Metal-specific differences of the exometabolites produced by serpentinite-associated *Pseudomonas chlororaphis* S15

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Abstract. The endolithic bacteria found in serpentinite rocks are a promising resource for biotechnological application. The present study investigates the metal-specific response of exometabolites produced by the serpentinite-isolated strain *Pseudomonas chlororaphis* S15. The integration of HPLC metabolomic profiling with CAS assay analysis has been demonstrated to reveal that the strain employs highly specific adaptive strategies. While both Co^{2+} and Ni^{2+} suppressed the core exometabolome, Co^{2+} was found to uniquely induce the *de novo* synthesis of a novel compound. In contrast, Fe^{3+} and Ga^{3+} were found to stimulate metabolite production and siderophore activity. These findings reveal metal-specific metabolic plasticity in *P. chlororaphis* S15, which has significant potential for the development of targeted environmental biotechnologies.

1 Introduction

Serpentinite is a metamorphic rock formed through the hydration and metamorphic transformation of ultramafic rocks from the Earth's mantle [1]. The serpentinite soils are characterized by high concentrations of heavy metals and deficient in essential plant nutrients like nitrogen, phosphorus, potassium, and sulfur [2, 3]. These extreme conditions provide a distinctive ecological niche for endolithic microbial communities. The endolithic bacteria adapted to serpentinite niche demonstrate a high resistance to heavy metals and the capacity to produce siderophores, and represent a valuable resource for understanding extremophile biology and for developing innovative biotechnological applications.

Pseudomonas chlororaphis is a well-known rhizobacterium that has been extensively studied for its potential application in plant growth-promoting (PGP) properties and its capacity in the context of bioremediation and agriculture [4]. Members of the *Pseudomonas* genus have been observed to produce a diverse secondary metabolite, including siderophores, antibiotics, and pigments [5]. Among these exometabolites, siderophores are of particular interest. The ability of bacteria to survive in conditions of iron limitation is entirely dependent upon their capacity to produce high-affinity iron-chelating compounds. The siderophores perform a variety of functions, including iron scavenging for the

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bacterium and plants, direct inhibition of phytopathogens by sequestering iron, and induction of systemic resistance in plants [6]. Furthermore, bacterial siderophores, through the process of complexation of heavy metals, have been demonstrated to reduce metal toxicity and enhance plant growth in contaminated soils [6]. Although the capacity of *P. chlororaphis* to produce siderophores such as pyoverdine, pyochelin, has been thoroughly documented [7], a detailed understanding how individual heavy metals impact on siderophore profile shifts remains largely unexplored. Therefore, the goal of this study was to investigate the metal-dependent alterations in the siderophore profile of the endolithic strain *Pseudomonas chlororaphis* S15 using a combination of HPLC and CAS assay analysis.

2 Materials and Methods

2.1 Isolation and Identification

The strain S15 was isolated from a colony on Luria Agar (LA) agar that had been plated with aqueous rinsate of crushed serpentinite. Genomic DNA from strain S15 was extracted from an overnight LB-grown culture using the phenol-chloroform method. The 16S rRNA gene (1500 bp) was amplified using polymerase chain reaction (PCR), and then sequenced using instrument ABI 3730 DNA Analyzer (Life Technologies, USA) following Sanger's method. The bacterial sequences were analyzed using the Basic Local Alignment Search Tool (BLASTn).

2.2 Cultivation conditions

P. chlororaphis S15 was cultivated in fresh Luria-Bertani (LB) broth (composition in g/L: tryptone (10), yeast extract (5), NaCl (5)) at 30°C with shaking (250 rpm) for overnight. Cells were harvested, washed with 0.9% NaCl solution and added into flasks containing M9 medium (composition in g/L: Na_2HPO_4 (6.77), KH_2PO_4 (3), NH_4Cl (1), NaCl (0.5), pH 7.0) with solutions of heavy metals. The initial cell concentration was adjusted to an optical density (600 nm) of 0.1.

2.3 Heavy metal resistance

P. chlororaphis S15 resistance to heavy metals was carried out in LB broth containing different concentrations (0.25 – 15.0 mM) of the test heavy metal salts ($CoCl_2 \times 6H_2O$, $AlCl_3$, $ZnSO_4$, $MnSO_4 \times H_2O$, $NiSO_4 \times 7H_2O$, $FeCl_3 \times 6H_2O$, $CuSO_4 \times 5H_2O$ and $GaBr_3$). Incubation was performed at room temperature for 24 h. All experiments were performed in duplicates.

