European Journal of Soil Biology 62 (2014) 15–21

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Genetic diversity of endophytic bacteria of the manganese-hyperaccumulating plant *Phytolacca americana* growing at a manganese mine

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ARTICLE INFO

Article history:
Received 27 June 2013
Received in revised form
22 January 2014
Accepted 17 February 2014
Available online 13 March 2014
Handling editor: Kristina Lindström

Keywords:
Bacterial endophytes
Community structure
Heavy metal pollution
Phytoremediation
Phytolacca americana

ABSTRACT

Endophytic bacteria have great potential for assisting metal-hyperaccumulating plants in remediation of contaminated soils. However, little information is available on the composition of the endophytic bacterial community of the manganese (Mn)-hyperaccumulator *Phytolacca americana*. In this study, PCR-denaturing gradient gel electrophoresis was used to analyze the endophytic bacterial diversity and community composition in *P. americana* growing at an Mn mining site. Results showed that Mn had a significant impact on the bacterial diversity and community structure. Phylogenetic analyses of the recovered DNA sequences classified the bacteria into 10 different divisions, indicating a high level of diversity amongst the endophytic bacterial species of *P. americana*. Sequencing results demonstrated that Proteobacteria, specifically the γ, δ and α subclasses, may be the dominant endophytic bacterial genera of *P. americana*. Some unique sequence types that occurred exclusively in heavily polluted sites were worth investigating their effects on phytoremediation under Mn-contaminated soils.

1. Introduction

Contamination of soil with heavy metals has become a worldwide environmental problem, posing a significant threat to human health and ecological security. Phytoremediation, which is the use of plants to extract pollutants from soils, is regarded as an effective, nonintrusive, inexpensive, and socially accepted technology to remediate polluted soils [3,5]. Despite these advantages, many metal-hyperaccumulating plants grow slowly and are inhibited by high concentrations of heavy metals [38,52]. To solve these problems, plant-associated microbes have attracted much attention for their close relationships with host plants. Many studies have demonstrated that they can accelerate seedling emergence and promote plant establishment under adverse conditions, as well as enhance plant growth and development [8,49,54]. Combining hyperaccumulators with plant-associated microbes has more advantages than independent use of hyperaccumulators, and was proposed as one of the most promising green remediation techniques [1,7,44].

Endophytic bacteria are plant-associated microbes that ubiquitously inhabit most plant species and do not harm the host plants [41]. Furthermore, recent research demonstrated that they may play an important role in enhancing the tolerance of plants to heavy metals, and increase heavy metal translocation factors, biomass, and trace element concentrations of hyperaccumulators [9,16,27,53]. Thus, endophytic bacteria have great potential for assisting their host plants in remediation of contaminated soils and water [27,50]. Screening for strains of bacteria resistant to heavy metals is a key factor for future application. Endophytic bacteria are highly diverse [45], and plants may be able to select those species that benefit from growth under specific environmental conditions [15,43]. Meanwhile, endophytic bacteria are influenced by the physicochemical properties of the soil and have evolved with the progress of heavy metal contamination [7,36]. Thus, studies of the abundance and composition of bacterial endophytes in the field are essential not only for understanding their interactions with the environment, but also for exploring the possible uses of these bacterial species for the bioremediation of heavy metals.

Exposure of animals to excess manganese (Mn) causes Mn toxicity, including Parkinson-like symptoms and abnormalities of the reproductive and immune systems [13,34,48]. Excess Mn in soil can also harm forest and agriculture ecosystems [11,21]. *Phytolacca americana* is a recently discovered Mn-hyperaccumulating plant

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http://dx.doi.org/10.1016/j.ejsobi.2014.02.011
1164-5563/

Table 1
Soil physicochemical properties from the three Mn mine spoils and one adjacent reference area.

Samples	TMn (mg/kg)	EMn (mg/kg)	pH	SOM (g/kg)	TP (%)	EP (mg/kg)	TN (%)	EN (mg/kg)
M1	501 ± 9 a	75 ± 6.6 a	7.41 ± 0.21 a	50.70 ± 4.59 a	0.14 ± 0.01 a	8.42 ± 0.58 a	0.24 ± 0.01 a	122.8 ± 6.0 a
M2	38,838 ± 1097 b	7165 ± 580 b	7.23 ± 0.18 a	48.71 ± 2.93 a	0.12 ± 0.01 a	8.57 ± 0.84 a	0.22 ± 0.01 b	116.8 ± 7.7 a
M3	76,897 ± 3269 c	10,672 ± 1998 c	7.52 ± 0.23 a	55.16 ± 4.59 a	0.14 ± 0.02 a	8.80 ± 1.85 a	0.11 ± 0.01 c	66.2 ± 5.4 b
M4	88,030 ± 1266 d	11,437 ± 1220 c	7.44 ± 0.09 a	54.52 ± 2.07 a	0.14 ± 0.01 a	8.50 ± 0.95 a	0.06 ± 0.01 d	33.9 ± 8.5 c

Abbreviations: TMn, total Mn concentration; EMn, extractable Mn concentration; SOM, soil organic matter; TP, total P; EP, extractable P; TN, total N; EN, extractable N. Only the means in the same column are compared. The values marked by different letters were significantly different at a 5% confidence level. Data are means ± S.E.M.

that has great potential for remediation of Mn-contaminated soils [12,31,35]. To our knowledge, there is little information on the composition of the endophytic bacterial community of *P. americana* growing in mines or soils highly polluted by Mn. Analysis of the endophytic bacterial diversity is conductive to the biotechnological application of *P. americana* in combination with endophytic bacteria in Mn-contaminated soil. Molecular techniques, such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and the combination of fingerprints with cloning/sequencing are proven to be more effective and less biased for the analysis of endophytic bacterial community than cultivation-dependent methods [37,40]. The aim of this study was to investigate the diversity of the endophytic bacterial populations in the tissues of *P. americana* growing in Mn mine by PCR-DGGE. The results of this study provide valuable information on the unique endophytic bacterial species of *P. americana*, and should aid in development of this hyperaccumulator for remediation of Mn-contaminated soils.

