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Effect of metals on a siderophore producing bacterial isolate and its implications on microbial assisted bioremediation of metal contaminated soils

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HIGHLIGHTS

- A unique catecholate siderophore producing *Bacillus amyloliquefaciens* strain.
- First report on *B. amyloliquefaciens* NAR38.1 siderophore binding to Fe^{+2} and Fe^{+3}.
- Variations induced by essential and abiotic metals on siderophore production.
- Implications of siderophore producing bacteria in improving phytoextraction.

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ABSTRACT

A bacterial isolate producing siderophore under iron limiting conditions, was isolated from mangroves of Goa. Based on morphological, biochemical, chemotaxonomical and 16S rDNA studies, the isolate was identified as *Bacillus amyloliquefaciens* NAR38.1. Preliminary characterization of the siderophore indicated it to be catecholate type with dihydroxy benzoate as the core component. Optimum siderophore production was observed at pH 7 in mineral salts medium (MSM) without any added iron with glucose as the carbon source. Addition of NaCl in the growth medium showed considerable decrease in siderophore production above 2% NaCl. Fe^{+2} and Fe^{+3} below 2 μM and 40 μM concentrations respectively, induced siderophore production, above which the production was repressed. Binding studies of the siderophore with Fe^{+2} and Fe^{+3} indicated its high affinity towards Fe^{+3}. The siderophore concentration in the extracellular medium was enhanced when MSM was amended with essential metals Zn, Co, Mo and Mn, however, decreased with Cu, while the concentration was reduced with abiotic metals As, Pb, Al and Cd. Significant increase in extracellular siderophore production was observed with Pb and Al at concentrations of 50 μM and above. The effect of metals on siderophore production was completely mitigated in presence of Fe. The results implicate effect of metals on the efficiency of siderophore production by bacteria for potential application in bioremediation of metal contaminated iron deficient soils especially in the microbial assisted phytoremediation processes.

1. Introduction

Siderophores, iron chelating agents, are produced by microorganisms under iron limiting conditions (Dhaenens et al., 1999; Vraspir and Butler, 2009; Braud et al., 2009a). Although iron is the key factor in regulating siderophore production, other factors such as pH, temperature, carbon source and other metals play an important role (Saha et al., 2012). While siderophores have an extremely high affinity for ferric iron they also form complexes with metals other than Fe^{3+}, although, with a lower affinity (Braud et al., 2009a). Metals other than iron are also reported to stimulate or repress siderophore production in bacteria (Braud et al., 2010). The production of siderophore by bacteria in presence of toxic metals implicates their potential in uptake, mobilization of heavy metals or developing metal resistance. Binding of siderophores to metals reduces the free metal concentration thereby restricting their diffusion across the porins. However, in the case where the receptor fails to recognize the actual complex of iron and siderophores, it can lead to intracellular metal accumulation (Schalk et al., 2011).

Heavy metal pollution of soils is a serious problem and demands efficient clean up of the polluted areas (McGrath et al., 1995). Most of the conventional methods used for soil remediation

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are uneconomical and result in deterioration of soil texture and its organic content (Rajkumar et al., 2010). One of the emerging technologies for bioremediation of metal contaminated soils is "microbial assisted phytoremediation", a process of utilizing plants to absorb, accumulate and detoxify contaminants in soil through physical, chemical and biological processes (Prasad et al., 2010) in the presence of Plant Growth Promoting Rhizobacteria (PGPR). These rhizhosphere bacteria are known to support the growth and sustenance of plants. Some of these bacteria are also metal resistant paving a way for the benign technology for reclamation of metal polluted soils. An important characteristic of PGPR for use as a bio-inoculant in this technology is their ability to produce siderophores (Jing et al., 2007). Therefore, for such a strategy to be viable, a better understanding of siderophore producing bacteria and their interaction with metals is required.

During our studies on siderophore producing bacteria from coastal ecosystems (Gaonkar et al., 2012), we isolated a *Bacillus* culture from mangrove sediments showing production of a siderophore in iron deficient conditions. We report here the characterization of the bacterial isolate and the alterations implicated by metals on growth and siderophore production. The metals selected were those which are essential for the metabolic activity like Zinc (Zn), Cobalt (Co), Copper (Cu), Molybdenum (Mo) and Manganese (Mn) and those which were non-essential toxic metals, termed as abiotic (Schalk et al., 2011), mainly, Arsenic (As), Lead (Pb), Aluminium (Al) and Cadmium (Cd).