2.4 Metabolites extraction and HPLC analysis

P. chlororaphis S15 was cultivated at an initial optical density of 0.1 at 600 nm in flasks containing M9 medium (composition in g/L: Na_2HPO_4 (6.77), KH_2PO_4 (3), NH_4Cl (1), NaCl (0.5), pH 7.0) with solutions of heavy metal salts at 30°C with shaking at 200 rpm for 120 h. All experiments were performed in duplicates.

Stock solutions of heavy metal salts ($CoCl_2 \times 6H_2O$, $AlCl_3$, $ZnSO_4$, $MnSO_4 \times H_2O$, $NiSO_4 \times 7H_2O$, $FeCl_3 \times 6H_2O$, $CuSO_4 \times 5H_2O$ and $GaBr_3$) were prepared and sterilized using a 0.22µm polyethersulfone (PES) filter. The concentrations of Fe^{3+}, Cu^{2+}, and Ga^{3+} were 10 µM; those of Co^{2+} and Al^{3+} were 20 µM; those of Zn^{2+} and Ni^{2+} were 100 µM; and those of

Mn^{2+} were 400 μM. Additionally, the M9 medium was enriched with 0.3% casamino acids, along with solutions of $MgSO_4 \times 7H_2O$ and $CaCl_2$ at final concentrations of 246 μM and 100 μM, respectively. The control experiment was performed in M9 supplemented with 50 μM 2,2'-bipyridyl. All experiments were performed in duplicates.

The exometabolites of *P. chlororaphis* S15 grown in the presence of heavy metals were extracted from a 120-hour culture liquid. The biomass was then removed by centrifugation (9000 rpm, 20 min), the supernatant was filtered through a 0.22 μm PES membrane (Millipore Express PLUS Stericup, USA) and subjected to solid-phase extraction on C18 cartridges (Supelco Discovery DSC-18, USA). The metabolites were eluted with 100% methanol. The samples were concentrated using a rotary evaporator (Concentrator Plus, Eppendorf, Germany) at a temperature not exceeding 45°C. The dry residue was resuspended in 50% aqueous methanol.

HPLC analysis was performed on an UltiMate 3000 UHPLC system (Thermo Scientific Dionex, USA) using an Acclaim® PolarAdvantage II (PA2) C18 reversed-phase column (250 × 4.6 mm, 5 μm). A binary gradient was applied at a flow rate of 1 mL/min using mobile phases: (A) water with 0.01% TFA and (B) acetonitrile:water (80:20, v/v) with 0.01% TFA. The elution profile was as follows: 0-40 min, linear increase of phase B from 0-60% vol; 40-50 min, increase 60-100% vol of phase B; 50-55 min, isocratic incubation at 100% B; 55-60 min, return to initial conditions (0% B) followed by column re-equilibration. Detection was performed using a photodiode detector (260 nm) and a fluorescence detector (470/530 nm).

2.5 Iron-binding activity of metabolites

The metal-binding activity of extracellular metabolites produced by *P. chlororaphis* S15 in the presence of heavy metals was analyzed using chrome azurol S (CAS) agar [8]. The appearance of orange halos surrounding the wells was interpreted as an indication of iron-binding activity.

3 Results and Discussion

The endolithic strain S15 was isolated from serpentinite from the Khalilovsky massif, Russia. The results of the BLAST analysis of the 16S rRNA sequencing indicated that S15 exhibited a high degree of similarity to *Pseudomonas chlororaphis* ATCC 174151, with a similarity score of 100%. The tolerance of *P. chlororaphis* S15 to heavy metals was determined by testing a range of concentrations for individual metals (Table 1). The strain was found to be most tolerant to aluminum and most sensitive to cobalt.

Table 1. The minimum inhibitory concentration (MIC) of heavy metals for *P. chlororaphis* S15.

