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SHORT REPORTS

Isolation, identification and characterization of arsenic transforming exogenous endophytic *Citrobacter* sp. RPT from roots of *Pteris vittata*

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Abstract The aim of the present study was to assess the arsenic (As) transformation potential of endophytic bacteria isolated from roots of *Pteris vittata* plant. The endophytic bacterium was tested for minimal inhibitory concentration (MIC) against As. The endophytic strain RPT exhibited the highest resistance to As(V) (400 mg/l). Phylogenetic analysis of the 16S rRNA sequence suggested that strain RPT was a member of genus *Citrobacter*. The As transformation assay revealed As(III) oxidation and As(V) reduction potential of *Citrobacter* sp. RPT. The As resistance mechanism was further confirmed by amplification of *arsC* and *aoxB* genes. The growth kinetics of strain RPT was altered slightly in the presence of different concentration (100–400 mg/l) of As stress. Temperature and pH influenced the As removal rate. The maximum As removal was observed at pH 7.0 (74%) and 37 °C (70.9%). The results suggest that strain RPT can survive under the As stress and has been identified as a potential candidate for application in bioremediation of As in contaminated environments.

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Introduction

Arsenic (As) contamination in environment is a major problem in all over the modern industrialized world. It has received increasing attention due to its carcinogenic impacts on human health. Mining and metallurgical activities lead to As pollution in the environment (Zhu et al. 2014). Due to negative clinical effects of As contamination to humans warrants urgent issue for mitigation and sustainable environmental management. Several physical and chemical techniques have been developed for mitigation of As pollution from soil and water (Yuan and Chiang 2007; Fu and Wang 2011). Although the physico-chemical methods have extensive removal application, the high cost solid and liquid waste generation from the remediation process causes unsustainable environment (Tiwari et al. 2016). Therefore, it is necessary to expand the remediation process to reduce its effects on ecosystem.

Bioremediation is a promising technology to remove As from contaminated soil and water, which is cost-effective and eco-friendly compared to physico-chemical methods (Govarthanan et al. 2013, 2015a). Microorganisms are primarily used in the bioremediation to degrade or detoxify the As into harmless and less toxic forms. Several microbial processes involve the removal of As in soil and water, including oxidation, reduction, adsorption and microbe-mediated electron transfer (Diesel et al. 2009; Guo et al. 2015). Generally As is toxic to microorganisms; however, several microorganisms gain the energy for rapid growth by metabolizing the As (Oremland and Stolz 2003, 2005). The As-resistant microorganisms utilize the As derivatives

as an electron donor and/or electron receptors during the As detoxification process (Johnson et al. 2003). Several studies have reported the role of microorganisms in the transformation of As in aqueous systems (Pepi et al. 2007; Chang et al. 2008; Lievremont et al. 2009; Mirza et al. 2014).

Microbial detoxification of As mainly occurs in two ways, such as oxidation and reduction. It has been established that the bacteria reduce the As(V) to As(III) inside the cells through the *ars* operon mechanism and exclude As(III) by As(III) efflux pump. However, microbial As(III) oxidation has been described either as a detoxification mechanism or energy source for chemolithoauthotrophic bacteria (Santini et al. 2000). So far most endophytic bacteria isolated from *P. vittata* are capable of either As(V) reduction or As(III) oxidation have been isolated from As contaminated soil/water (Stolz and Oremland 1999; Stolz et al. 2002). However, to our knowledge, there is no report on the endophytic Gram-negative coliform bacterium *Citrobacter* sp. isolated from *P. vittata* grown in non-polluted soil that is able to reduce As(V).

