescence levels after bacterial
281 growth in the presence of asbestos and differences in iron extraction from chrysotile-gypsum
282 after incubation in the presence of WT or mutant *P. aeruginosa* PAO1. Values with the same
283 letter are not significantly different ($p > 0.05$).

284

285 **3. Results and Discussion**

286 **3.1 Microbial growth in the presence of FAW**

287 Wildtype cultures of *Pseudomonas aeruginosa* were grown in medium depleted of
288 magnesium and iron, with or without FAW. This waste contains asbestos fibers of chrysotile and
289 gypsum, potentially containing iron and magnesium that could be used as nutrient sources by *P.*
290 *aeruginosa*. We observed no growth after 24 h of incubation in minimal succinate medium
291 depleted of magnesium and iron (Succ-Mg) (**Figure 1A**). Growth was restored after 8 h of

292 incubation in the positive control in succinate medium (Succ). Thus, magnesium is essential for
293 optimal bacterial growth. The number of bacteria decreased in the first hours of culture in the
294 Succ-Mg medium supplemented with FAW, probably due to the adsorption of bacterial cells to
295 the waste. Microbial attachment to mineral surfaces leads to the formation of a
296 microenvironment that confers protection to microorganisms against environment stress
297 (Beveridge et al., 1997; Liermann et al., 2000; Ojeda et al., 2006). As a consequence, mineral
298 nutrients can be extracted directly by some microorganisms. *Pseudomonas* shares the same
299 mechanism as many soil bacteria that promote mineral weathering by the production of
300 siderophores to overcome iron limitation. After 12 h, the number of planktonic cells reached the
301 same level as that of the initial inoculum and showed growth similar to the positive control at 24
302 h. This shows the ability of *P. aeruginosa* to use magnesium present in the FAW as a nutrient
303 source. Thus, bacteria can alter asbestos waste, showing the feasibility of the biological treatment
304 of asbestos.

305 We determined the amount of the exogenous siderophores pyoverdine and pyochelin for
306 all conditions. Similar amounts of pyochelin were observed in Succ-Mg medium, with or without
307 FAW, after 24 h (110-130 μM) (data not shown), whereas the levels of pyoverdine varied widely
308 between conditions (**Figure 1B**). *P. aeruginosa* cultures grown in Succ medium produced a
309 cumulative pyoverdine concentration of 183 μM as a consequence of iron depletion of the
310 medium. In contrast, only 2 μM of pyoverdine was produced in Succ-Mg medium related to the
311 absence of bacterial growth. Supplementation with FAW resulted in a lower pyoverdine
312 concentration (27 μM) than that of the positive control, whereas growth was similar. The
313 pyoverdine pathway is well known to be repressed by the presence of iron. Our results show a
314 decrease of pyoverdine production, suggesting that dissolved iron was released from the FAW,

315 repressing the pyoverdine system. Indeed, *P. aeruginosa* has already been reported to use iron
316 from smectite, a type of clay, by the siderophore acquisition system (Ferret et al., 2014), similar
317 to the repression of the pyoverdine pathway and stimulation of growth that we observed.

318 We measured the amount of iron and magnesium in the bacterial cells and supernatant
319 (**Figure 1C**). The concentration of total iron was significantly higher in the presence of bacteria
320 (0.27 mg/L) than the control (0.06 mg/L), FAW incubated in sterile medium. The supernatant
321 contained 0.09 mg/L iron, whereas more iron accumulated in the bacterial cells (0.18 mg/L). As
322 iron is an essential element required for bacterial metabolism, bacteria regulate the intracellular
323 iron concentration and can store it, as well several other elements. Cunrath et al. (2016) focused
324 on the metallome of *P. aeruginosa* and showed that the ratio between the metal concentration in
325 bacteria and that in growth medium are not equivalent. They showed that all metals are not taken
326 up from the medium by *P. aeruginosa* cells with the same efficiency. Metals accumulated in the
327 cells depending on the surrounding environment. Among many elements, one of the most
328 efficiently extracted metals in succinate medium was Fe, with a concentration factor reaching
329 3,200. Certain toxic elements, such as nickel or chromium, can be incorporated into the mineral
330 structure of asbestos, depending on the metals present in the mines from which it is extracted.
331 Serpentine soils often contain large amounts of nickel, which can be found in the chemical
332 composition of chrysotile fibers through substitution with Mg (Chardot-Jacques et al., 2013).
333 These toxic elements can be dissolved together with Fe during bacterially-promoted dissolution
334 of minerals. Schalk et al. (2011) showed that siderophores produced by *P. aeruginosa* can
335 chelate other metals, although at a lower efficiency. Thus, these toxic metals can be solubilized
336 due to bacterial activity. In addition to their role in iron acquisition, siderophores can also
337 prevent the accumulation of toxic metals in *P. aeruginosa* cells, thus preserving the equilibrium

338 of the metallome in a polluted environment. Thus, siderophore-producing *Pseudomonas* could be
339 used to remove toxic heavy metals from asbestos waste, avoiding environmental pollution.

