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Biodeterioration of asbestos cement by siderophore-producing *Pseudomonas*

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Abstract

Since the ban on the use of asbestos due to its carcinogenic properties, the removal of asbestos cement, representing the major asbestos-containing waste, has proven to be a challenge in most industrial countries. Asbestos-containing products are mainly disposed of in landfills and have remained untreated. Bioremediation involving bacteria previously reported the ability of *Pseudomonas aeruginosa* to release iron from flocking asbestos waste through a siderophore-driven mechanism. We examined the involvement of siderophore-producing *Pseudomonas* in the biodeterioration of asbestos cement. Iron and magnesium solubilization were evaluated by specific siderophore-producing mutants. The absence of one of the two siderophores affected iron extraction, whereas equivalent dissolution as that of the control was observed in the absence of siderophore. Both pyoverdine and pyochelin biosynthesis was repressed in the presence of asbestos cement, suggesting iron bioavailability from the waste. We compared the efficiency of various pyoverdines to scavenge iron from asbestos cement waste that revealed the efficiency of all pyoverdines. Pyoverdines were efficient in iron

removal extracted continuously, with no evident extraction limit, in long-term weathering experiments with these pyoverdines. The optimization of pyoverdine-asbestos weathering may allow the development of a bioremediation process to avoid the disposal of such waste in landfills.

Keywords

asbestos cement; *Pseudomonas*; pyoverdine; iron; weathering

1. Introduction

Asbestos comprises a family of natural compounds that have been intensively used for many purposes, such as heat-insulating boards, plastic and spun products, tar, and asbestos cement (AC), due to their insulating, chemical, and mechanical properties. Exposure to asbestos fibers by inhalation results in pleural calcification, pulmonary fibrosis, pleural, lung and peritoneal mesothelioma, and ovarian cancer. The toxicity of asbestos is typically attributed to the presence of the transition metal iron as a catalyst of the Fenton reaction, leading to the generation of free radicals and reactive oxygen species (ROS) (Kamp et al., 1995; Toyokuni, 2009; Valko et al., 2015). Consequently, asbestos has been banned since the beginning of the 80's in many countries, but some still use asbestos, such as Russia, India, China, and Brazil. Asbestos removal from buildings, its main area of application, generates a vast amount of asbestos-containing waste (ACW). ACW management is obliged to follow current safety recommendations, which can include rendering it inert by heating to 1,300 to 1,500°C. However, the most widely used technique is disposal in landfills, although this does not provide a definitive solution to health risks (Paolini et al., 2018; Spasiano and Pirozzi, 2017). Moreover, damage to landfill storage sites could potentially release asbestos fibers into the surrounding environment. Thus, alternative treatments are required to alter asbestos fibers and convert this waste into non-harmful compounds. Various treatment technologies have been investigated, such as thermal, chemical, hydrothermal, and mechanochemical approaches, which differ in terms of cost, energy consumption, and management of the end-products (Paolini et al., 2018; Spasiano and Pirozzi, 2017). Bioremediation strategies have also been explored, as asbestos consists of minerals originating from soil, with a worldwide distribution. Indeed, Asbestos consist of fibrous silicate minerals belonging to amphibole or

serpentine families. Amphiboles include five types of minerals (actinolite, anthophyllite, tremolite, amosite (brown asbestos), and crocidolite (blue asbestos)), whereas serpentine consists of only chrysotile (white asbestos). Among the six species, chrysotile, crocidolite, and amosite have the most commercial applications, with chrysotile representing 95% of world production, as it is the most common type found in many asbestos-containing products (Kanarek, 2011). Chrysotile is a magnesium phyllosilicate, which may carry iron as an integral part of the chemical structure, depending on soil composition. Indeed, substitution can occur between Si^{4+} and Mg^{2+} , which can be replaced by Fe^{3+} and Fe^{2+} , respectively (Bernstein et al., 2013; Virta, 2002). The structure of chrysotile, which consists of a double layer comprised of a silicate sheet and brucite ($\text{Mg}(\text{OH})_2$), is sensitive to acid attack, whereas it is well known that basic solutions have no effect.

Iron and magnesium are two essential elements present in asbestos that are required by many microorganisms. Bacteria are well-known to interact with minerals through various mechanisms (Ferret et al., 2014; Kraemer, 2004), amongst which the use of siderophores is a common strategy to overcome iron deprivation, as iron is poorly bioavailable in aerobic circumneutral environments. Siderophores are highly Fe(III)-specific ligands (Neilands, 1981) present in significant amounts in soil (Hersman et al., 1995). Fluorescent *Pseudomonas* are soil bacteria that produce the siderophore pyoverdine (PVD), which is a green fluorescent compound produced in large quantities under iron starvation (Cocozza and Ercolani, 1997). PVD is composed of three distinct structural components i) a dihydroxyquinoline chromophore, which confers the color and the fluorescence of the molecule, ii) a peptide chain, comprised of 6 to 12 amino acids, bound to its carboxyl group, and iii) a small dicarboxylic acid (or its monoamide) connected to the NH_2 -group (Budzikiewicz, 2004, 1997; Budzikiewicz et al., 2007; Demange et al., 1990; Fuchs and Budzikiewicz, 2001). *Pseudomonas aeruginosa* is a ubiquitous fluorescent *Pseudomonas* species (Goldberg, 2000) known to produce, aside from PVD, a second siderophore, called pyochelin (PCH), a low-molecular-weight thiazoline derivative [2(2-*o*-hydroxy-phenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid] that has a lower affinity for iron ($K_a = 10^{28.8} \text{M}^{-2}$) (Brandel et al., 2012) than PVD ($K_a = 10^{32} \text{M}^{-1}$) (Albrecht-Gary et al., 1994). Siderophore-producing *Pseudomonas* can mobilize iron from various minerals, such as Fe(III)(hydr)oxide (Hersman et al., 2001) or aluminosilicate clay minerals (Ams et al., 2002) by *Pseudomonas mendocina*. Furthermore, Ferret et al. (2014) showed that PVD and PCH, produced by *P. aeruginosa*, release structural iron from smectite. Asbestos weathering related to bacterial and fungal siderophores is a common mechanism that has been previously described (Daghino et

