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Potential application of selected metal resistant phosphate solubilizing bacteria isolated from the gut of earthworm (*Metaphire posthuma*) in plant growth promotion

Jayanta Kumar Biswas[a,b,*], Anurupa Banerjee[a], Mahendra Rai[c], Ravi Naidu[d,e], Bhabananda Biswas[d,f], Meththika Vithanage[g], Madhab Chandra Dash[h], Santosh Kumar Sarkar[i], Erik Meers[j]

[a] *Enviromicrobiology, Ecotoxicology and Ecotechnology Research Unit, Department of Ecological Studies, University of Kalyani, Kalyani, Nadia, 741235, West Bengal, India*
[b] *International Centre for Ecological Engineering, University of Kalyani, Kalyani 741235, West Bengal, India*
[c] *Department of Biotechnology, SGB Amravati University, Amravati 444602, Maharashtra, India*
[d] *Global Centre for Environmental Remediation (GCER), The University of Newcastle, Callaghan, NSW 2308, Australia*
[e] *Cooperative Research Centre for Contamination Assessment and Remediation of the Environment, ACT Building, The University of Newcastle, Callaghan 2308, NSW, Australia*
[f] *Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095, Australia*
[g] *Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka*
[h] *Sambalpur University, Jyoti Vihar, Sambalpur, Odisha 768019, India*
[i] *Department of Marine Science, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, West Bengal, India*
[j] *Laboratory of Analytical Chemistry and Applied Ecochemistry, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*

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ABSTRACT

The present study focuses on the isolation of three phosphate solubilizing bacteria (PSB), PSB1, PSB2 and PSB3 from the gut of earthworm *Metaphire posthuma*. The three stains were identified as *Bacillus megaterium* (MF 589715), *Staphylococcus haemolyticus* (MF 589716) and *Bacillus licheniformis* (MF 589720) through 16 S rRNA gene sequencing and biochemical characterization. The strains showed resistance to the metals Cu and Zn at significant concentrations and could solubilize phosphate even in the presence of metals. Maximum phosphate was solubilized by strain PSB3 with a production of 222 ± 2.0 mg L^{-1} soluble phosphate followed by PSB1 (213.7 ± 1.3 mg L^{-1}) and PSB2 (193.5 ± 1.5 mg L^{-1}) at 96 h of incubation. The strains were able to produce indole acetic acid (IAA) in presence of L-tryptophan and possessed ammonium ion production potential in the order PSB3 > PSB1 > PSB2 ($P < 0.05$). The sterilized seeds of mung beans (*Vigna radiata*) displayed greater germination rate and higher growth under bacterium-enriched conditions. The effect on seed germination traits by the isolated strains followed the order of PSB3 > PSB1 > PSB2 ($P < 0.05$). Our results suggest that the three isolated PSB strains from earthworm gut possess intrinsic abilities of growth promotion, metal resistance and solubilization of phosphate which could be exploited for plant growth promotion and bioremediation even under metal-stress conditions.

1. Introduction

Earthworm is an important zoological group of soil macrofauna that dominates in the contribution of soil biomass of invertebrates (Liu et al., 2017). The fundamental role of earthworms in the decomposition of organic matter and nutrient cycling and its impact on agriculture is well established (Andriuzzi et al., 2016; A. Singh et al., 2016; P. Singh et al., 2016; Thomason et al., 2017). The feeding behavior of earthworm, which is the ingestion of soil and litter, can ultimately host other potential soil biological components "microorganisms" in their guts (Eisenhauer et al., 2012; Ali et al., 2015). These gut-dwelling microbes play an important role in biogeochemical processes of soil elements through gut-passage processes, such as producing cast (Sruthy et al., 2013; Aria and Dominguez, 1998; Clause et al., 2015). Thus, the earthworms together with the microbiota are responsible for decomposition and turnover of substances in nature and thereby regulate the

* Corresponding author at: Department of Ecological Studies, University of Kalyani, Kalyani, Nadia, 741235, West Bengal, India.
E-mail address: biswajoy2008@gmail.com (J.K. Biswas).

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biogeochemistry of terrestrial soils (Byzov et al., 2015; Arai et al., 2017). Soil enzymes along with microbial biomass are important biochemical parameters that define biological activities in soils (A. Singh et al., 2016; P. Singh et al., 2016). Although there are some reports on earthworm gut epithelium serving as a source of luminal enzyme activities (Sanchez-Hernandez et al., 2009), majority of the enzymatic activities in the earthworm gut are actually originated from the ingested, activated microbes, especially those of nitrogen fixing (Hussain et al., 2016), nitrifying and denitrifying (Horn et al., 2005; Drake and Horn, 2006), and phosphate solubilizing categories (Hussain et al., 2016).

Phosphorus (P) plays an important role in plant maturation and is required for photosynthesis, root establishment, energy transfer, good flowering, fruit quality etc. (Bhat et al., 2017). Of the various chemical forms of P, plants take up only negatively charged primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) as nutrient, but most of P in nature exists in various organic and inorganic forms. The insoluble and inaccessible forms of P are hydrolyzed to soluble and available forms through the process of solubilization of inorganic P and mineralization of organic P (Koch et al., 2018; Khan et al., 2014). The insoluble forms of P such as tricalcium phosphate ($Ca_3PO_4)_2$, aluminium phosphate ($AlPO_4$), or iron phosphate (Fe_3PO_4), may be converted to soluble P by phosphate-solubilizing microorganisms and enzymes (e.g. phosphatase, phosphotriesterases) that are present in soils (Sharma et al., 2013). Such release of P can be performed by earthworm gut phosphatases and phosphate-solubilizing microbes present in the gut (Bhat et al., 2017). Some studies suggest that the phosphatase activity in the casts was significantly higher than the soil; the major contribution of this enzyme was the earthworm gut microorganisms than the epithelium of the gut itself (Vinotha et al., 2000).