Heavy metal ions	Co^{2+}	Ni^{2+}	Ga^{3+}	Cu^{2+}	Fe^{3+}	Zn^{2+}	Mn^{2+}	Al^{3+}
MIC (mM)	≥ 3.0	≥ 4.0	≥ 5.0	≥ 6.0	≥ 7.0	≥ 7.0	≥ 7.0	≥ 9.0

Despite the high heavy metal tolerance exhibited by *P. chlororaphis* S15, concentrations that have previously been demonstrated to be optimal for siderophore production by *P. aeruginosa* ZGKD3 were used [9]. The HPLC analysis of the exometabolome of *P. chlororaphis* S15 revealed a conserved core profile under most conditions, yet with distinct, metal-specific alterations (Figure 1A). The metal-free control exhibited six major metabolites. A key finding was the universal suppression of the metabolite at RT 13.0 min by all metals tested, suggesting it represents a highly metal-sensitive pathway. In contrast, the profile was largely unaltered by 20 μM Al^{3+}, 10 μM each of Fe^{3+} and Ga^{3+} with the latter two even slightly stimulating the production of metabolites

at RT 16.3 and 17.7 min. Notably, 20 µM Co^{2+} and 100 µM Ni^{2+} triggered the most significant shifts, including the inhibition of core metabolites and, in the case of cobalt, the de novo synthesis of a compound at RT 42.0 min, indicating a potential detoxification or adaptive response. The exposure of *P. chlororaphis* S15 to 10 µM Cu^{2+} and 100 µM Zn^{2+} resulted in a general reduction of its exometabolites.

Fluorescence detection revealed that Co^{2+} and Ni^{2+} ions similarly inhibited metabolite production relative to the control (Figure 1B). Furthermore, Zn^{2+}, Mn^{2+}, and Cu^{2+} ions exhibited a mixed effect, simultaneously inhibiting one metabolite while stimulating another (Figure 1C). In contrast, Al^{3+}, Fe^{3+}, and particularly Ga^{3+} ions significantly stimulated the production of metabolites (Figure 1D).

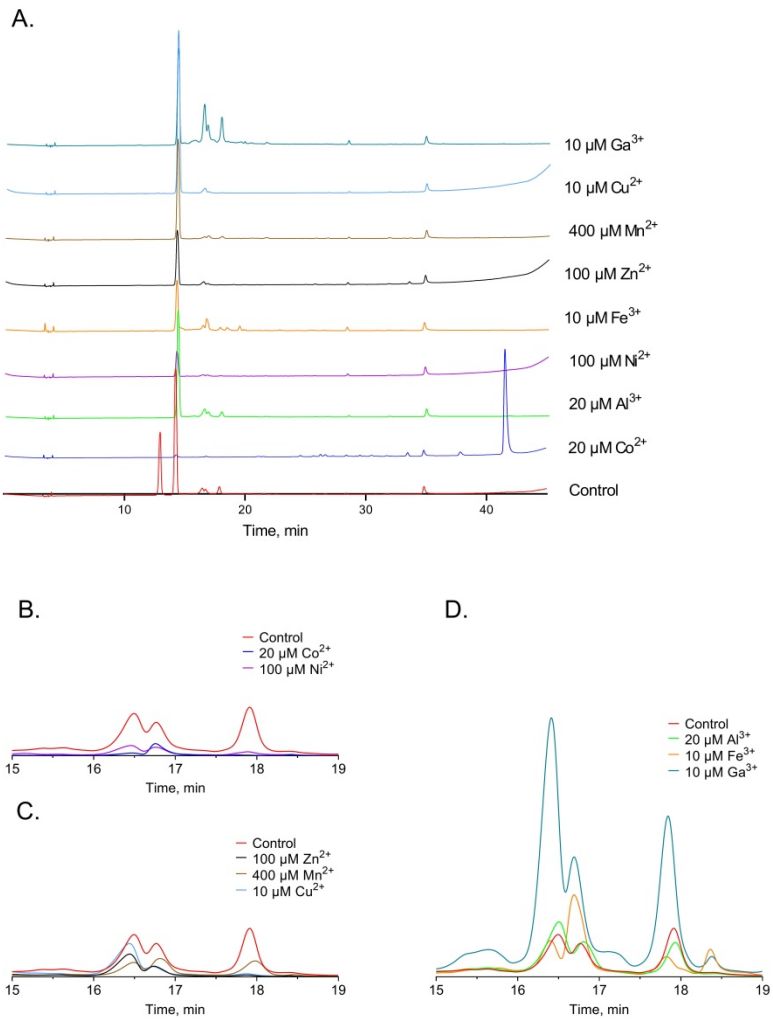

Fig. 1. The exometabolomic profile of *P. chlororaphis* S15 grown in the presence/absence of heavy metals. A. – The HPLC chromatogram detected at 260 nm, and B, C, D – fluorescence emission profile (excitation/emission 470/530 nm detection). Lines correspond to the following treatments: Control (red); Co^{2+} (blue); Al^{3+} (bright green); Ni^{2+} (violet); Fe^{3+} (orange); Zn^{2+} (black); Mn^{2+} (brown); Cu^{2+} (light blue); Ga^{3+} (turquoise).

