Communication

Phymabactin Facilitates the Growth of the Legume Symbiont *Paraburkholderia phymatum* in Aluminium-Rich Martian Soil and Acts as a Bioremediation Agent

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Posted Date: 8 April 2025

doi: 10.20944/preprints202504.0592.v1

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Phymabactin Facilitates the Growth of the Legume Symbiont *Paraburkholderia phymatum* in Aluminium-Rich Martian Soil and Acts as a Bioremediation Agent

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Abstract: Beneficial interactions between nitrogen-fixing soil bacteria and legumes offer a solution to increase crop yield on Earth and potentially in future Martian colonies. In this study, we assessed the growth of the beta rhizobium *Paraburkholderia phymatum* in Martian simulant soil using Enhanced Mojave Mars Simulant 2 (MMS-2) that contains a high amount of iron (18.37 percent by weight) and aluminium (13.07 percent by weight). We observed that while *P. phymatum* wild-type's growth was not affected by exposure to MMS-2, a mutant strain impaired in siderophore biosynthesis (Δ*phmJK*) grew less than *P. phymatum* wild-type on gradient plates prepared with increasing proportions of MMS-2 or aluminium concentration. This result suggests that the *P. phymatum* siderophore phymabactin alleviates aluminium-induced heavy metal stress. Using Ultra-high performance liquid chromatography-mass spectrometry (UHPLC MS), we showed that phymabactin can bind to aluminium more efficiently than iron. These results not only deepen our understanding of the behaviour of rhizobia in simulated extraterrestrial environments but also provide new insights into the potential use of *P. phymatum* for bioremediation and the multiple roles of the siderophore phymabactin.

Keywords: siderophore; rhizobium; bioremediation; metal; space; agriculture

1. Introduction

The prospect of extraterrestrial colonization requires the implementation and optimization of space farming to ensure that human settlements are as self-reliant as possible [1,2]. Indeed, supplying essential nutrients or goods to space settlers is increasingly difficult and expensive because of the planet's great distance from Earth [1]. In addition, access to fresh and vitamin-rich food is crucial to human physical and psychological health [3,4]. Single-cell-based nutrition is critical in the long term due to its high nucleic acid content, which leads to uric acid formation and health problems such as gout or kidney stone formation in humans [5,6]. These concerns, therefore, motivate the need to develop and professionalize space farming. Yet, crops typically require the supplementation of nitrogen-based synthetic fertilizers to produce sufficient yields for human consumption [7,8]. One way to circumvent this issue and provide plants with soluble nitrogen is to take advantage of the beneficial relationship between legumes and nitrogen-fixing rhizobia [9]. Rhizobia are soil bacteria that can intracellularly colonize specialized legume root structures called nodules, where they convert atmospheric nitrogen into ammonia that can be used by the plant [9,10]. Nitrogen is the most limiting factor for plant growth and development since it is essential for synthesizing nucleic and

 doi:10.20944/preprints202504.0592.v1

amino acids, proteins, and chlorophyll [11,12]. Certain rhizobia also produce phytohormones like auxins and brassinosteroids that stimulate plant growth and root development [13–17]. Additionally, rhizobia can also have a health-protective effect on crops, either by directly stimulating their immune system or indirectly by preventing phytopathogen growth [18–20]. For example, some rhizobia produce siderophores that sequester iron from their microenvironment and prevent phytopathogens from obtaining it, thus inhibiting their growth [21]. Moreover, siderophores-producing soil bacteria were shown to protect plants against heavy metals-induced oxidative stress [22]. Indeed, certain types of siderophores can bind to heavy metals like aluminium, cadmium, copper, lead, and zinc, hence alleviating the stress induced by heavy-metal contamination in soil and thereby improving plant growth [23,24].