Fern *Pteris vittata* L. is a native and widespread paleotropic As hyperaccumulator, considered the suitable candidate plant for the phytoremediation of As-contaminated soil (Ma et al. 2001). Its rapid growth, large biomass, wide and extensive root system, as well as a wide geographic distribution, justify the successful application of As remediation (Danh et al. 2014). However, the endophytic bacterial population in the roots of *P. vittata* from non-polluted soil has not been reported. Hence, the objectives of this study were (1) to isolate As-resistant endophytic bacteria from roots of *Pteris vittata* (2) to evaluate minimal inhibitory concentration of As, (3) to screen As transformation and oxidation and reduction assay, and (4) to optimize experimental variables, such as pH and temperature for enhanced As removal.

Materials and methods

Isolation of arsenic resistant endophytic bacteria from *Pteris vittata*

Healthy *Pteris vittata* plants were collected from Kolli Hills, part of the Eastern Ghats of Tamil Nadu, India. The site is located at 11.2485° latitude and a 78.3387° longitude ranges about 1000–1300 m in height. The collected *Pteris vittata* plant sample was washed with tap water followed by several rinses with sterile water (Govarthanan et al. 2016). The washed plant was dissected into roots and stems with a sterile scalpel. The root samples were surface sterilised with 95% ethanol and 3% hypochlorite solution for 2–3 min. The surface sterilised root samples were again washed several times in sterile water to remove the sterilization agents. The sterilised root tissue (1 g fresh weight) was ground in a mortar and pestle with 5 ml saline (0.85% NaCl). The saline suspension was serially diluted and plated using the spread plate technique onto Nutrient Agar (Hi-Media, India) plates. The plates were incubated at 35 ± 2 °C for 48 h and observed for bacterial growth on the agar surface. Morphologically distinct colonies were purified and stored at 4 °C for further studies.

Minimal inhibitory concentration of As

Arsenic tolerance levels of the isolates were assessed by agar-dilution method according to Kamala-Kannan and Krishnamurthy (2006). Briefly, the isolates were inoculated in nutrient agar plates containing different concentrations of As ranging from 50 to 400 mg/l. The plates were incubated overnight at 35 °C and observed for bacterial growth. The lowest concentration of the metal that completely inhibited the growth of the bacteria was considered as the minimal inhibitory concentration (MIC). Arsenic metal salts were added to the nutrient agar after autoclaving and cooling to 50 °C from filter-sterilised stock solutions.

Screening of arsenic transformation assay

Arsenite to As(V) transforming potential of the isolate was performed as described by Simeonova et al. (2004). Briefly, the isolate pre-grown on As(V) and As(III) nutrient agar plates were flooded with 0.1 M $AgNO_3$. In the presence As(III) in the medium was detected by a bright yellow precipitate formation. Negative test was represented by white colour formation (Tiwari et al. 2016).

Genomic DNA extraction and identification of As-resistant bacteria

DNA was extracted from the potential isolate RPT using QIAGEN (CA, USA) DNA extraction kit, and its concentration was determined using a UV–Vis spectrophotometer (NanoDrop 2000). Fragments of 16S rRNA were amplified using universal primers 27f (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492r (5′-CCCCGTCAATTCATTTGA GTTT-3′). The PCR product was purified using a QIAGEN PCR purification kit and sequenced using an automated ABI PRISM 3700 sequencer (USA). The obtained 16S rRNA sequence was compared against the available sequence database using BLAST program in NCBI website. The phylogenetic tree was constructed using neighbour-joining distance method by software Mega 6.0.

Effect of As on bacteria growth

Five millilitres of the mid-log phase cultures (10^8 cells/mL) of the isolate grown individually in nutrient broth is supplemented with different concentrations of As (100–400 mg/l). The flasks were incubated in a shaking incubator (200 rpm) at 37 °C, and the growth was measured at different time intervals according to the increase in optical density at 600 nm in a UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). Cultures grown in the absence of As were used as a control (Govarthanan et al. 2015b).