Also such P solubilizing microbes exhibit multifunctional activities that benefits plants by synthesizing siderophores, indole acetic acid (IAA), and gibberellic acid etc. (Khan et al., 2013). However, in soils which are contaminated with high levels of metals like copper (Cu) and/or zinc (Zn), microbial functions and earthworm actions may be adversely affected resulting in decreased P release (Doelman and Haanstra, 1989; Wang et al., 2007). In recent years, metal resistant microbes have been employed because they display a high potential to alter the metal mobility and bioavailability (Park et al., 2010; Mohamed and Almaroai, 2017). However, multifunctional microorganisms that are capable of P solubilization and are resistant to high level of metals (e.g. Cu, Zn) should be available in the soil and soil-inhabiting organisms have rarely been explored. We hypothesize that if the specific gut resident bacteria of the earthworms are found to be endowed with unique attributes like phosphate solubilization and metal resistance they could elucidate earthworm's role in enhancing the soil fertility and metal remediation. This could establish such gut inhabiting bacteria as ‘connecting link’ and ‘behind the screen’ role players in those ecological services. Hence, the present study focuses on the isolation of PSB from the gut of endogeic earthworm *Metaphire posthuma*, and investigates their properties as a plant growth promoter and also their resistance towards metals.

2. Materials and methods

2.1. Source of the earthworm sample

Endogeic geophagous earthworms (*Metaphire posthuma*) used in this study were adult with a medium body size (Length: ~10 cm, Diameter: ~5 mm).Earthworms were collected from the garden soil of Kalyani University campus (22.9862°N, 88.4464°E) and stored in sterile bags and used for further examination and isolation of bacteria from its gut.

2.2. Isolation of PSB from earthworm gut

The earthworms were surface sterilized with 70% ethanol and washed with sterile water. The gut contents were released from the anterior to the posterior end by aseptically squeezing intact worms. Gut contents were then stored at 4 °C unless used immediately for the isolation of target bacteria. The isolation of potential PSB was executed following the pour plate technique using Pikovskaya's agar medium (dextrose 10 g; tri calcium phosphate (TCP) 5 g; yeast extract 0.5 g; ammonium sulphate 0.5 g; potassium chloride 0.2 g; sodium chloride 0.2 g; magnesium sulphate 0.1 g; ferrous sulphate trace; manganese sulphate trace; agar 15 g; distilled water 1 L; the pH was adjusted to 7.0 ± 0.2 before sterilization). After 48 h of incubation at 28 ± 2 °C, three bacterial colonies showing discrete hallo zones were isolated and sub-cultured for further characterization.

2.3. Identification and characterization of PSB

Several biochemical tests of the isolated PSB were carried out for the identification of strains (Benson, 2015).

The 16S rRNA gene sequencing was performed to identify the strains (Biswas et al., 2017). To sequence the 16S rRNA gene, genomic DNA of the bacterial strain was extracted and amplification of 16S rRNA gene was performed by polymerase chain reaction (PCR). For PCR bacterial genomic DNA was used as the template and bacterial universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR reaction mixture (20 μL) contained 60 ng templates, 2 μL of 10× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.6 μL of dNTP mix (10 mM each), 0.2 μL of 5 U μL^{-1} Taq DNA polymerase and 3.75 pmol primers (each). The PCR performed (Applied Biosystems Thermo Cycler) with an initial heat step for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension step at 72 °C for 90 s, and final extension at 72 °C for 7 min. Electrophoresis of the PCR product was done in 1% agarose stained with ethidium bromide and; 1.5 kb band was purified by HiPurA Quick Gel Purification Kit (HiMedia Laboratories, India). Then the purified 16S rRNA gene was transformed into JM109 competent *E. coli* cells using pGEM-T Easy Vector System I (Promega Corporation, USA). Plasmid DNA was isolated from the transformed cell by QIAprep Spin Miniprep Kit (Qiagen, Germany) and was sent for sequencing at Eurofins Genomics, Bengaluru, India. Using BLAST (Basic local alignment search tool, BLAST at NCBI) 16S rRNA gene sequence was compared to the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Effect of pH and NaCl on PSB strains isolated

The bacterial isolates were incubated into Luria Bertani (LB) medium consisting of casein enzymic hydrolysate 10 g; yeast extract 5 g; sodium chloride 10 g and distilled water 1 L in order to determine the optimum pH and salinity tolerance (halotolerance) for those isolates. The pH of the medium was adjusted to obtain different pHs (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0) using drops of 0.05 M HCl or NaOH. In the other case, LB medium was supplemented with various concentrations of NaCl (0, 2, 4, 6, 8, 10, 12, 14, and 16) %, (w/v) in order to assess the salinity tolerance of the strains. The experiments were performed in triplicates. Optimal growth in the LB medium was evaluated by measuring the increase in optical density (OD) at 600 nm with a spectrophotometer (UV-1800 UV–Vis Shimadzu).

2.5. Examining the resistance of PSB strains to metals

The resistance of the isolated PSB strains to Cu and Zn was tested. Stock solutions (metal concentration 500 mM) of the elements in the forms of $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$ were sterilized using 0.45 μm pre-sterile syringe filter (Millipore filter paper). Further, increasing concentrations (0.5 mM to 8 mM) of the metal salts were added from the stock solution to sterile nutrient agar (NA) medium (composition: peptic digest of animal tissue 5 g; beef extract 3 g; sodium chloride 5 g;