al., 2010; Rajkumar et al., 2009). *Aspergillus tubingensis* and *Coemancia reversa*, two filamentous fungi isolated from a Rajasthan Indian mine, efficiently remove iron from asbestos fibers (Bhattacharya et al., 2016a). These isolates are able to produce siderophores and were considered to be potential bioremediators for asbestos, as previously demonstrated for *Fusarium oxysporum* (Daghino et al., 2005; Martino et al., 2004), *Verticillium leptobactrum*, and *Aspergillus fumigatus* (Daghino et al., 2008). Bhattacharya et al. (2015) showed a decrease in iron content from asbestos by several Rajasthan mine bacterial isolates identified as Gram-positive and -negative bacteria, with two Gram-positive siderophore producers (Bhattacharya et al., 2016b). Bacterial weathering of asbestos has been recently investigated. Several studies focusing on raw asbestos demonstrated that certain Gram-positive and Gram-negative bacterial isolates decrease the iron content of asbestos rocks (Bhattacharya et al., 2015), whereas bacterial metabolites, such as organic acids and ligands produced by another Gram-positive bacterium, *Bacillus mucilaginosus*, promote the dissolution of serpentine (Yao et al., 2013). Bhattacharya et al. (2016b) suggested that the observed iron dissolution from fibers probably correlates with siderophore production by the mine bacterial isolates. Several studies have focused on desferrioxamine, the siderophore produced by actinomycetes. Mohanty et al. (2018) showed that iron can be removed from chrysotile raw asbestos by desferrioxamine, similarly to the findings of Chao and Aust (1994), who observed the same for amphibole fibers, such as crocidolite and amosite. We previously showed the involvement of the bacterial siderophores produced by *P. aeruginosa*, PVD and PCH, in the weathering of chrysotile, crocidolite, and amosite raw fibers, resulting in iron depletion from the fibers (David et al., 2020b). These findings have provided new insights into the bacterial degradation of asbestos but further studies are still required to understand how these materials can be transformed into a non-toxic form when the fibers are mixed in a matrix inside the waste. The matrices can be very different, depending on the ACW, consisting of plastic, tar, gypsum, or cement, and the percentage of asbestos fibers can also vary, from 10 to more than 90%. In addition, the fibers can be free or embedded in the matrices, which can potentially impede the biodeterioration process. Thus, the challenges of waste degradation are highly specific for each type of waste. The bacterial alteration of flocking asbestos waste (FAW) has been recently investigated. Indeed, David et al. (2020a) showed the ability of *P. aeruginosa* to use FAW as iron and magnesium nutrient sources, with iron removal linked to a siderophore-driven mechanism. In addition, iron removal from FAW by PVDs has been demonstrated, showing varying efficiency depending on the PVD structure (David et al., 2020c). AC materials have been widely used in pipelines, flat sheets,

corrugated roof sheeting, and insulation boards, which represent 80% of the world production of asbestos (Bordebeure, 2017; Dériot and Godefroy, 2005; Douguet et al., 1997; *Fiche toxicologique amiante*, 2018). These materials contain a ratio corresponding to approximately 10% asbestos fibers embedded in 90% cement (Douguet et al., 1997), leading to compact compounds, in contrast to FAW, which contains 90% free fibers. Wasserbauer et al. (1988) showed that nitrifying bacteria, such as *Nitrosomonas* and *Nitrobacter*, increase the porosity of AC roofs and reduce their strength due to the production of nitrous and nitric acid. Moreover, slime-forming bacteria and heterotrophic aerobic bacteria lead to the corrosion of the inside surface of AC pipes of water distribution systems due to acid production resulting from bacterial metabolism (Wang et al., 2011; Wang and Cullimore, 2010). Balducci et al. (2012) also demonstrated that lactic-acid production by lactic bacteria causes the efficient dissolution of the cement phase, while Spasiano et al. (2017, 2018, 2019) showed that dark fermentation can degrade the cement phase together with a partial dissolution of brucitic layer of chrysotile fibers of AC wastes thanks to volatile fatty acids metabolized during the process (Race et al. 2019) .

We aimed to investigate the involvement of siderophore-producing bacteria in AC biodeterioration and examine the role of PVD and PCH in mineral dissolution. The induction of the PVD- and PCH-biosynthetic pathways was measured in the presence of AC waste. Given the low bacterial requirement for iron and the release of iron from AC, which would repress siderophore production, we also tested the supplementation of several representative PVDs to AC to determine their ability to scavenge iron and selected the most efficient. The best PVDs were used in long-term weathering experiments to determine whether complete degradation could be achieved. A better understanding of the bacterial weathering of AC could lead to its definitive conversion into non-harmful compounds and the development of an eco-compatible alternative technology, avoiding the continual disposal of such waste in landfills.

2. Materials and Methods

2.1. Asbestos waste preparation

The AC used in this study was obtained from the Center for Studies and Training in Asbestos and Chemical Safety (CEFASC ENVIRONNEMENT) and consisted of chrysotile associated with a cement matrix. Scanning Electron Microscopy (SEM) was performed on AC waste as described in Talbi et al. (2019). This analysis method was coupled with Energy

Dispersive X-ray (EDX) to determine the chemical composition of the samples. Matrix composition was 48 % CaO, 24.3 % SiO₂, 11.7% MgO, 8 % Na₂O; 2.3% Al₂O₃, 1.3 % Fe₂O₃ and the fiber composition was 57.9 % MgO, 42.1 % SiO₂. The Mg/Si ratio measured in the fiber found in the roof tile range from 1.0 to 1.4, which is very close to the Mg/Si ratio of pure chrysotile. The diameter of the fibers was approximately 0.05 to 0.50 µm. Due to the grinding, a heterogeneity in fiber length was obtained ranging from 0.5 µm to 500 µm.