340 In contrast to iron, there was no difference in the Mg content in solution in the absence or
341 presence of bacteria, suggesting that the bacteria may not influence Mg dissolution from asbestos
342 fibers, whereas the medium was likely responsible for solubilization of the magnesium. We thus
343 determined the magnesium requirements for *P. aeruginosa*. The number of bacteria gradually
344 increased when the medium was supplemented with Mg (**Figure 2**). Bacterial growth was
345 restored in 0.05 mg.L⁻¹ Mg under the tested condition, whereas optimal growth required 0.5 to 2
346 mg.L⁻¹ Mg. The incubation of FAW in succinate medium without bacteria, as a control in the
347 experiment of Figure 1, resulted in a dissolved Mg concentration of 1.5 mg.L⁻¹ (data not shown).
348 Given that the media was Mg-poor, the bacteria could only grow if Mg was obtained from the
349 solid phase FAW and at a sufficient concentration to sustain their growth.

350 **3.2 Long-term alteration of chrysotile gypsum by *Pseudomonas aeruginosa***

351 Our results show that bacteria can access Fe and Mg from FAW over a short period of
352 time (24 h). This suggest that the materials were altered under these conditions. We thus tested
353 whether repeated cycles could continuously provide these elements for bacterial growth. Short
354 contact cycles of 8 h were performed to conform to the exponential growth phase and avoid
355 biofilm formation. We observed a higher number of bacteria when the media was supplemented
356 with FAW until the fourth renewal cycle (C1 to C4), whereas similar growth to that in media
357 without FAW was observed for C5 and C6. The following two cycles (C7 and C8) showed a
358 second stimulation of growth, which no longer occurred at later cycles until the end of the
359 experiment (C16) (**Figure 3A**). Such enhancement of growth could correlate with various sites
360 of extraction from the FAW. Siderophores often bind to minerals and their high affinity for iron

361 binding or adsorption is a prerequisite to dissolve iron. During the first cycles, iron and
362 magnesium absorbed or present in the brucite layer could be easily dissolved, whereas elements
363 present in the silica layer could be slow to be released. Our results tend to confirm this
364 hypothesis, as already suggested by Mohanty et al. (2018) on the chrysotile ore. In the following
365 cycles, there was not ready access to these nutrients present in the FAW, as there was no
366 stimulation of growth for two cycles (C5/C6). More cycles or longer exposure appears to be
367 required to restore the benefit provided by FAW to bacterial growth, probably due to the seeking
368 of the essential elements in the deeper layers of the asbestos fiber. The absence of an effect after
369 the ninth cycle may be attributed to the time of contact, which may have been too short.

370 Analysis of chrysotile fibers by STEM-EDX (Figure 3B, C, D, E) showed a decrease in
371 iron content after long-term alteration with *P. aeruginosa* and confirms that iron was removed
372 from the fiber structure. These results confirm the active dissolution of iron driven by
373 *Pseudomonas*. Iron removal could reduce the toxicity of asbestos fibers as showed by Mohanty
374 et al. (2018). The potential detoxification of asbestos waste by bacteria could be considered to
375 reduce the health problems associated with exposure to toxic fibers at contaminated sites.
376 However, a similar magnesium content was observed, with or without *P. aeruginosa*. This is
377 likely associated with equivalent dissolution between the two conditions, corroborating the
378 impact of the succinate medium in solubilizing magnesium.

379 **3.3 Role and involvement of pyoverdine and pyochelin in altering FAW**

380 *P. aeruginosa* is a bacterium that produces the two siderophores pyoverdine and
381 pyochelin. We showed the ability of this bacterium to extract elements from FAW and next
382 investigated the involvement of these two siderophores in this process. Given the ability of
383 succinate medium to solubilize magnesium from FAW, further experiments were performed

384 using another minimal medium restricted in iron, CAA. When needed, the medium was not
385 supplemented with magnesium (CAA-Mg). This medium showed weak magnesium dissolution
386 (1 mg.L⁻¹) relative to the succinate medium (11 mg.L⁻¹) after 24 h of incubation with FAW
387 (data not shown).

388 Among the biosynthetic enzymes for pyochelin and pyoverdine, we tagged PvdJ and
389 PchA with the fluorescent protein mCherry to follow the synthesis of proteins involved in
390 siderophore production in the presence of FAW (**Figure 4**). Allelic exchange was used to
391 produce a strain expressing PvdJ with mCherry fused to its C-terminus (*PvdJmcherry*).
392 Chromosomal insertion was chosen to maintain expression levels close to those of the wildtype
393 strain. This strain grew as well as the parental PAO1 strain and showed the same level of
394 pyoverdine production as that of the parental strain in iron-depleted CAA medium
395 (Supplementary Figure 1).

396 After 24 h of incubation in CAA medium, we observed high fluorescence for both
397 biosynthetic enzymes PvdJ (1,064) and PchA (1,331) due to iron depletion from the medium.
398 However, the fluorescence of PvdJ-mCherry (143) and PchA-mCherry (309) was low in CAA-
399 Mg medium, related to the absence of bacterial growth. Both biosynthetic enzymes were highly
400 expressed (547 for PvdJ and 785 for PchA) in CAA medium supplemented with FAW, but the
401 levels remained significantly lower than those of the positive control (CAA). This is consistent
402 with the repression of siderophore production shown in **Figure 1A**, related to iron release from
403 FAW. These results confirm expression of the siderophore pathways in the presence of asbestos.