Amplification of As-resistant genes

The As transporting gene *arsC* was amplified using the primers amlt42-F (5′-TCG CGT AAT ACG CTG GAG AT-3′), and amlt376-R (5′-ACT TTC TCG CCG TCT TCC TT-3′) (Sun et al. 2004). The PCR conditions for the amplification were initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 45 s, 57 °C for 1 min, 72 °C for 2 min and a final extension step of 72 °C for 7 min. Arsenic oxidizing gene *aoxB* was amplified using the primers 69-F (5′-TGY ATY GTN GGN TGY GGN TAY MA-3′) and 1374-R (5′-TAN CCY TCY TGR TGN CCN CC-3′) (Rhine et al. 2006).

Removal of As at different pH and temperature conditions

The effect of pH on the removal of As was determined at various pH levels ranging from 6 to 9. In brief, 10 ml of the bacterial suspension was aseptically inoculated into 250 ml Erlenmeyer flasks containing 100 ml of As (initial metal concentration 50 mg/l). The flasks were incubated in a shaking incubator (200 rpm) at 37 °C and the samples were collected after 48 h of incubation. The samples were centrifuged (10,000 rpm for 5 min), filtered (0.22 µm) and analysed for the residual heavy metal concentration using atomic adsorption spectroscopy (AAS) (Thermo Scientific™ iCE™ 3500). Similarly, the effect of temperature was measured by incubating the flasks at different temperatures (30, 35, 37 and 40 °C). Flask without bacteria was used as a control for this experiment. All the experiments were repeated for three times and each sample was tested in triplicate.

Results and discussion

The As-resistant mechanism of the endophytic isolate *Citrobacter* sp. RPT isolated from roots of *P. vittata* grown in unpolluted soil was investigated in this present study. A total of three morphologically different endophytes were isolated. The isolated endophytes were further tested for As resistance by determining its minimal inhibitory concentration. The minimal inhibitory concentration of the As showed that the isolate RPT exhibited maximum resistance to As (400 mg/l). The other two isolates showed inadequate resistance to the As. Based on the minimal inhibitory concentration results, the isolate RPT was selected for further As resistance characterization studies. Morphological and 16S rRNA gene sequencing was carried out to identify the isolate. The 16S rRNA sequence of this strain showed 99% identity with *Citrobacter* sp. The partial 16S rRNA of the isolate RPT was deposited in GenBank (Accession no.: KX348779). A phylogenetic tree was derived from the partial 16S rRNA sequences of the isolate *Citrobacter* sp. RPT with existing sequences in the NCBI database, and the results are shown in Fig. 1. Several studies reported the As resistance mechanism of the endophytes isolated from *P. vittata* (Xu et al. 2014, 2016). However, there is no information about arsenic resistant endophytic *Citrobacter* sp. isolated from the roots of *P. vittata.*

The isolate RPT was further screened by silver nitrate assay on LB agar plates. Silver nitrate assay is aimed to check the As(III) to As(V) and As(V) to As(III) transformation ability of the isolate RPT. The quantitative reaction between AgNO3 and As(III) or As(V) results in the formation of coloured precipitates on the LB agar plates. The reaction between $AgNO_3$ with AsO_3 (AsIII) plates showed that the yellow precipitation on the LB plates. Whereas, the reaction between AsO_4^{3-} with $AgNO_3$ showed the brownish precipitation on the surface of the LB agar plates (Simeonova et al. 2004; Tiwari et al. 2016). The As transformation assay results indicated that the isolate RPT is able to oxidize As(III) and reduce As(V). The growth rate of the isolate RPT was evaluated in LB (1/4 strength) broth supplemented with different concentrations (100 and 400 mg/l) of As. The results (Fig. 2) indicate that the growth rate of the strain RPT decreases with increasing concentrations of As. The results are in agreement with previous studies reported the growth rate of As-resistant *Herbaspirillum* sp. GW103 was decreased by the increasing concentration of As in batch experiments (Govarthanan et al. 2015b). It is suggested that the direct exposure of the strain RPT to As and limited nutrient availability in the LB broth might have reduced the growth rate of the organism.