For laboratory scale experiments, AC was ground for 10 min at 500 rpm in a planetary ball mill (Pulverisette 7, Fritsch). Asbestos samples (0.2 g) were sterilized by autoclaving (121°C for 20 min) and incubated at 70°C for 14 days before experiments for complete sterilization of the material. Samples were washed with 20 mL sterile casamino acids medium (CAA) without magnesium (CAA-Mg) before each experiment to remove free iron and magnesium.

2.2. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Tables 1 and 2. Strains were stored in LB-20% glycerol at -80°C. *Pseudomonas* cells were grown routinely overnight at 30°C in Luria Bertani medium (LB) with shaking (220 rpm). CAA (composition in g.L⁻¹: low-iron-casamino acids, 5.0; K₂HPO₄.3H₂O, 1.46; MgSO₄.7H₂O, 0.25) was used as iron-restricted medium and sterilized using 0.2-µm polyethersulfone membrane filter units (Stericup Merck). For iron and magnesium-depleted cultures, cells were harvested from a LB pre-culture (24 h incubation) and washed once with sterile CAA medium without magnesium. Cultures were incubated for 24 h at 25°C with shaking (200 rpm) (Geoffroy and Meyer, 2004). For bacterial suspension, cells were washed once with sterile CAA medium without magnesium. Cell density was adjusted to an OD₆₀₀ of 0.1, corresponding to 1-2.10⁸ CFU.ml⁻¹, and diluted to obtain a started inoculum between 6.10⁴ and 1.10⁵ CFU.ml⁻¹.

2.3. Pyoverdine production

PVDs used in this study are listed in Table 2. First, 1 mL overnight LB culture of *Pseudomonas* strains was centrifuged (3 min at 9,871 x g). For iron starved cells, iron-deficient CAA medium was used. The pellet was washed twice with 1 ml sterile CAA medium, suspended in 1 ml sterile CAA medium, used to inoculate 15 mL of sterile CAA medium, and the suspension incubated at 25°C with shaking (220 rpm). After 24 h of growth, 7.5 mL of the culture was transferred to an Erlenmeyer flask containing 2 L sterile CAA medium. After 48 h of incubation at 25°C with shaking (220 rpm), the culture was centrifuged

at 2,664 x g for 40 min. The supernatant was filtered twice with glass microfiber filters (Whatman, GF/C) and once with a nitrocellulose filter (0.45 µm porosity) (Meyer and Abdallah, 1978). The PVD concentration was determined by measuring the OD₄₀₀ using a Specord 205 spectrophotometer (Analytik Jena). When needed, the PVD concentration was adjusted with PVD purified according to the method described thus far. Siderophore-containing supernatant was loaded, after acidification (pH 6.0) onto an XAD-4 column, washed with two volumes of purified water, and eluted with one volume of 50% ethanol. The eluate was concentrated under vacuum on a rotary evaporator and lyophilized.

2.4. Expression of the pyoverdine and pyochelin pathways in the presence of AC

Expression of the biosynthetic enzymes for siderophore production was analyzed using *Pseudomonas aeruginosa* PAO1 strains with the biosynthetic enzymes for PCH and PVD, PvdJ (David et al., 2020a) and PchA (Cunrath et al., 2015), tagged with the fluorescent protein mCherry. Bacterial suspensions (20 mL) prepared in CAA medium without magnesium, as described in paragraph 2.2, were incubated with AC samples. After 40 h of incubation at 25°C with shaking (220 rpm), the number of bacteria was determined by serial dilution-plating on LB-agar plates and incubation at 30°C for 24 h. For the expression analysis, the samples were gently centrifuged for 5 min at 67 x g to separate the bacteria from the asbestos fibers. Bacterial cells (100 µL) were dispensed into individual wells of a 96-well plate (Greiner, PS flat-bottomed microplate). mCherry fluorescence (excitation/emission wavelengths: 570 nm/610 nm) was measured in a Tecan microplate reader (Infinite M200, Tecan). Assays were performed in triplicate.

2.5. Asbestos dissolution by *Pseudomonas* and pyoverdines

The involvement of PVD and PCH in iron and magnesium removal from AC was evaluated using the wildtype *Pseudomonas aeruginosa* PAO1 strain (Stover et al., 2000) and three siderophore mutants: i) a PVD-deficient strain ($\Delta pvdF$) (Hoegy et al., 2009), ii) a PCH-deficient strain ($\Delta pchA$) (Cunrath et al., 2015), and iii) a PVD- and PCH-deficient strain ($\Delta pvdF\Delta pchA$) (Gasser et al., 2016). Bacterial suspensions (20 mL) prepared in CAA medium without magnesium, as described in paragraph 2.2, were added to AC samples. Controls without bacterial cells were included. After 40 h of incubation at 25°C with shaking (220 rpm), the number of bacteria was determined by serial dilution-plating on LB agar plates and incubation at 30°C for 24 h (Ferret et al., 2014). The assays were gently centrifuged for 5 min at 67 x g to separate the bacterial cells from the asbestos fibers. Bacterial cells (10 mL) were centrifuged 10 min at 9,871 x g and the supernatants filtered (0.22 µm porosity). Bacterial

pellets were mineralized by washing them once with ultrapure water and drying at 50°C for 48 h. Cells were mineralized by incubation in 77 µL 65% (v/v) HNO₃ for 48 h at room temperature (Ferret et al., 2014). The volume was brought to 5 mL with ultra-pure water to obtain 1% HNO₃ and the samples filtered through a membrane with a 0.22 µm filter unit. The iron and magnesium content were measured. Assays were performed in triplicate.