Paraburkholderia phymatum STM815^T is a good model to study legume symbiosis, as this beta-rhizobium can nodulate more than 50 different legume species, including crops of human interest such as common bean and cowpea [15,25–27]. *P. phymatum* is also highly competitive against other soil bacteria in nodulating legume roots and shows remarkable abilities to survive abiotic stresses like those induced by salt or drought [15,26,28]. Importantly, we recently showed that *P. phymatum* can grow well in simulated microgravity and identified the *phm* gene cluster responsible for producing the hydroxamate-type siderophore phymabactin [29].

To determine the survival of *P. phymatum* under conditions that mimic extraterrestrial life, we tested its growth on a Martian soil simulant using the Enhanced Mojave Mars Simulant 2 (MMS-2). Indeed, data collected by different Mars exploration programs (Viking, Pathfinder, Spirit, and Opportunities) showed that heavy metals are present in high amounts on the surface of Mars [30,31]. It is estimated that there is between 18.5 and 21.7 percent by weight (wt%) iron on the Martian crust, while aluminium accounts for between 7.3 and 12.3 wt% [30]. By comparison, iron accounts for roughly 7 wt% and aluminium for 8 wt% of Earth's crustal composition [32,33]. This study shows that a previously constructed *P. phymatum* strain, unable to produce the siderophore phymabactin, grew less than the wild-type strain when exposed to iron and aluminium-rich MMS-2. However, while the *phm* mutant was also growth-impaired in a medium supplemented with aluminium (102.4 mM), in a medium supplemented with 92 mM iron, the *phm* mutant was not affected in growth, suggesting that phymabactin is mainly neutralizing the toxic effects of aluminium. Finally, mass spectrometry analyses revealed that phymabactin extracted from the supernatant of *P. phymatum* can bind to aluminium even stronger than to iron. These results suggest that phymabactin production is not only beneficial for iron scavenging but also for binding to other heavy metals, thereby making *P. phymatum* a suitable candidate for space farming in Martian soil and bioremediation of aluminium-rich soils.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Cultivation

The bacterial strains and the antibiotics used in this study are listed in Supplementary Table S1. *P. phymatum* STM815 strains were grown in Luria-Bertani (LB) rich medium prepared without salt (LB-NaCl) or in AB minimal medium [34] with 15 mM of succinate (Sigma-Aldrich, St. Louis, MO, USA) as a carbon source. LB-NaCl was supplemented with 40 g/L of enhanced Mojave Mars Simulant 2 (MMS-2; The Martian Garden, Austin, Texas) to grow *P. phymatum* in artificial Martian soil. Trimethoprim was used to select the *P. phymatum phmJK* mutant (100 μg/mL).

2.2. Preparation of Linear Gradient Plates

P. phymatum's growth was tested on MMS-2 using the linear gradient plate technique [35]. For this, two agar layers were poured into 12 cm square plates. First, 20 mL of LB-NaCl supplemented with 40 g/ L of MMS-2 or with 102,4 mM of $AlCl_3$ (Sigma-Aldrich, St. Louis, MO, USA) or 92 mM $FeCl_3$ (Sigma-Aldrich, St. Louis, MO, USA) was poured into tilted squared plates to form the bottom layer. After roughly 20 minutes, the second layer containing 20 mL of LB-NaCl was poured on the

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plate horizontally to cover the bottom layer. In this way, the proportion of MMS-2 or the concentration of $AlCl_3$ or $FeCl_3$ gradually increased along the horizontal axis. Pre-cultures of tested strains were grown in LB-NaCl media, washed twice, and set to an OD_{600} of 0.5. Sterile cotton swabs were used to draw lines on the gradient plates, alternating the strains to avoid variations due to plate irregularities. The plates were incubated for 48 hours at 28 °C.