The strain RPT was screened for *arsC*, *aoxB* genes reported in the As oxidizing and reducing bacteria. Visible *arsC* band was clearly observed on the agarose gel surface, which indicates the presence of As transporting (arsenate reductase) in the strain RPT. The arsenate reductase gene arsC is the most important genes in association with As(III) oxidation and

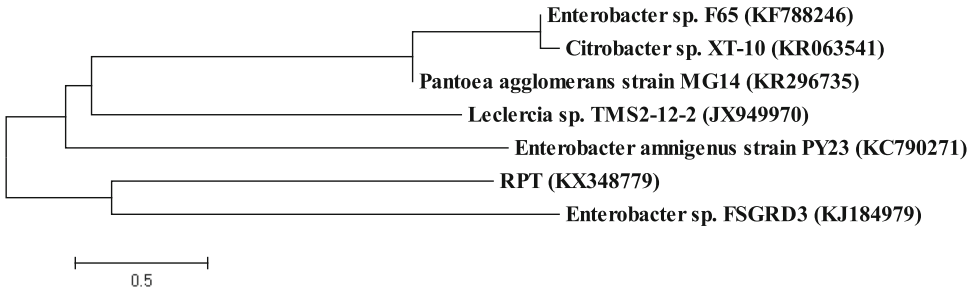

Fig. 1 A neighbour-joining tree constructed using Mega 6.0 showing the phylogenetic relationship of 16S rRNA sequence of isolated strain *Citrobacter* sp. RPT from closely related sequences from GenBank. Accession numbers at the GenBank of National Centre for Biotechnology Information (NCBI) are shown in *parenthesis*

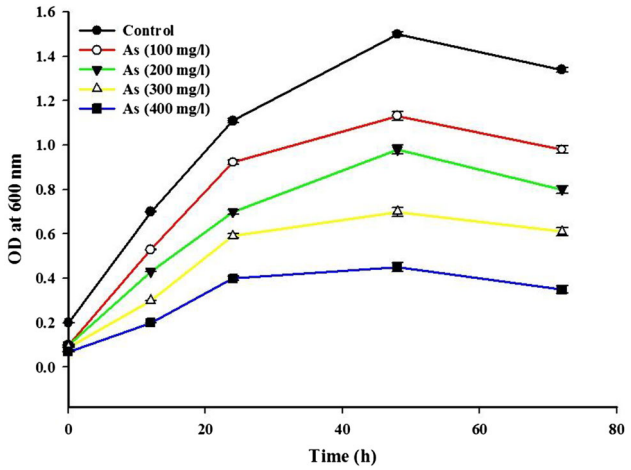

Fig. 2 Growth kinetics of *Citrobacter* sp. RPT using As at different concentrations (100–400 mg/l)