To study the effect of various PVDs on the dissolution of iron from AC, 20 mL of PVD-containing supernatants adjusted to 100 µM at pH 7 were incubated with the materials. The kinetics of iron dissolution from AC were followed for 24 h with regular sampling of the solution at 0, 2, 4, 8, 12, and 24 h. After 24 h of incubation at 30°C with shaking (220 rpm), the assays were centrifuged 30 min at 9,871 x g to recover the supernatants. Two additional cycles were repeated for 48 h and 96 h under the same conditions to prolong biodeterioration. The kinetic samples and cycle supernatants were filtered (0.22 µm porosity) to eliminate the material from the solutions and the iron content measured. Each assay was performed in five replicates.

Long-term weathering of AC by PVD-containing supernatants of *P. syringae* and *P. aeruginosa* was performed by renewal cycles at 30°C with shaking (220 rpm). For each renewal, 20 mL PVD-containing supernatant was added to the samples. Twenty cycles of 24 hours were realized in the presence of 100 µM PVD-containing supernatant. After each cycle, the assays were centrifuged 30 min at 9,871 x g and the supernatants filtered (0.22 µm porosity). The iron content was measured after each cycle and analysis of the fibers was performed by transmission electron microscopy at the end of the experiment. Assays were performed in five replicates.

2.6. Determination of Fe and Mg content

We determined iron content using the bathophenanthroline method, a sensitive colorimetric method (adapted from Bauman et al., 2019) for determining ferrous iron content. Total iron was determined by converting ferric iron to ferrous iron using thioglycolic acid. Each sample was analyzed in triplicate. Twenty microliters of sample was dispensed into each well of a 96-well plate (Greiner, PS flat-bottomed microplate), to which were added 40 µL saturated sodium acetate (5.5 M) (Sigma-Aldrich), 80 µL cold distilled water, 10 µL of a thioglycolic acid solution diluted 10 times in water (Sigma-Aldrich), and 10 µL bathophenanthroline (5 mg.mL⁻¹) (Sigma-Aldrich). After shaking, microplates were stored overnight at 4°C. Absorbance was measured at 535 nm in a Tecan microplate reader (Infinite

M200, Tecan) and iron concentration was determined by comparing the values to those of a standard reference curve.

The calmagite method was used to measure magnesium content (ELITech magnesium calmagite kit). Magnesium forms a colored complex with calmagite. EGTA (Ethylene Glycol Tetraacetic Acid) was used to eliminate any calcium interference. Each sample was analyzed in triplicate. The reactive solution consisted of one volume of reagent 1 (1 mol.L⁻¹ 2-Methyl-2-Amino-1-Propanol and 215 µmol.L⁻¹ EGTA) and one volume of reagent 2 (300 µmol.L⁻¹ calmagite). Three microliters of sample was dispensed into each well of a 96-well plate (Greiner, PS flat-bottomed microplate) and 300 µL reactive solution added. After shaking and 30 s of incubation, absorbance was measured at 500 nm in a Tecan microplate reader (Infinite M200, Tecan) and the magnesium concentration determined by comparing the values to those of a standard reference curve.

2.7. STEM-EDX of asbestos fibers

TEM (Transmission Electron Microscopy) images were recorded with a JEOL 2100 microscope, with a 200-kV potential applied to a LaB6 filament as an electron source. Resolution of the TEM was 0.21 nm. TEM mapping was performed in STEM (scanning transmission electron microscopy) mode (resolution 2 nm), using an SSD-EDX (silicon drift detector-energy dispersive X-ray) spectrometer to determine the chemical composition.

2.8. Statistical analysis

Results presented in all figures correspond to the mean values of three to five replicates. Significant statistical differences between values were determined using the Kruskal-Wallis test (R version 1.0.153) followed by a Conover post-hoc analysis. Values with the same letter are not significantly different ($p \geq 0.05$).

3. Results and discussion

3.1. Role of pyoverdine and pyochelin in iron and magnesium dissolution from AC

We investigated the role of the *P. aeruginosa* siderophores PVD and PCH in iron and magnesium removal from AC using *P. aeruginosa* strains producing both endogenous siderophores (WT), no PVD (Δ PVD), no PCH (Δ PCH), or neither of the two siderophores (2Δ). Bacteria were incubated in iron-minimal CAA medium depleted of magnesium, supplemented or not with AC. In this medium, inoculated with an average started inoculum of

7.10⁴ CFU.ml⁻¹, weak growth occurred for all strains (7.10⁶ to 5.10⁷ CFU.ml⁻¹), whereas more significant growth occurred in the presence of AC (3 to 7.10⁸ CFU.ml⁻¹) (Supplementary Fig. 1). AC provided sufficient magnesium to sustain bacterial growth equivalent to that in the CAA control with sufficient magnesium. However, no difference between the growth of the WT and mutant strains was observed in the presence of AC. The iron and magnesium measurements presented in Fig. 1 are represented as the concentration of the element measured in the presence of AC subtracted from that measured in the presence of bacteria in CAA-Mg. Iron and magnesium dissolved from the AC was evaluated both in the supernatants and bacteria. Iron was dissolved from the AC in the cultures with WT, ΔPVD, or ΔPCH, with 0.54, 0.33, and 0.17 mg. L⁻¹ respectively, whereas no difference was observed for the 2Δ strain (0.08 mg. L⁻¹) relative to the CAA-Mg control (0.13 mg. L⁻¹) (Fig. 1A). The theoretical iron content after mineralization is 2.3 mg of Fe for 0.2 g of AC, which represents 117 mg.L⁻¹. The evolution of the pH varied from 7 at the beginning of the culture to 8.5 at the end of the cycle which favor the precipitation of iron hydroxydes, leading to a very low iron solubility in those conditions. During the culture, bacterial metabolism synthesizes various small organics acids and siderophores that will complex iron and other metals in order to keep them solubilized. In our experiment, the solubility of metal is correlated mainly to the presence of siderophore which will maintain metal in solution thanks to their high affinity. The absence of one of the two siderophores affected iron removal, with a stronger effect in the absence of PCH. There was thus a synergistic effect on iron dissolution in the WT strain in which both siderophores were produced. These results show the involvement of both siderophores in iron dissolution from waste.