404 We next investigated the role of each siderophore in iron and magnesium dissolution
405 from FAW using *P. aeruginosa* strains producing both endogenous siderophores (WT), no
406 pyoverdine (Δ PVD), no pyochelin (Δ PCH), or neither of the two siderophores (2Δ). FAW

407 samples were incubated in CAA-Mg with the WT or mutants and the iron and magnesium
408 content in the supernatants and bacterial cells measured. There was no difference between the
409 growth of the WT and mutant strains (Supplementary Figure 2). Weak iron dissolution (0.08
410 mg.L-1) occurred in the control CAA-Mg medium, whereas we observed significantly higher
411 iron concentrations during the growth of the WT (0.28 mg.L-1) or single mutants Δ PVD (0.26
412 mg.L-1) or Δ PCH (0.23 mg.L-1) (**Figure 5A**). The absence of a difference in iron extraction
413 between these strains shows that the absence of one of the two siderophores can be compensated
414 by the production of the other. Only 0.09 mg.L-1 iron was measured in the absence of both
415 pyoverdine and pyochelin, similar to the concentration in the control CAA-Mg. This result
416 shows that both siderophores are involved in iron removal from fibers and that the absence of the
417 siderophores greatly reduces iron dissolution. One of the common weathering processes for
418 fungi, plants, and bacteria in the interaction with minerals is siderophore production. For
419 example, siderophore production by *Pseudomonas mendocina* can solubilize iron from goethite,
420 hematite, and ferrihydrite (Hersman et al., 2001). Ferret et al. (2014) reported the ability of
421 *Pseudomonas aeruginosa* to extract iron from smectite, linked with siderophore production.
422 Bacterial metabolism can therefore mobilize elements from various minerals and the use of
423 siderophores as chelating compounds can be considered to be a major mechanism of such
424 alterations. Asbestos minerals, although highly resistant to weathering, have been reported to be
425 biodegraded by a few species of bacteria. Yao et al. (2013) showed, for example, that *Bacillus*
426 *mucilaginosus* was able to accelerate serpentine powder dissolution due to ligand production.
427 Recently, Gram-negative bacteria, including some identified by the authors as probably a
428 *Staphylococcus*, were isolated from asbestos rock and asbestos-contaminated soil samples. These
429 isolates showed their ability to reduce the iron content of asbestos fibers (Bhattacharya et al.,

430 2015). Further study of previously isolated Gram-positive bacteria showed that these bacteria
431 synthesize siderophores, with no indication of the structure of the produced compounds
432 (Bhattacharya et al., 2016). Siderophore production is a common mechanism already described
433 in the bacterial and fungal weathering of asbestos (Daghino et al., 2010; Rajkumar et al., 2009).
434 Several studies have focused on the effect of the siderophore deferoxamine, produced by
435 *Streptomyces pilosus*, on raw asbestos fibers and showed this siderophore to efficiently remove
436 iron (Chao and Aust, 1994; Mohanty et al., 2018). Our results confirm the involvement of
437 siderophores in altering asbestos and show that the pyoverdine and pyochelin pathways play an
438 important role in iron removal from FAW. The use of siderophore pathways in a bacterial
439 bioremediation process could allow the development of eco-friendly asbestos treatment, avoiding
440 the continuing disposal of such waste in landfills. In addition to iron, we investigated the role of
441 these two siderophores in magnesium dissolution from FAW (**Figure 5B**). The concentration of
442 dissolved magnesium measured in experiments with the WT and mutants of *P. aeruginosa* was
443 not significantly different. A well-known parameter described to influence the dissolution of the
444 chrysotile brucite layer is pH (Choi and Smith, 1972; Gronow, 1987). Here, the pH changed
445 from 7.50 to 8.40. Thus, it is likely that it did not influence the process, whereas it is probably
446 linked to the involvement of other mechanisms, such as organic-acid production. Indeed, during
447 growth, bacteria may secrete various metabolites, such as oxalic, succinic, formic, or lactic acid,
448 depending on the carbon substrate present in the medium. The dissolution of chrysotile by
449 organic acids is well known and has already been described in several studies. Oxalic acid, for
450 example, can dissolve the brucite layer in chrysotile fibers (Rozalen et al., 2014; Thomassin et
451 al., 1977). In our study, the alteration of asbestos may be the effect of a ligand-controlled
452 dissolution mechanism driven by the siderophores and other organic ligands, depending on a

453 surface-controlled process that is a function of the surface concentration of the adsorbed ligands
454 (Furrer and Stumm, 1986; Mohanty et al., 2018) and the solution saturation state.