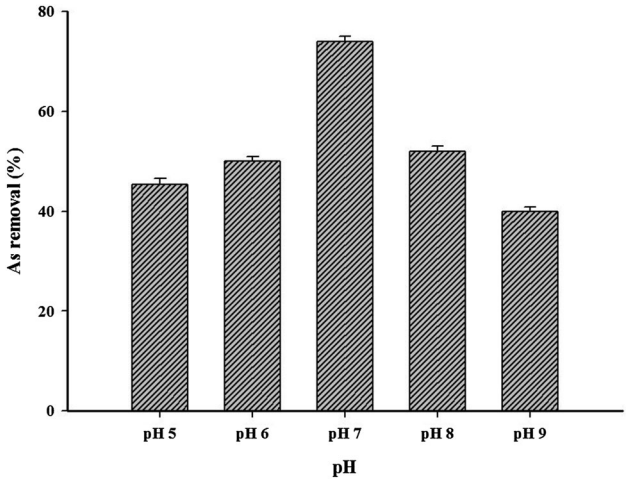

Fig. 3 Influence of pH on As removal by the isolate *Citrobacter* sp. RPT

As(V) reduction in soils by microbes (Han et al. 2017). For most bacteria, arsC-mediated As(V) reduction, followed by As(III) efflux to the external environment, is an important mechanism for As detoxification (Bhattacharjee and Rosen 2007). However, As detoxification in some bacteria is associated with As(III) oxidation by converting As(III) to less toxic As(V) (Stolz et al. 2010). No visible *aoxB* band was observed on the gel, which indicates that the strain RPT may harbour another type of As oxidizing protein or the primers (69-F and 1374-R) were inappropriate for the amplification of the *aoxB* gene present in the strain RPT.

Arsenic removal efficiency of the strain *Citrobacter* RPT at different pH levels was assessed and the results are presented in Fig. 3. The highest rate of As removal (74%) showed at pH 7.0, whereas the average levels of As removal showed at pH 5.0 (45.3%), pH 6.0 (50%), pH 8.0 (52%) and pH 9.0 (40%). It has been reported that in acidic pH, association of hydronium ions with the cell surface creates reduction of negative charge intensity on the bacterial cell wall results in the reduction or inhibition of the binding of metal ions (Khalid et al. 2011). Fourest et al. (1994) reported that the functional groups on the bacteria may become positively charged and may not interact with the metal ions at acidic pH. The decreased removal rate at alkaline pH can be attributed to the growth response of the isolate under pH stress. The limited growth of isolates at alkaline pH reduces the overall uptake of metals (Guo et al. 2010). The As removal efficiency of *Citrobacter* sp. RPT was tested with different temperature conditions, as temperature is an important parameter in biological oxidation and/or reduction of As, and the results are presented in Fig. 4. The results showed that the removal of As increased with increasing temperature up to 37 °C (70.9%), but the removal efficiency of As decreased slightly at 40 °C (25.3.9%). The biological removal of metals was highly influenced by temperature (Zhou et al. 2013). The results from this study indicated that pH and temperature play a major role in the survival of and As removal potential of the *Citrobacter* sp. RPT. The bioremediation rate of living metal-resistant bacteria should be strongly dependent on the population of cells at optimal growth conditions (Guo et al. 2010).

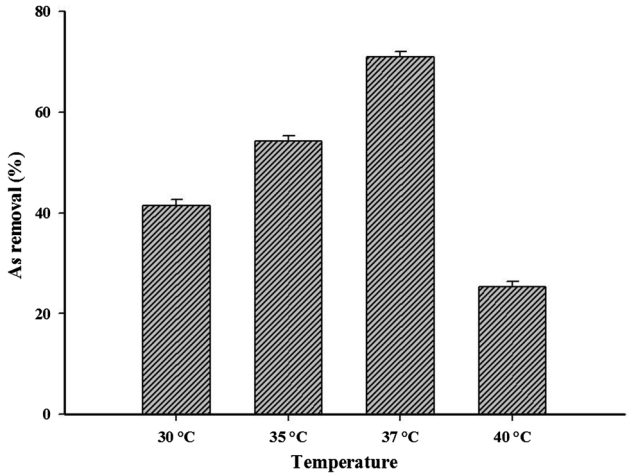

Fig. 4 Influence of temperature on As removal by the isolate *Citrobacter* sp. RPT

Conclusion

The endophytic bacteria *Citrobacter* sp. RPT isolated from *P. vittata* showed a significant resistance to As. The strain RPT grew in culture media with high As concentration (400 mg/l) under 37 °C at pH 7.0. The strain had ability to both As(III) oxidation and As(V) reduction. Bioremediation studies with batch experiments showed that the isolate RPT caused high removal of As (74%) at pH 7.0 and (70.9%) at 37 °C. The obtained results suggested that the exogenous endophytic bacteria would affect the As transformation in As-contaminated systems, and play a major role in the mitigation of As in the ecosystem.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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