The findings of the present study indicate an inhibitory effect of the tested concentrations of Co^{2+}, Ni^{2+}, and Cu^{2+}, on the exometabolome of *P. chlororaphis* S15. As demonstrated in previous studies, the presence of cadmium and zinc has been shown to induce the production of pyoverdine by *P. aeruginosa* strains [10-12]. As well, ion metals such as Al^{3+}, Cu^{2+}, Ga^{3+}, Mn^{2+}, Ni^{2+} and Zn^{2+} have been reported to induce pyoverdine I type production in *P. aeruginosa* PAOI [10]. Conversely, ferric iron and gallium have been observed to enhance *P. chlororaphis* S15 exometabolite production, a process that is likely to involve siderophores such as pyoverdine.

In recent studies, exposure to lead has been observed to enhance the synthesis of pyoverdine in *Pseudomonas* spp. [13]. It has been demonstrated that the production of a pyoverdine in *P. chlororaphis* O6 is increased upon treatment with ZnO nanoparticles (NPs), while the production of siderophores is inhibited by CuO NPs [14]. However, the synthesis of pyochelin in *P. aeruginosa* PAO1 was suppressed by 10 μM of Fe^{3+} and Co^{2+}, as well as by 100 μM of Mo^{6+}, Ni^{2+} and Cu^{2+} [15].

The CAS agar plate assay of the exometabolites revealed a metal-dependent variation in siderophore-mediated chelating activity (Table 2). No clearance zones were observed from the extracted exometabolites of *P. chlororaphis* S15 grown in the presence of Co^{2+}, Ni^{2+}, or Cu^{2+}, indicating a suppression of iron-chelating activity under these conditions. In contrast, exometabolites produced by S15 in the presence of Al^{3+}, Mn^{2+}, or Zn^{2+} formed a zone of uniform coloration, suggesting active siderophore production. A distinctive dual-zone pattern comprising an inner orange halo and an outer yellow zone was observed for exometabolites produced by S15 in the presence of Ga^{3+}. A similar pattern was also observed in the case of Fe^{3+} suggests that siderophore biosynthesis was not inhibited at this particular iron concentration.

Table 2. The CAS agar plate assay of siderophore activity of exometabolites extracted from *P. chlororaphis* S15 cultures supplemented with heavy metals.

Heavy metal ions	CAS assay phenotype
Control (no metals), Al^{3+}, Mn^{2+}, Zn^{2+}	Uniform yellow coloration
Ga^{3+}, Fe^{3+}	Dual-zone pattern (orange halo + yellow zone)
Co^{2+}, Ni^{2+}, Cu^{2+}	No color change or clearance zones

Thus, the integration of HPLC and CAS assay data reveals a graded response of *P. chlororaphis* S15 to metal stress. While Co^{2+} and Ni^{2+} act as general inhibitors of secondary metabolism. The effect of other metals, such as Cu^{2+} and Zn^{2+}, suppress the process to a certain extent and alter the specific output of chelating agents. Exposure to Al^{3+}, Ga^{3+}, and Mn^{2+} has been demonstrated to induce metabolic modification, resulting in the synthesis of a wide array of siderophores, as evidenced by the characteristic dual-zone patterns. This specificity may highlight the complex regulatory mechanisms employed by the strain to adapt to metal-contained media.

4 Conclusion

The findings of the present study demonstrate highly specific, metal-dependent changes to the exometabolites of *Pseudomonas chlororaphis* S15, thus revealing a spectrum of distinct metabolic responses. The strain demonstrates significant metabolic plasticity, exhibiting a complete inhibition of siderophore production in the presence of Co^{2+}, Ni^{2+}, and Cu^{2+}, and, conversely, active synthesis of chelators in the presence of Al^{3+}, Mn^{2+}, and Zn^{2+}. Of particular interest is the observation of Co^{2+}-induced *de novo* synthesis of a novel

compound (RT 42.0 min), suggesting the presence of a specialized detoxification mechanism. The observed gradient of metabolic responses, ranging from inhibition to stimulation, reflects the strain's evolutionary adaptation to its serpentinite niche. This finding offers promising avenues for developing metal-specific bioremediation strategies and other biotechnological applications.

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