2.3. Sample Preparation for Siderophore Screening Analysis

Bacterial supernatants of *P. phymatum* wild-type and Δ*phmJK* were prepared in triplicates and subjected to a siderophore screening analysis by UHPLC-MS. Therefore, 200 µL of bacterial supernatant was frozen using liquid nitrogen and lyophilized overnight in a vacuum concentrator at 8 °C. The dry lyophilizate was reconstituted in 300 µL of H_2O/CH_3OH (3:2) and centrifuged for 10 min at 5 °C and 14'000 rpm. Three LC-MS vials were filled with 100 µL of supernatant. One vial was spiked with 1 µL of an aqueous 100 mM $FeCl_3$ solution, another vial was spiked with 1 µL of an aqueous 100 mM $AlCl_3$ solution, and the last vial was not spiked. These samples were analysed by UHPLC-MS to investigate the formation of iron-phymabactin and aluminium-phymabactin complexes.

2.4. UHPLC-MS Method

Samples were analysed using a Vanquish Horizon UHPLC system (Thermo Fisher, Waltham, MA, USA) connected to a timsTOF Pro HR-QTOF mass spectrometer (Bruker, Bremen, Germany). The Vanquish Horizon UHPLC system was built from a binary pump H, a split sampler HT, and a temperature-controlled column compartment. Chromatographic separation was performed at 40 °C with an ACQUITY HSS T3 UPLC column (100 Å, 1.8 µm particle size, 2.1×100 mm, Waters, Milford, USA). The injection volume was 1 µL. The mobile phase consisted of A: Ultrapure H_2O + 0.1% HCOOH and B: CH_3CN + 0.1% HCOOH. A constant flow rate of 0.5 mL/min was applied using the following gradient: (I) 5% B isocratic from 0.0 to 0.5 min; (II) linear increase to 45% B from 0.5 to 6.0 min; (III) linear increase to 100% B from 6.0 to 6.1 min; (IV) 100% B isocratic from 6.1 to 9.0 min; (V) linear decrease to 5% B from 9.0 to 9.1 min; (VI) 5% B isocratic from 9.1 to 12.0 min. The mass spectrometer was operated in the positive ESI mode at 4500 V capillary voltage and 500 V endplate offset with an N_2 nebulizer pressure of 2.2 bar and a dry gas flow of 10 L/min at 220 °C. Mass spectra were acquired in a mass range from *m/z* 20 to 1300 at a resolution of 40'000 (*m/z* 431 full width at half maximum) and a 12 Hz acquisition rate. Mass measurements were externally calibrated between *m/z* 118 and 1222 using an ESI-L Low Concentration tuning mix (Agilent, Santa Clara, USA). Internal mass calibration was performed at the beginning of each LC run between *m/z* 91 and 1247 using a 10 mM solution of sodium formate that was injected using a 6-port valve with a 20 µL loop giving a mass accuracy below 2 ppm.

3. Results

3.1. The Siderophore Phymabactin Is Important for the Growth of P. phymatum in Martian Soil and in Aluminium-Rich Medium

We previously showed that *P. phymatum* STM815^T grows in simulated microgravity. In this study, we explore i) the ability of this strain to grow on an iron and aluminium-rich Martian soil simulant (MMS-2) and ii) the importance of *P. phymatum* siderophore phymabactin under these conditions. For this purpose, wild-type and the siderophore mutant Δ*phmJK* were grown on LB-NaCl gradient plates containing an increasing proportion of the Martian soil simulant MMS-2. In contrast to *P. phymatum* wild-type, a strain unable to produce the siderophore phymabactin (Δ*phmJK*) was not able to grow when cultivated with a high proportion of MMS-2 (Figure 1a), as well as in the presence of high concentrations of aluminium (Figure 1b). However, *P. phymatum* wild-type and Δ*phmJK* showed a similar growth on gradient plates prepared with 92 mM of $FeCl_3$ (Figure 1c). As a control,

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both wild-type and mutant strains were inoculated on LB-NaCl plates and showed no difference in growth (data not shown).