455 **4. Conclusion**

456 Here, we report, for the first time, the acquisition of iron and magnesium from flocking
457 asbestos waste by *Pseudomonas aeruginosa* and the involvement of siderophores. The presence
458 of chrysotile-gypsum promoted the growth of *P. aeruginosa* in correlation with greater iron
459 dissolution in the presence of the bacteria. Active dissolution of iron content from asbestos fibers
460 was driven by *Pseudomonas* in a long-term alteration experiment of chrysotile-gypsum.
461 Moreover, we clearly demonstrate the role and involvement of the siderophores, pyoverdine and
462 pyochelin, in altering flocking asbestos. Indeed, the siderophore biosynthetic pathways were
463 expressed in the presence of chrysotile-gypsum and the absence of both siderophores
464 significantly reduced iron removal from asbestos waste. Our results show that FAW can serve as
465 nutrient sources for bacteria using siderophores for iron release. The biodegradation of asbestos
466 by siderophore-producing *Pseudomonas* could be an eco-friendly bioremediation process that
467 could be used to treat loose asbestos waste and reduce asbestos-related environmental and health
468 problems.

469 **Acknowledgements**

470 This work was supported by the French Environment and Energy Management Agency
471 (ADEME) and the SOMEZ (Société Méditerranéenne des Zéolithes).

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673 **Figure legends**

674 **Figure 1.** Growth of *Pseudomonas aeruginosa* PAO1 during 24 h in succinate medium with
675 (Succ) or without magnesium (Succ-Mg), in the presence or absence of chrysotile-gypsum
676 (CHR-GY) (A). Quantification of pyoverdine in supernatants after 24 h of *P. aeruginosa* PAO1
677 growth in Succ or Succ-Mg, with or without CHR-GY (B). Evolution of iron concentration
678 extracted from chrysotile-gypsum waste over 24 h in the presence of *P. aeruginosa* PAO1 or
679 Succ-Mg (C). Total concentration of iron dissolved from chrysotile-gypsum after 24 h in the
680 presence of *P. aeruginosa* PAO1 or Succ-Mg. Iron was measured in the bacterial cells (Pellet)
681 and supernatants (D). Error bars indicate the standard deviations of the means of three or five
682 replicates. Bars with the same letter are not significantly different ($p > 0.05$, Kruskal-Wallis test
683 and Wilcoxon signed rank test, three or five replicates).

684 **Figure 2.** Growth of *Pseudomonas aeruginosa* PAO1 during 37.5 h in succinate medium
685 containing various concentrations of magnesium (0 to 2 mg.L⁻¹). Error bars indicate the standard
686 deviations of the means of three replicates.

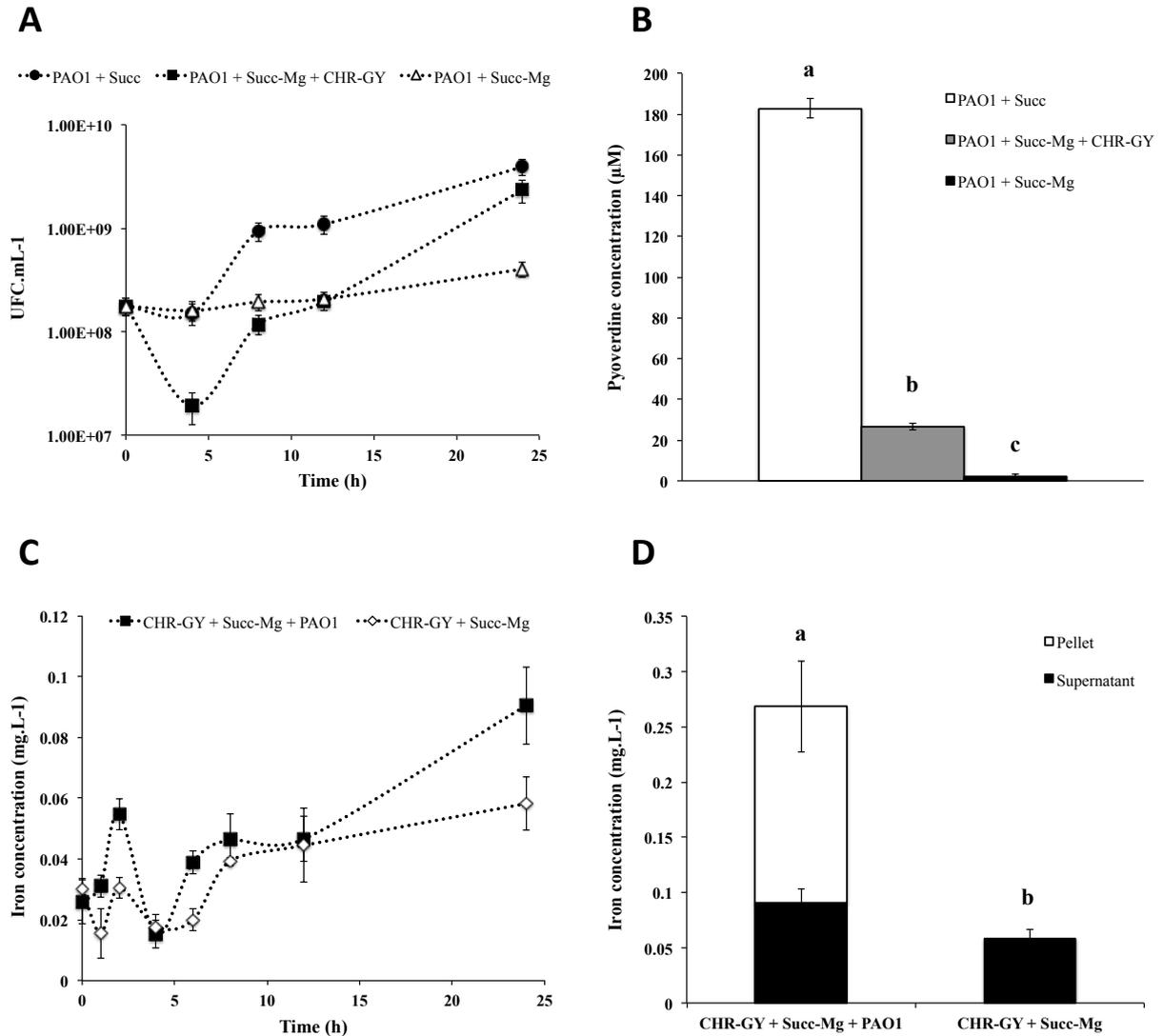
687 **Figure 3.** Enumeration of *Pseudomonas aeruginosa* PAO1 during 16 growth cycles of 8 h in
688 succinate medium without magnesium (Succ-Mg), in the presence or absence of chrysotile-
689 gypsum (CHR-GY) (A). STEM images of chrysotile fibers before (B) and after (C) bacterial
690 contact. Atomic ratios of Mg/Si and Fe/Si before (control) and after contact of chrysotile-gypsum
691 with *P. aeruginosa* PAO1 (D). Mass percentage of iron, magnesium, and silicon before and after
692 contact of chrysotile-gypsum with *P. aeruginosa* PAO1 (E). Error bars indicate the standard
693 deviations of the means of three replicates.