Induction of the PVD and PCH pathways was followed by measuring the fluorescence of mCherry fused to PvdJ as an indicator of expression of the PVD biosynthetic pathway and PchA for that of the PCH biosynthetic pathway. There was weak induction after 40 h of incubation in CAA-Mg medium (1.10⁻⁷ and 3.10⁻⁸ U.A. fluorescence/UFC.mL⁻¹ for PvdJ and PchA respectively), which correlated with weak bacterial growth, as shown before. The CAA control showed optimal induction for both siderophore pathways (1.1 10⁻⁵ and 4.2 10⁻⁶ U.A. fluorescence/UFC.mL⁻¹ for PvdJ and PchA, respectively), whereas supplementation with AC resulted in strong repression for both PVD and PCH (4. 10⁻⁷ and 1.10⁻⁷ U.A. fluorescence/UFC.mL⁻¹ for PvdJ and PchA respectively) (Fig. 2). These results are in accordance with those for iron solubilization shown in Fig. 1A, which repressed the production of both siderophores. Moreover, these results are also consistent with the WT siderophore production measured in the previous experiment. Indeed, AC supplementation

decreased PVD and PCH production (6.5 μM and 9.7 μM , respectively) relative to that of the positive control in CAA (112.4 and 82.4 μM respectively) (Data not shown).

David et al. (2020b) studied mineral interactions with siderophore-producing *Pseudomonas* for the main asbestos species, chrysotile and amphiboles. Results showed direct evidence of the involvement of the bacterial siderophores PVD and PCH on fiber weathering for the three tested asbestos species, chrysotile, crocidolite, and amosite. Such a processes of bio-weathering could lead to a reduction in asbestos toxicity due to iron removal (Daghino et al., 2006; Martino et al., 2003) and may represent an alternative bioremediation strategy to solve this environmental problem. However, these studies focused only on raw asbestos and rocks. David et al. (2020a) recently investigated these new bio-weathering perspectives on asbestos waste. The same results were successfully obtained for FAW, with iron removal linked to a siderophore-driven mechanism. Moreover, new insights into asbestos waste management were obtained, with magnesium and iron being an elemental source to sustain bacterial growth. Despite the highly different matrix composition and texture of AC from that of FAW, our results demonstrate the ability of *Pseudomonas* to use AC as a nutrient source to acquire the necessary iron and magnesium required for bacterial metabolism.

Yao et al. (2013) suggested that ligands and organic acids, such as carboxylic acids or alcohols, metabolites produced by *Bacillus mucilaginosus*, could be responsible for the increased mineral dissolution of serpentine. The resulting interaction with this soil bacterium dissolved magnesium from the brucite portion of the chrysotile, inducing an effective bio-weathering process and degradation of the serpentinic mineral. In our study, if the magnesium contained in the asbestos assay (0.2 g) would be dissolved completely, it would represent 7.2 mg of magnesium corresponding to 360 mg.L^{-1} . In our experiment, we measured 0.95 to 1.86 mg.L^{-1} which is far lower than the magnesium content that could be released from asbestos fibers. However, there were no significant differences in magnesium solubilization between any of the strains and the control (Fig. 1B), confirming previous results obtained for FAW weathering, in which siderophores did not appear to influence magnesium dissolution (David et al., 2020a). Bacterial organic acids have already been shown to act primarily on rocks and therefore allow better accessibility to siderophore-specific chelation (Ahmed and Holmström, 2014). This synergistic effect may play an important role in altering asbestos.

3.2. Efficiency of various pyoverdines in iron extraction from AC

Our results showed the siderophore pathways of *P. aeruginosa* to be repressed in the presence of AC. We studied the effect of PVD on iron removal from AC waste by incubating

various PVDs, differing in their structure, with the material and measuring the dissolved iron after various times of incubation. PVDs belong to a large family that contains more than a hundred structures. Little data is available related to the specificity of each PVD towards iron and more generally to metal complexation. Indeed, PVDs are well-known to chelate iron but have also been shown recently to chelate other metals (Braud et al., 2009; Schalk et al., 2011). This may be beneficial if, for example, the asbestos came from a nickel-rich soil. Indeed, varying efficiency of iron removal from FAW by various PVDs has already been demonstrated (David et al., 2020c). It is thus important to understand the effect of PVD on AC waste to extend the bioremediation process, as AC represents most of the waste collected. PVDs bind to Fe^{3+} in a 1:1 stoichiometry (Albrecht-Gary et al., 1994) and structural differences correspond to variations in the length, nature, and presence of cyclic rings in the peptide chain, as well as the radical R (Meyer et al., 1998). We selected 10 PVDs from a bank of more than 100 and measured the kinetics of iron extraction from AC after adjusting them to a final concentration of 100 μM to determine the efficiency and optimal time of incubation. Assays were carried out with PVD-containing supernatants, since previous experiments showed the same iron extraction efficiency between purified PVDs and PVD-containing supernatants from chrysotile (David et al., 2020b). We measured the dissolved iron of the samples after 0, 2, 4, 8, 12, and 24 h. All tested PVDs dissolved iron within the first hours of incubation relative to the CAA medium (Fig. 3A). Within the first 2 h, the dissolution pattern distinguished two groups of PVDs, with one group showing rapid iron removal followed by a decrease between 2 and 4 h of incubation, probably linked to the binding of PVD to the fibers, as previously observed (Data not shown). The other group showed time-dependent dissolution for 24 h. After 2 h of incubation, the PVD from *P. syringae* dissolved 2.6 mg.L^{-1} and *P. putida* 2.3 mg.L^{-1} , whereas the concentration for the others varied from 0.9 to 1.9 mg.L^{-1} . At the end of the experiment, *P. syringae* was still the most efficient (3.1 mg.L^{-1}), whereas iron dissolution reached a plateau after 24 h for *P. putida* 2.2 mg.L^{-1} . Our results show that certain PVDs exhibit the same dynamics as the siderophore desferrioxamine, as the study of Mohanty et al. (2018) showed an iron extraction equilibrium at 24 h for raw chrysotile fiber weathered by desferrioxamine. Iron removal from AC was faster than that from FAW (David et al., 2020c), which can be explained by the granulometry of the waste, which was ground, thus increasing the capacity for surface exchange between the PVDs and asbestos, improving the extraction and accessibility of iron release. At the end of the experiment, 50% of the siderophore was in a PVD-Fe complex. Thus, the reaction involved