2. Materials and methods

2.1. Sampling of plants and soil

Sampling was carried out in the Xiang Tan Mn mining area situated in south central Hunan Province, China (27°53′–28°03′N, 112°45′–112°55′E), where mining and smelting operation ceased in 1913. Based on the distribution of the slag heaps, three Mn mine spoils were selected: a slag heap (site M4), a tailing dam with smelting wastes and wastewater (site M3), and an ore charge heap (site M2). One area (M1), 10 km from the mine area, was chosen as an uncontaminated reference site. Soil and plant samples (fruiting stage) were collected in September 2012 from these areas. Three plots of 10 m × 10 m were randomly selected on each site. Each plot was then divided into four 5 m × 5 m sampling subplots. The mature leaves and corresponding rhizosphere soils from *P. americana* were sampled from each subplot. The samples from the four subplots were pooled and homogenized to form a composite sample.

The soils and plants were immediately transported to the laboratory and stored at 4 °C until further analysis. Portions of the soils were air-dried, ground in a ceramic mortar, and then sieved (2 mm mesh) for analysis of metal and physicochemical traits, following the methods described by Ref. [24]. Leaves were immersed in 70% ethanol for 1 min, washed with sodium hypochlorite solution (2.5% available Cl^-) for 20 min, rinsed with 70% ethanol for 30 s, and washed three times with sterile distilled water. To confirm complete sterilization, aliquots of the sterile distilled water used in the final rinse were spread on tryptic soy agar plates. The plates were examined for bacterial growth following incubation at 28 °C for 7 days. Plant samples that were negative for contamination in this test were used for further analysis.

2.2. Extraction of total bacterial DNA from P. americana

Surface-disinfected leaves obtained as described above were used for DNA extraction. Approximately 1 g of leaves was frozen with liquid nitrogen and ground to a fine powder in a sterilized and precooled mortar. Total DNA was extracted using a DNA Extraction kit following the manufacturer's protocol (Axygen Biosciences, China). DNA was dissolved in 100 μL of elution buffer.

2.3. PCR amplification of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE) analysis

A nested PCR was used to investigate the structure of the total endophytic bacterial community. To avoid interference of plant chloroplast DNA, amplification of the 16S rRNA gene was carried out using primers F27 and R1530 in the first round of PCR [23]. Each 25 μL PCR mixture contained 1 μL template solution, 10 mM Tris–HCl (pH 8.3), 9.5 μL ddH2O, 10 pmol of each primer (1 μL), and 12.5 μL 2× Master mix (Promega, USA). PCR reactions were performed in a PTC220 gradient DNA thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following procedure: 94 °C for 5 min, followed by 43 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 8 min. The PCR products were checked by agarose gel electrophoresis (1.0% (w/v) agarose,

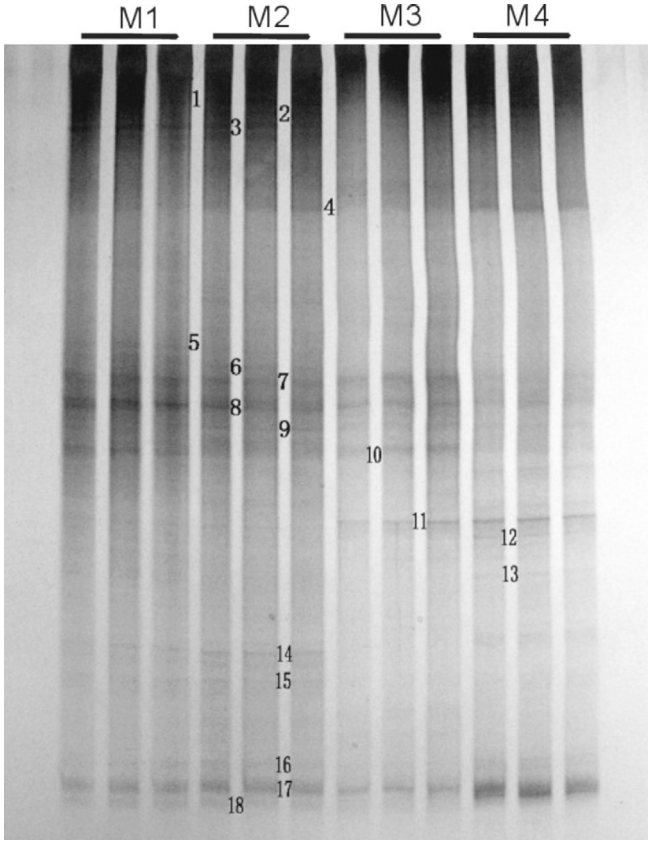

Fig. 1. DGGE pattern of nested PCR-amplified 16S rDNA fragments of endophytic bacteria from the leaves of *P. americana* from four sampled sites. The labeled bands in the gel correspond to the sequenced clones.