694 **Figure 4.** Fluorescence of PvdJ-mCherry and PchA-mCherry after 24 h of *P. aeruginosa* PAO1
695 growth in casamino acids medium with (CAA) or without magnesium (CAA-Mg), in the

696 presence or absence of chrysotile-gypsum (CHR-GY). Error bars indicate the standard deviations
697 of the means of three or five replicates. Bars with the same letter are not significantly different (p
698 > 0.05 , One-way ANOVA, three or five replicates).

699 **Figure 5.** Concentration of iron (A) and magnesium (B) dissolved from chrysotile-gypsum after
700 18 h in the presence of casamino acids medium without magnesium (CAA-Mg) as a control or
701 inoculated with the wildtype *Pseudomonas aeruginosa* PAO1 strain (WT), pyoverdine-deficient
702 strain (Δ PVD), pyochelin-deficient strain (Δ PCH), or pyoverdine- and pyochelin-deficient strain
703 (2Δ). Elements were measured in the bacterial cells (Pellet) and supernatants. Error bars indicate
704 the standard deviations of the means of three replicates. Bars with the same letter are not
705 significantly different ($p > 0.05$, One-way ANOVA and Kruskal-Wallis test, three replicates).

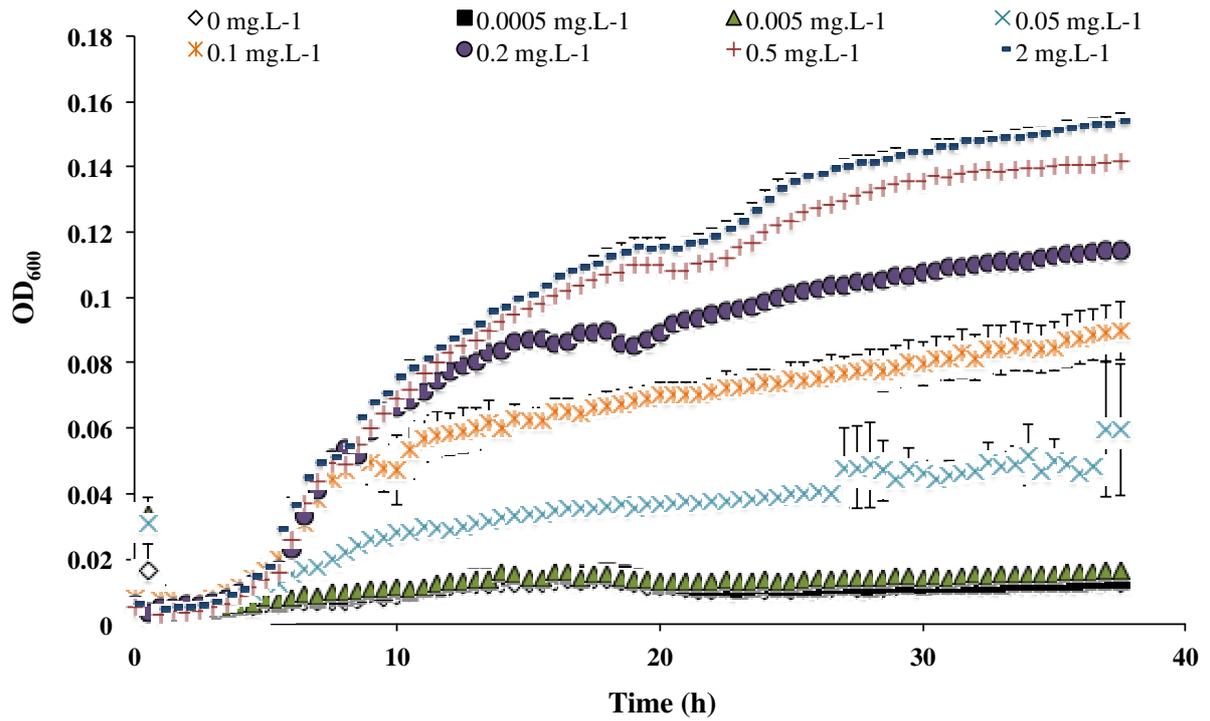