sufficient apo-PVD to extract iron, indicating that a limiting factor could be the time of contact.

We therefore continued the exposure with renewal cycles to 48 and 96 h with the PVD-containing supernatants. The PVD supernatant was maintained at a final concentration of 100 μM . We had already noticed the greater effect of the three PVD_{syringae}, PVD_{aeruginosa} and PVD_{monteilii} at 24 h, corresponding to the end point of the kinetic study, with no statistical differences between the three conditions (Fig. 3B). However, PVD_{syringae} and PVD_{aeruginosa} tended to be more efficient than the other tested PVDs, although the efficiency was not statistically different (PVD_{fluorescens 13525} and PVD_{mosselii} for 48 h and PVD_{fluorescens 13525} and PVD_{lini} for 96 h). At 48 h, the dissolved iron in the presence of PVD_{syringae} and PVD_{aeruginosa} was 2.7 and 2.8 mg.L^{-1} , respectively, whereas lower concentrations (1.7 to 2.1 mg.L^{-1}) were extracted for the others. For the renewal cycle of 96 h, PVD_{syringae} and PVD_{aeruginosa} resulted in iron removal of 2.9 and 3.2 mg.L^{-1} versus 1.3 to 1.7 mg.L^{-1} for the other PVDs. At the end of the renewal cycles, the siderophore treatment dissolved a total of 172 μg iron for PVD_{syringae} and PVD_{aeruginosa} and 96 μg for the less efficient PVD_{mandelii} versus 2 μg for the medium control. Interestingly, the efficiency of the PVDs varied depending on the type of ACW. Indeed, we previously showed that PVD_{mandelii} was the most efficient for iron removal from FAW (David et al., 2020c), whereas it showed poor activity with AC. The affinity constant is well known for the well-studied PVD_{aeruginosa} but there is little available data for the other PVDs. Our unpublished data lead us to believe that the affinity constant between PVDs is not sufficiently different to explain our results. However, the 3D structures vary between PVDs and there may be structures that can more easily access the structural iron present in the layers of chrysotile. Longer exposure did not increase the iron extracted but the efficiency was almost constant, even though the quantity of apo-PVD remained sufficiently high (Fig. 3B). Therefore, the limiting factor is the availability of iron from the fibers and matrix.

The asbestos fibers contained in FAW and AC consist of chrysotile. The amount of iron solubilized from AC (3 mg.L^{-1}) was higher than that from FAW (1 mg.L^{-1}). These results were confirmed by mineralization of each type of waste, which showed that the amount of iron in AC (12 mg of iron per waste gram) is higher than in FAW (5 mg of iron per waste gram). FAW contains 90% chrysotile, whereas AC contains only 10%. Thus, part of the iron necessarily comes from the cement matrix of AC, which appears to also be bioavailable.

3.3. Long-term weathering of AC by pyoverdines

Short-term experiments showed that iron can successfully be extracted from AC after each renewal with a PVD solution. Iron still appeared to be bioavailable after three renewal cycles, raising the question of how many cycles could still be performed to reach the limit of extraction. We conducted a long-term AC-weathering experiment by renewing the apo-PVD solution every 24 h for 20 cycles. We performed this experiment using PVD_{syringae} and PVD_{aeruginosa}, given their previously observed equivalent iron release. Not surprisingly, iron was poorly dissolved in the CAA medium (0.01 to 0.5 mg.L⁻¹), as already shown. Both PVDs dissolved iron from AC throughout the 20 cycles, with a steady decrease from T24-1 to T24-11 cycles from 3.4 to 1.6 mg.L⁻¹ for PVD_{aeruginosa} and 3.0 to 1.4 mg.L⁻¹ for PVD_{syringae} (Fig. 4). The rate of dissolution then remained stable from T24-11 to T24-20 (the end of the renewals) at 1.0 mg.L⁻¹ iron. The cumulative iron amount removed from waste was 0.75 mg solubilized by PVD_{aeruginosa} and 0.65 mg by PVD_{syringae} at the end of the experiment. Thus, such a weathering process could be pursued for some time, as iron was still being extracted after 20 cycles. This batch experiment was not continued, given that it is time consuming, but this process provides interesting perspectives for a bioremediation strategy and the development of eco-friendly asbestos treatment, avoiding the continuous disposal of landfills with ACW. The same experiments carried out on FAW showed that PVD extraction was limited to 16 renewal cycles with 100 µM PVD. When the exposure time was increased to 96 H, dissolution was higher for seven cycles and the extraction limit was rapidly attained (David et al., 2020c). For the sampling used in our assay, PVD-mediated degradation resulted in extraction of 34 and 27% of the iron from AC by PVD_{aeruginosa} and PVD_{syringae}, respectively, whereas the extraction of 12% for FAW was measured with PVD_{aeruginosa} and PVD_{mandelii}. This higher efficiency may be due to the grinding of AC waste, as already discussed for the kinetics experiments. Considering the heat-energy consumption of grinding, the effectiveness of this biological treatment should be tested on ungrinded samples to develop an ecological process. Several studies have focused on siderophore dissolution mechanism for asbestos fibers. Mohanty et al. (2018) suggested that the siderophore concentration could have an effect on the efficiency of iron extraction. Indeed, at low concentrations, most of the siderophore may be adsorb onto the asbestos, as already demonstrated for other minerals, such as PVD on smectite (Ferret et al., 2014). Increasing the siderophore concentration would allow better siderophore surface coverage and may increase iron removal. Renewal cycles primarily dissolve the iron from the matrix, the iron from the fiber surface, and that in the brucite layer, with later access to the silica layer, for which iron may be slow to be released, as suggested by Mohanty et al. (2018) and David et al. (2020c). Further studies would provide