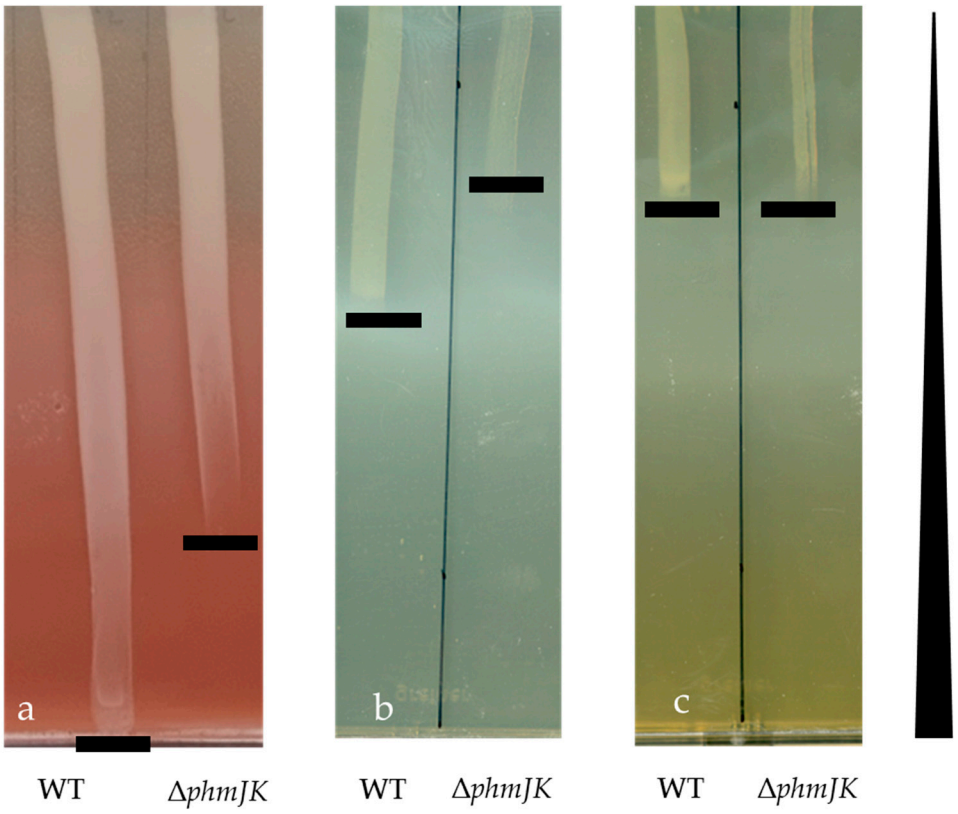

Figure 1. Representative figure of P. phymatum wild-type (WT) and a siderophore mutant (ΔphmJK) grown on linear gradient plates prepared with LB-NaCl agar medium supplemented with **(a)** 40 g/L of MMS-2, **(b)** 102.4 mM of $AlCl_3$, and **(c)** 92 mM $FeCl_3$ for the bottom layer. LB-NaCl agar medium was used for the upper layer. The plates were incubated for 48 hours at 28°C. Three (n=3) biological replicates were performed. The black line shows where the cells stop growing, referring to a specific concentration on a steadily increasing gradient from the top to the bottom.

3.2. Phymabactin Chelates Fe(III) and Al(III)

Supernatants of P. phymatum wild-type and ΔphmJK cells grown in minimal medium ABS prepared without iron were subjected to a siderophore screening analysis by UHPLC-MS. As shown in Figure 2, specific retention times, mass-to-charge ratio (m/z) shifts, and characteristic isotopic pattern distributions were observed for the complexation of phymabactin (Figure 2a) with iron (Figure 2b) and aluminium (Figure 2c). While the unbound siderophore had a retention time of 4.25 minutes, the Fe(III)-phymabactin complex displayed a retention time of 4.14 min, and the aluminium-phymabactin complex had a retention time of 3.90 min. The Al-phymabactin complex was detected in the P. phymatum sample spiked with $FeCl_3$ or $AlCl_3$, whereas the iron-phymabactin complex was only observed in the P. phymatum sample spiked with $FeCl_3$. No siderophores or siderophore-metal complexes were detected in the supernatant of ΔphmJK cells (Figure 2d). These results suggest that phymabactin is binding to both metals with a stronger affinity to aluminium.