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Figure 1: Growth of *Pseudomonas aeruginosa* PAO1 during 24 h in succinate medium with (Succ) or without magnesium (Succ-Mg), in the presence or absence of chrysotile-gypsum (CHR-GY) (A). Quantification of pyoverdine in supernatants after 24 h of *P. aeruginosa* PAO1 growth in Succ or Succ-Mg, with or without CHR-GY (B). Evolution of iron concentration extracted from chrysotile-gypsum waste over 24 h in the presence of *P. aeruginosa* PAO1 or Succ-Mg (C). Total concentration of iron dissolved from chrysotile-gypsum after 24 h in the presence of *P. aeruginosa* PAO1 or Succ-Mg. Iron was measured in the bacterial cells (Pellet) and supernatants (D). Error bars indicate the standard deviations of the means of three or five replicates. Bars with the same letter are not significantly different ($p > 0.05$, Kruskal-Wallis test and Wilcoxon signed rank test, three or five replicates).

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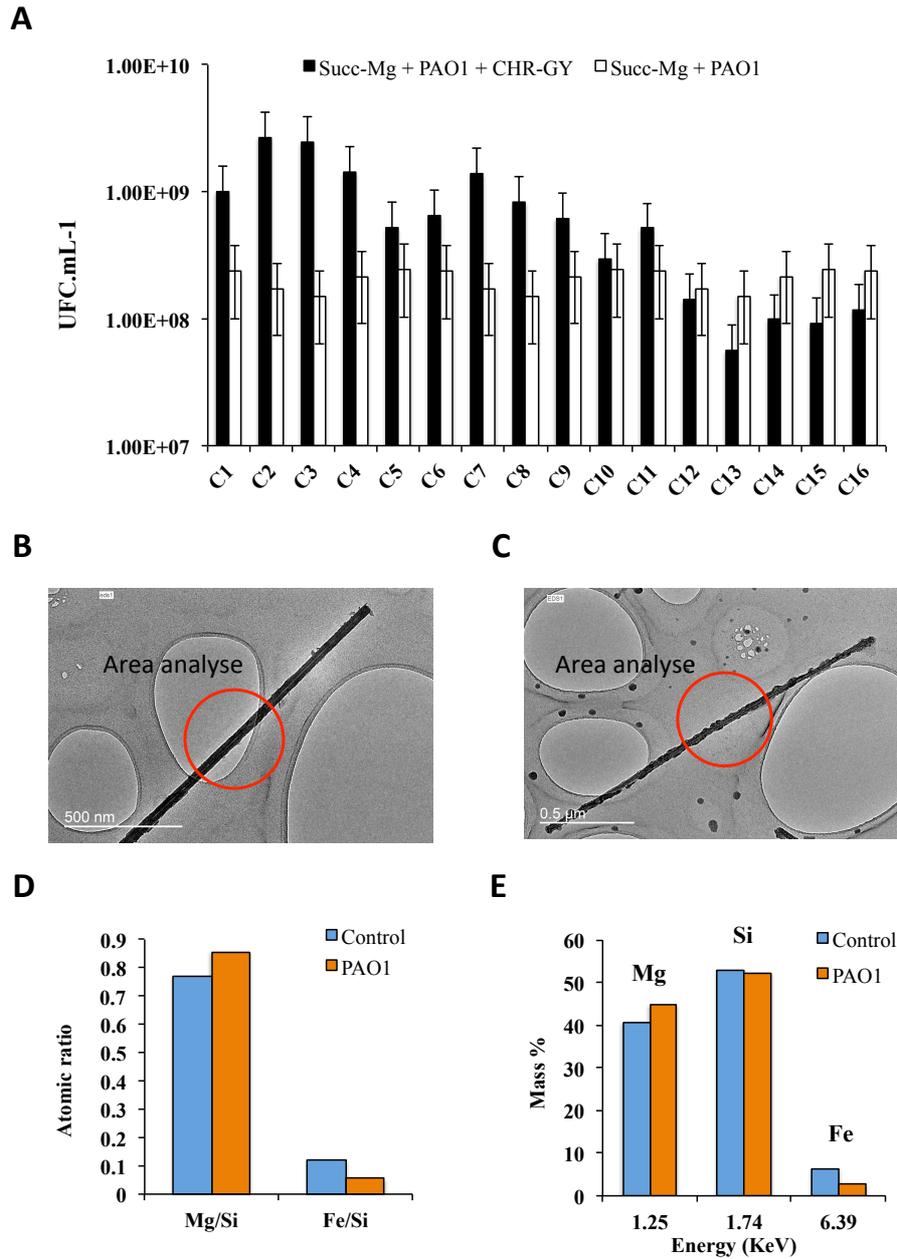
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Figure 2: Growth of *Pseudomonas aeruginosa* PAO1 during 37.5 h in succinate medium containing various concentrations of magnesium (0 to 2 mg.L⁻¹). Error bars indicate the standard deviations of the means of three replicates.