a better understanding of iron dissolution and confirm these hypotheses that suggest a prolonged incubation time and higher siderophore concentrations.

Analysis by STEM-EDX of the chrysotile fibers resulting from the long-term weathering of AC (Fig. 5) showed a large decrease of iron content for each PVD treatment. In AC waste, iron can be present in both chrysotile fibers and the cement matrix. Our results (Fig. 3) already demonstrate the bioavailability of iron from AC and these results confirm that iron is released from the fiber structure, as well as that of the matrix, thus confirming the active removal of iron driven by PVD. The magnesium content also decreased after treatment with PVD_{aeruginosa}. This can be explained by a synergistic effect between iron and magnesium dissolution. Indeed, iron extraction from chrysotile fibers may destabilize the brucite layer and thus allow the release of magnesium. However, the magnesium content did not decrease after treatment with PVD_{syringae}, probably linked to the heterogeneity of iron substitution in fibers. However, given the heterogeneity of iron substitution in fibers, the iron extraction does not necessarily affect the dissolution of magnesium as observed with PVD_{syringae} treatment.

4. Conclusion

AC is a worldwide concern due to the considerable amount of waste generated as a consequence of asbestos removal. We aimed to investigate the role of siderophore-producing *Pseudomonas* in AC bio-weathering and to evaluate the efficiency of various PVDs to release iron from AC waste. *Pseudomonas* was able to use AC as a nutrient source for its required iron and magnesium for bacterial metabolism, as already demonstrated for FAW. We clearly showed the involvement of the siderophores PVD and PCH in iron dissolution from AC waste. Indeed, the absence of one of the two siderophores significantly reduced the iron removal from asbestos waste. The interaction of the bacteria with AC waste resulted in repression of the siderophore biosynthetic pathways, demonstrating the bioavailability of iron. Moreover, all 10 PVDs selected from PVD diversity were able to dissolve a significant amount of iron from AC, but with differences in efficiency depending on the PVD. Overall, PVD_{aeruginosa} and PVD_{syringae} extracted the highest amount of iron from AC waste. Long-term treatment of AC by these two PVDs showed a large decrease in iron content from the chrysotile fiber structure, with no extraction limit after 20 days of renewal cycles. In AC waste, crocidolite and amosite can also be found in low amounts, in addition to chrysotile fibers. Given the ability of PVDs to progressively extract iron from both amphibole and chrysotile fibers, the biodeterioration of ACW by siderophore-producing *Pseudomonas* could contribute to the development of the eco-friendly management of asbestos waste to reduce

asbestos-related environmental and health problems together with the disposal of ACW in landfills.

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Figure and table legends

Fig. 1. Concentration of iron (A) and magnesium (B) dissolved from asbestos cement after 40 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), a pyoverdine-deficient strain (Δ PVD), a pyochelin-deficient strain (Δ PCH), or a pyoverdine- and pyochelin-deficient strain (2Δ). Elements were measured in the bacterial cells (pellet) and supernatants. Error bars indicate the standard errors of the means of three replicates. Bars with the same letter are not significantly different ($p \geq 0.05$, Kruskal-Wallis test, three replicates).

Fig. 2. Fluorescence of PvdJ-mCherry and PchA-mCherry after 24-h growth of *Pseudomonas aeruginosa* PAO1 in casamino acids medium with (CAA) or without magnesium (CAA-Mg), in the presence or absence of asbestos cement (FIBRO). Error bars indicate the standard errors of the means of three replicates. Bars with the same letter are not significantly different ($p \geq 0.05$, Kruskal-Wallis test, three replicates).

Fig. 3. Concentration of iron dissolved from asbestos-cement waste in the presence of 100 μ M of various pyoverdine-containing supernatants (SN) during a 24-h kinetics experiment (a) and after renewal cycles to 48 h and 96 h (b). Histogram bars of the 24 h timepoint (b) correspond to the endpoint of the kinetics experiment. Error bars indicate the standard errors of the means of five replicates. Bars with the same letter are not significantly different ($p \geq 0.05$, Kruskal-Wallis test, five replicates).

Fig. 4. Iron removal from asbestos-cement waste after 24-h renewal cycles in the presence of 100 μ M (T24-1 to T24-20) *Pseudomonas syringae* or *Pseudomonas aeruginosa* pyoverdine-containing supernatants (SN). Error bars indicate the standard errors of the means of five replicates.

761

762 **Fig. 5.** STEM images and STEM mapping of chrysotile fibers from asbestos-cement waste
763 after 24-h renewal cycles in the presence of casamino acids medium (A and B), *Pseudomonas*
764 *syringae* (C and D), or *Pseudomonas aeruginosa* (E and F) pyoverdine-containing
765 supernatants (SN). Large images obtained from the combination of the distributions of Mg,
766 Si, and Fe, with analysis areas (A, C and E). Atomic ratios of Mg/Si and Fe/Si of various
767 areas (B, D and F) and total area (H). Mass percentage of magnesium, silicon, and iron after
768 renewal cycles with casamino acids medium, *Pseudomonas syringae* or *Pseudomonas*
769 *aeruginosa* pyoverdine-containing supernatants (G).