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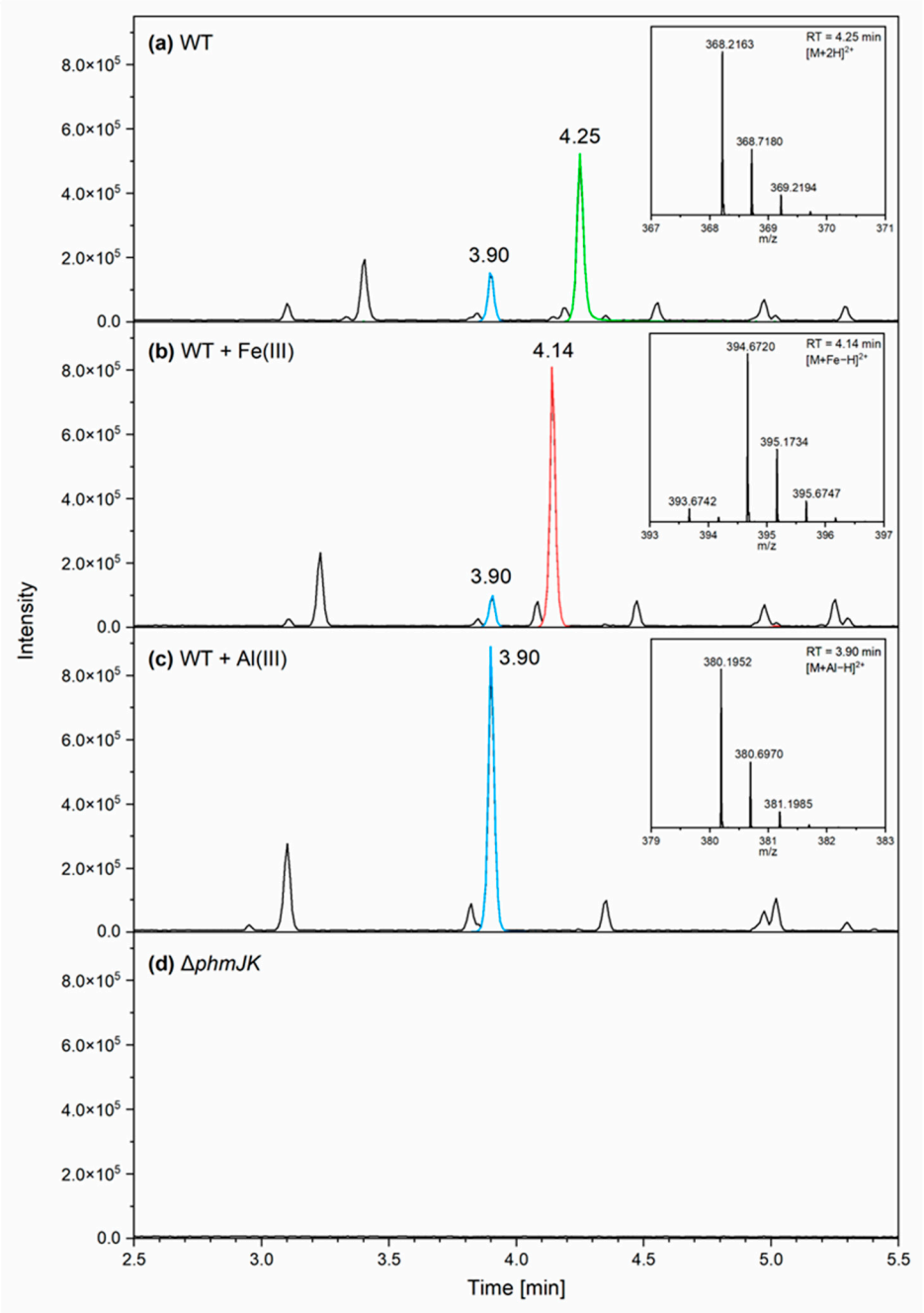