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Figure 3: Enumeration of *Pseudomonas aeruginosa* PAO1 during 16 growth cycles of 8 h in succinate medium without magnesium (Succ-Mg), in the presence or absence of chrysotile-gypsum (CHR-GY) (A). STEM images of chrysotile fibers before (B) and after (C) bacterial contact. Atomic ratios of Mg/Si and Fe/Si before (control) and after contact of chrysotile-gypsum with *P. aeruginosa* PAO1 (D). Mass percentage of iron, magnesium, and silicon before and after contact of chrysotile-gypsum with *P. aeruginosa* PAO1 (E). Error bars indicate the standard deviations of the means of three replicates.

In color print

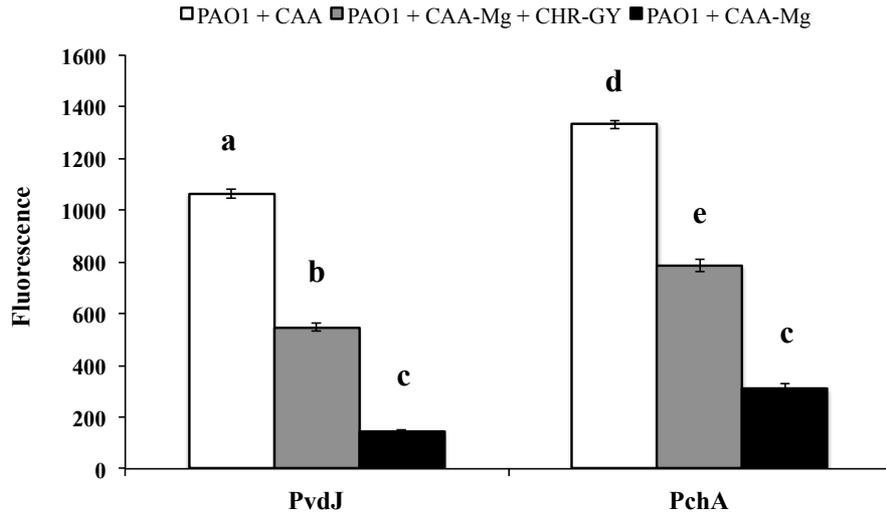
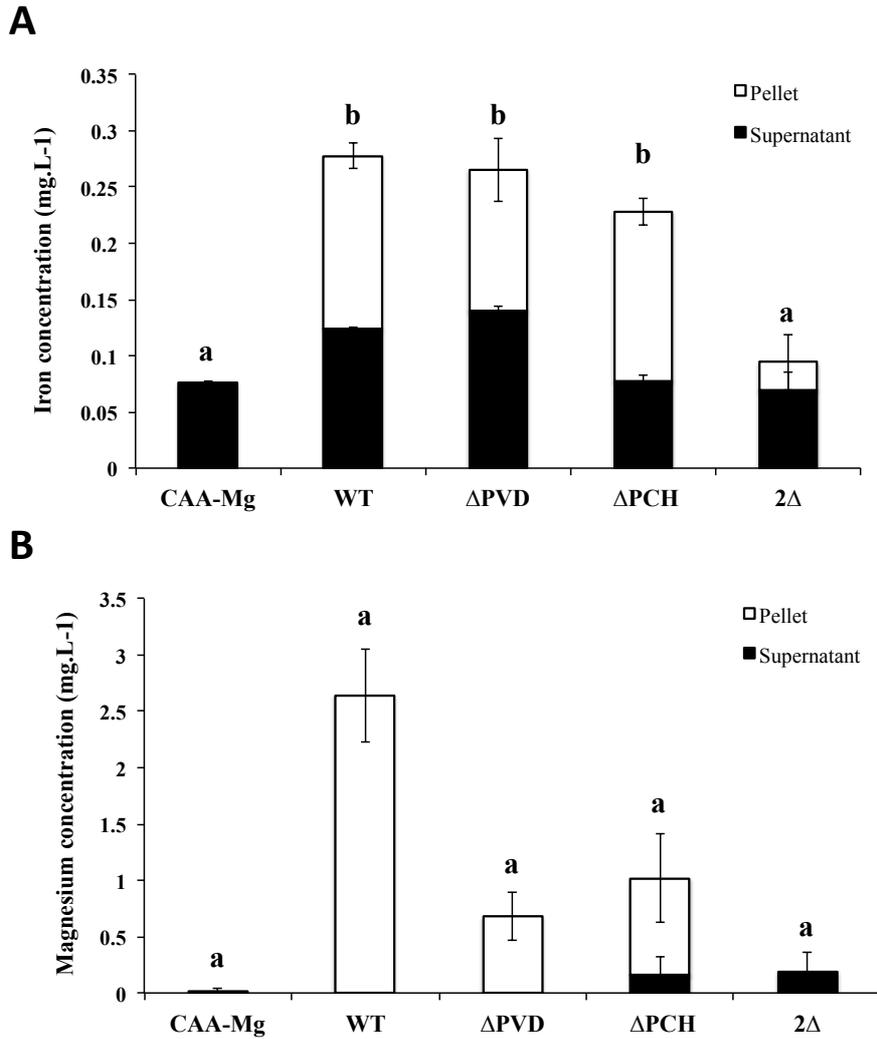