770

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

772

773 **Table 2.** List of pyoverdines used, with their molecular mass, isoelectrofocusing
774 characteristics, and peptide composition.

775

- Pyoverdine and pyochelin are important in iron removal from asbestos cement.
- Pyoverdines exhibited various weathering efficiency in iron extraction.
- No extraction limit reached after pyoverdine alteration cycles.
- Asbestos waste weathering by pyoverdine decrease Fe content in fibers.
- Pyoverdines could be used in bioremediation process of asbestos waste.

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

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Strains	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>	PAO1; <i>pvdjmcherry</i>	(David <i>et al.</i> , 2020b)
<i>pchAmcherry</i>	PAO1; <i>pchAmcherry</i>	(Cunrath <i>et al.</i> , 2015)

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Table 2. List of pyoverdines used, with their molecular mass, isoelectrofocusing characteristics, and peptide composition.

Species	Siderovars	MM	Number of isoforms and pHi values		Pyoverdine peptide chain	References
<i>P. mandelii</i>	SB8.3	1 046	3	9,0/8,8/7,6	<u>Ala</u> -Lys-Thr- <u>Ser</u> -AOHOrn-cOHOrn	(Budzikiewicz <i>et al.</i> , 2014)
<i>P. fluorescens</i>	CHA0	1 287	3	8,5/7,5/5,1	Asp-FOHOrn-Lys-(Thr- <u>Ala</u> - <u>Ala</u> -FOHOrn-Lys)	(Wong-Lun-Sang <i>et al.</i> , 1996)
<i>P. putida</i>	12633	1 336	3	4,6/4,2/4,0	Asp-εLys-OH <u>Asp</u> -Ser-Thr- <u>Ala</u> - <u>Glu</u> -Ser cOHOrn	(Persmark <i>et al.</i> , 1990)
<i>P. mosselii</i>	Lille 17	-	3	9,0/7,4/5,2	ND	(Dabboussi <i>et al.</i> , 2002)
<i>P. lini</i>	B10/PL9/7SR1/A225	989	3	7,5/5,2/4,6	εLys-OH <u>Asp</u> -Ala-a <u>Thr</u> -Ala-cOHOrn	(Teintze <i>et al.</i> , 1981)
<i>P. putida</i>	90-51	1 234	3	7,3/7,0/5,3	Asp-εLys-OH <u>Asp</u> -Ser-Gly-a <u>Thr</u> -Lys-cOHOrn	(Sultana <i>et al.</i> , 2000)
<i>P. fluorescens</i>	13525	1 160	3	8,7/7,3/7,1	<u>Ser</u> -Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser)	(Hohlneicher <i>et al.</i> , 2014)
<i>P. monteilii</i>	Lille 1	1 291	2	7,3/4,6	Asp-Lys-AcOHOrn-Ala-Ser-Ser-Gly-Ser-cOHOrn	(Meyer <i>et al.</i> , 2008)
<i>P. aeruginosa</i>	PAO1	1 333	2	8,8/7,0	<u>Ser</u> -Arg- <u>Ser</u> -FOHOrn-(Lys-FOHOrn-Thr-Thr)	(Briskot <i>et al.</i> , 1989)
<i>P. syringae</i>	Syr	1 123	2	5,0/4,0	εLys-OH <u>Asp</u> -Thr-(Thr-Ser-OH <u>Asp</u> -Ser)	(Jülich <i>et al.</i> , 2014)

εLys: lys linked by its ε-NH₂; OHAsp: threo-β-hydroxy-aspartic acid; aThr: allo-Thr; cOHOrn: cyclo-hydroxy-ornithine; FOHOrn: δN -formyl- δN -hydroxy-ornithine; AOHOrn δN -acetyl- δN -hydroxy-ornithine.

Parentheses indicate cyclic structures. D-amino acids are underlined. A broken line means that the two enantiomers were detected among the underlined residues. ND: not determined.

Fig. 1. Concentration of iron (A) and magnesium (B) dissolved from asbestos cement after 40 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), a pyoverdine-deficient strain (Δ PVD), a pyochelin-deficient strain (Δ PCH), or a pyoverdine- and pyochelin-deficient strain (2 Δ). Elements were measured in the bacterial cells (pellet) and supernatants. Error bars indicate the standard errors of the means of three replicates. Bars with the same letter are not significantly different ($p \geq 0.05$, Kruskal-Wallis test, three replicates).

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A

B

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In color online

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Fig. 5. STEM images and STEM mapping of chrysotile fibers from asbestos-cement waste after 24-h renewal cycles in the presence of casamino acids medium (A and B), *Pseudomonas syringae* (C and D), or *Pseudomonas aeruginosa* (E and F) pyoverdine-containing supernatants (SN). Large images obtained from the combination of the distributions of Mg, Si, and Fe, with analysis areas (A, C and E). Atomic ratios of Mg/Si and Fe/Si of various areas (B, D and F) and total area (H). Mass percentage of magnesium, silicon, and iron after renewal cycles with casamino acids medium, *Pseudomonas syringae* or *Pseudomonas aeruginosa* pyoverdine-containing supernatants (G).

In color online

Fig. 1: Enumeration of wildtype *Pseudomonas aeruginosa* PAO1 strain (WT), a pyoverdine-deficient strain (ΔPVD), a pyochelin-deficient strain (ΔPCH), or a pyoverdine- and pyochelin-deficient strain (2Δ) after 40 h in casamino acids medium without magnesium (CAA-Mg) in the absence or presence of asbestos cement (FIBRO). Error bars indicate the standard deviations of the means of three replicates.

1 **Graphical Abstract**

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5 **In color online**