2. Materials and methods

2.1. Isolation and identification of the isolate

Bacterial strain used in this study was isolated from sediment sample obtained from mangroves located at Ribander, Goa. The production of siderophore by the isolate was determined using Chrome azurol sulphonate (CAS) assay as described by Schwyn and Neiland (1987). To prepare 100 ml of CAS solution, 60.5 mg of CAS was dissolved in 50 ml of deionised water to which 10 ml of $FeCl_3{\cdot}6H_2O$ solution was added. 72.9 mg HDTMA (Hexadecyl Trimethyl Ammonium bromide) dissolved in 40 ml of deionised water was added to CAS to make the volume to 100 ml. The selected isolate NAR38.1 was grown on nutrient agar and its cultural, morphological and biochemical characteristics were noted. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1413 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16 S rDNA sequence was used to carry out BLAST (Altschul et al., 1990) with the nrdatabase of NCBI genbank database. Sequences were selected and aligned using multiple alignment software program Clustal X (Thompson et al., 1997). Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.2.1. Chemotaxonomic characterization by FAME analysis was performed by Gas Chromatography using MIDI Sherlock Microbial Identification System software, version 6.1.

2.2. Characterization of siderophore

All the glassware used in the study were soaked in 6 M HCl overnight and then repeatedly washed with deionised water to remove any metal traces. Mineral salts medium (MSM) (Parulekar and Mavinkurve, 2006) with 0.2% glucose was used for growth and siderophore production. The isolate was inoculated in MSM without $FeSO_4$ and incubated on shaker at 150 rpm and 28 °C for 24 h, the culture broth centrifuged at 4480 g for 10 min and the supernatant was subjected to following tests for determination of the functional group: Hydroxamate group was checked using Neiland's spectrophotometric assay (Neilands, 1981), Tetrazolium salt test (Snow, 1954) and Csaky assay (Gilliam et al., 1981). For Catecholate nature Neiland's spectrophotometric assay (Neilands, 1981) and Arnow's assay (Arnow, 1937) were used. Carboxylate nature was tested using Vogel's chemical test (Yeole et al., 2001) and spectrophotometric assay (Shenker et al., 1992).

2.3. Effect of pH and NaCl on growth and siderophore production

Effect of NaCl (1–5%) and pH (5–9) on growth and siderophore production was determined using MSM with variations in pH/NaCl and analyzed after 24 h of incubation. Growth was measured as increase in turbidity at 600 nm using a Shimadzu UV–Visible spectrophotometer (UV-2450). For siderophore analysis the supernatant was centrifuged at 4480 g for 10 min and cell free supernatant was analyzed using Arnow's test (Arnow, 1937). The presence of catechol was noted by the development of a red color and an increase in absorbance at 515 nm. The concentration of siderophore was determined using catechol as the standard.

2.4. Effect of iron on siderophore production

Flasks containing MSM with 0.2% glucose as the sole carbon source, supplemented with iron, in increasing concentrations (Fe^{+3} = 5, 10, 20, 30, 40 and 50 μM; Fe^{+2} = 1, 2, 3, 4 and 5 μM) were inoculated with 5% of exponential cells grown in the respective medium. All the culture flasks were incubated at 150 rpm at 28 °C and growth and siderophore production was monitored over a period of 72 h as described above.

To determine binding of siderophore to Fe^{+2} and Fe^{+3}, the siderophore was extracted from cell free supernatant of overnight grown culture. The pH of the supernatant was adjusted to pH 2 and siderophore was extracted with one-fifth the volume of ethyl acetate. The ethyl acetate was evaporated to obtain crude siderophore (Nair et al., 2007). The siderophore was dissolved in deionised water at a concentration of 10 μg/ml. To 0.5 ml of siderophore, 0.5 ml of Fe^{+2} (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) or Fe^{+3} (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) was added and the solution was centrifuged at 6797 g for 10 min to remove iron bound siderophore. The unbound siderophore in the solution was estimated using Arnow's test.

2.5. Effect of metal concentration on growth and siderophore production

Stock solutions of following metal forms: $MnSO_4$, $Co(NO_3)_2{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$, $Na_2HAsO_4{\cdot}7H_2O$, $Pb(NO_3)_2$, $Al_2(SO_4)_3{\cdot}16H_2O$, $CuSO_4$, $CdSO_4{\cdot}8H_2O$ and $Na_2MoO_4{\cdot}2H_2O$ were prepared and sterilized with 0.22 μM filters under aseptic conditions. These stock solutions were incorporated in the sterile medium (MSM without $FeSO_4{\cdot}7H_2O$) to adjust metal concentration to predetermined levels. Mn, Mo and Zn were tested at 125, 250, 500 and 1000 μM and Co, Cu, Cd, As, Pb and Al were tested at lower concentrations of 10, 20, 50 and 100 μM. The flasks were inoculated with 5% of exponential cells grown in MSM without any metal ions. All the culture flasks were incubated at 150 rpm at 28 °C and growth and siderophore production was monitored at 24 h as mentioned above.