Figure 2. UHPLC-MS base peak chromatograms of **(a)** P. phymatum wild-type (WT), **(b)** WT spiked with $FeCl_3$, **(c)** WT spiked with AlCl3, and **(d)** ΔphmJK. Phymabactin was eluted at a retention time (RT) of 4.25 min, the Fe(III)-phymabactin complex at an RT of 4.14 min, and the Al(III)-phymabactin complex at an RT of 3.90 min. The respective mass spectra of the double-charged molecular ions are shown in the boxes on the right side.

4. Discussion

P. phymatum is a soil bacterium that displays important traits such as reducing atmospheric nitrogen into ammonium in symbiosis with plants and in free-living conditions. Moreover, this strain can enter symbiosis with an unusually large number of plants, produces plant-like hormones, and is very resistant to abiotic and biotic stresses. We have recently shown that *P. phymatum* can grow well even under simulated microgravity conditions, making it an ideal candidate for space farming. In this study, we tested the growth of *P. phymatum* strains in Martian simulant soil (MMS-2), which is rich in iron (18.4 wt%) and aluminium (13.1 wt%). Indeed, Mars contains a high percentage of metals such as iron (Fe_2O_3; 19.2 wt%), aluminium (Al_2O_3; 9.4 wt%), chromium (Cr_2O_3; 0.5 wt%), and

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magnesium (MgO; 8.7 wt%) [36–38]. In comparison, on Earth, there are 4.7 wt% of Fe, 8.1 wt% of Al, 0.008 wt% of Cr, and 1.9 wt% of Mg [39]. In our study, we observed that the siderophore produced by *P. phymatum* (phymabactin) contributed to the growth of this strain in the Martian soil (Figure 1a). To test if the difference in growth between *P. phymatum* wild-type and a phymabactin mutant was due to the high concentration of heavy metal present in MMS-2, we grew the strains on linear gradient plates containing increasing concentrations of iron and aluminium, up to the same final concentration present in MMS-2. To our surprise, the siderophore phymabactin was found to be important for growth with high aluminium concentrations but did not play a role in *P. phymatum* fitness in an iron-rich environment (Figure 1c). This result may be explained by an inhibitory effect of high iron concentrations on *P. phymatum* siderophore production. Indeed, a potential ferric uptake regulation (Fur) binding sequence is localized upstream of the gene cluster responsible for phymabactin production [29], suggesting that the transcriptional repressor Fur binds to the *phm* promoter and regulates intracellular iron homeostasis. A similar effect has been observed in *Burkholderia cenocepacia* 715J, where the production of the siderophore ornibactin is negatively affected by iron supplementation [40]. The fact that Δ*phmJK* grows less than the wild-type strain in aluminium-rich media (Figure 1b) suggests that the metal does not hinder siderophore production and that aluminium is the component that prevents the growth of Δ*phmJK* in Martian simulant soil. Furthermore, we also observed that Δ*phmJK* grew less than *P. phymatum* wild-type when cultured in an aerated liquid medium and exposed to other metals such as zinc, lead, and copper, showing a similar behaviour after the addition of aluminium (Supplementary figure 1a-1d). Previous studies showed that a *Burkholderia cenocepacia* H111 siderophore mutant (not producing pyochelin and ornibactin) was affected in growth in the presence of these divalent cations [41]. *P. phymatum* wild-type grew even in the highest concentration of iron- and aluminium-rich Martian soil (Figure 1a-1c). This result can be explained by the fact that, in MMS-2, iron and aluminium are present as ferric oxide (Fe_2O_3) and aluminium oxide (Al_2O_3). In contrast, in the gradient plates assay, iron and aluminium were added as $FeCl_3$ and $AlCl_3$, respectively. Indeed, ferric oxide and aluminium oxide are less soluble than their chloride counterpart at neutral pH and, therefore, release fewer ions in their microenvironment, reducing their toxicity to microorganisms [42,43]. We showed here that phymabactin binds to aluminium and iron with retention times of 3.90 minutes and 4.14 minutes, respectively (Figures 2 b and 2c), suggesting that -in addition to the iron scavenging function. *P. phymatum* phymabactin relieves the cell of metal-induced stress.