Figure 4: Fluorescence of PvdJ-mCherry and PchA-mCherry after 24 h of *P. aeruginosa* PAO1 growth in casamino acids medium with (CAA) or without magnesium (CAA-Mg), in the presence or absence of chrysotile-gypsum (CHR-GY). Error bars indicate the standard deviations of the means of three or five replicates. Bars with the same letter are not significantly different ($p > 0.05$, One-way ANOVA, three or five replicates).

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Figure 5: Concentration of iron (A) and magnesium (B) dissolved from chrysotile-gypsum after 18 h in the presence of casamino acids medium without magnesium (CAA-Mg) as a control or inoculated with the wildtype *Pseudomonas aeruginosa* PAO1 strain (WT), pyoverdine-deficient strain (Δ PVD), pyochelin-deficient strain (Δ PCH), or pyoverdine- and pyochelin-deficient strain (2 Δ). Elements were measured in the bacterial cells (Pellet) and supernatants. Error bars indicate the standard deviations of the means of three replicates. Bars with the same letter are not significantly different ($p > 0.05$, One-way ANOVA and Kruskal-Wallis test, three replicates).

1 **Table 1:** Strains and plasmids used in this study.

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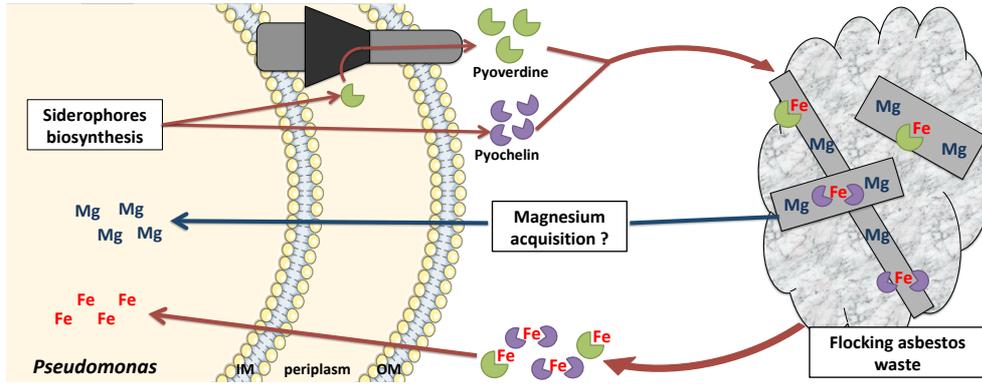
Strains and plasmids	Relevant characteristics	References
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild-type strain	(Stover <i>et al.</i> , 2000)
$\Delta pvdF$	PAO1; $\Delta pvdF$	(Hoegy <i>et al.</i> , 2009)
$\Delta pchA$	PAO1; $\Delta pchA$	(Cunrath <i>et al.</i> , 2015)
$\Delta pvdF\Delta pchA$	PAO1; $\Delta pvdF\Delta pchA$	(Gasser <i>et al.</i> , 2016)
<i>pvdJmcherry</i>	Derivate of PAO1; <i>pvdJmcherry</i> chromosomally integrated	This study
<i>pchAmcherry</i>	PAO1; <i>pchAmcherry</i>	(Cunrath <i>et al.</i> , 2015)
<i>Escherichia coli</i>		
SM10	thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λ pir	(Herrero <i>et al.</i> , 1990)
Plasmids		
pLG45	pME3088 <i>pvdJmcherry</i> with 700bp flanking region relative to <i>mcherry</i>	(Gasser <i>et al.</i> , 2015)
pEXG2	allelic exchange vector with pBR origin, gentamicin resistance, <i>sacB</i>	(Rietsch <i>et al.</i> , 2005)
pEXG2 <i>pvdJmcherry</i>	pEXG2 carrying the sequence to insert <i>mcherry</i> tag in C-terminus of <i>pvdJ</i>	This study

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1 **Graphical Abstract**

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