Siderophores-producing soil bacteria have been shown to reduce heavy metal toxicity by capturing the metal in soil or increasing plants' systemic resistance, making them more resilient [22]. Heavy metals such as copper, cadmium, or lead are human activity's common byproducts and phytotoxic compounds that cause plant growth inhibition and even death [44]. Yet, soybean grown in lead-supplemented growth media and inoculated with the siderophore-producing and plant growth-promoting rhizobacteria (PGPR) *Pseudomonas putida* K9P9 showed a 70.6% and 28.6% increase in shoot and root length, respectively, compared to the uninoculated control [45]. A similar effect was observed in *Paraburkholderia fungorum* BRRh-4 that alleviated the stress of rapeseed grown in cadmium-contaminated soil and induced a 40% increase in height compared to the uninoculated control [46].

In the future, studies looking at the symbiotic and nitrogen-fixation efficiency of *P. phymatum* with legumes cultivated in MMS-2 or growth media containing heavy metals will help to understand the role of PGPR in bioremediation. Finally, this study provided new insights into the potential of *P. phymatum* as an inoculant in extraterrestrial environments such as Martian soil. It also revealed the role of the siderophore phymabactin as a possible bioremediation tool to protect plants from heavy metals, both on Earth and in extraterrestrial environments.

5. Conclusions

Our study unveiled the significance of *P. phymatum*'s siderophore production in its ability to thrive in Martian-like soil. We used Martian soil simulant media and showed that *P. phymatum* strains

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capable of producing phymabactin had a growth advantage compared to a mutant strain lacking this ability. This growth advantage is attributed to the high aluminium content in the simulated Martian soil. Notably, phymabactin demonstrated a superior capacity to bind aluminium ions compared to iron, as confirmed through UHPLC-MS analysis. These findings reveal that beyond its nitrogen-fixing capabilities across diverse legume species, *P. phymatum* demonstrates remarkable potential for thriving in the metal-rich soils characteristic of Mars and in decontaminating soils with high metal content.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Supplementary Figure S1; Supplementary Table S1; Supplementary Table S2.

Author Contributions: The following author contributions were made. Conceptualization: D.G. and G.P. Methodology: D.G, L. Bu., L. Bi and G.P. Investigation: D.G. and L. Bu. Data analysis: D.G., L. Bu., M.E., L. Bi. and G.P. Visualization: D.G., L. Bu., M.E. L. Bi. and G.P. Funding acquisition: M.E., L. Bi. and G.P. Writing: D.G., L. Bu., M.E., L. Bi. and G.P.

Funding: This work was supported by the Swiss Space PRODEX proposal FISURI (ESA PRODEX ARRANGEMENT No 4000144510) to Marcel Egli and the Swiss National Science Foundation (SNFS) with project number 310030_215282 to Gabriella Pessi.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Bernd Rattenbacher for providing MMS soil. We acknowledge Yilei Liu and Julie Ahrens for their help in preparing microbiology material. We would like to thank Christian Schwander for his support in optimizing the chromatographic method as part of his bachelor's thesis.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MMS-2	enhanced Mojave Mars Simulant 2
UHPLC MS	Ultra-high performance liquid chromatography-mass spectrometry
wt%	Percent per weight

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