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A unique Pb-binding flagellin as an effective remediation tool for Pb contamination in aquatic environment

Baowei Chen[a,1], Linchuan Fang[b,1], Xueting Yan[c], Aiqian Zhang[c], Ping Chen[d], Tiangang Luan[d,e], Ligang Hu[c,*], Guibin Jiang[c]

[a] Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering, School of Marine Sciences, Sun Yat-Sen University, Guangzhou, 510275, PR China
[b] State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Northwest A&F University, Yangling, 712100, PR China
[c] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center of Eco-Environmental Sciences, Chinese Academy of Sciences, P. O. Box 2871, Beijing, 100085, PR China
[d] School of Life Sciences, Sun Yat-Sen University, Guangzhou, 510275, PR China
[e] School of Environmental Science and Engineering, Sun Yat-sen University, Guangzhou, 510275, PR China

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ABSTRACT

Metal contaminants present persistent and deleterious threats to environmental ecosystems and human health. Microorganisms can rapidly develop protective mechanisms against metal toxicity, such as metallothionein production. The identification of biological factors related to these protective mechanisms is essential for effective metal remediation. This study presents a robust pathway to rapidly locate and characterize a Pb-binding flagellin in *Serratia* Se1998, which can bind Pb at a 16:1 Pb: protein ratio. A column gel electrophoresis system hyphenated with inductively coupled plasma mass spectrometry (ICP MS) was constructed to efficiently separate and identify Pb-binding proteins from the whole bacterial proteome. PCR and transgenic assays were used to elucidate the exact sequences and biological function of Pb-binding proteins and heterogeneous expression of Pb-binding flagellin in *E. coli* could significantly enhance Pb removal from aqueous solution by approximately 45%. This method provides a benchmark procedure to rapidly identify biological factors responsible for metal biosorption. Identification of this unique Pb-binding flagellin highlights that microorganisms can survive high metal stresses due to various complex biological pathways for metal detoxification and remediation.

1. Introduction

The levels of toxic metals can be enriched in the environment, either naturally due to weathering of parent rocks or as a consequence of anthropogenic activities such as mining, smelting and fuel combustion [1–3]. Metal contaminants are often difficult to remove from the environment, as they cannot be chemically or biologically degraded [4]. Elevated levels of toxic heavy metals such as mercury (Hg), lead (Pb) and cadmium (Cd), have been found to cause detrimental effects on the both ecosystem and public health [5,6]. Some organisms have been found to undergo various adaptations to survive stresses posed by toxic metal exposure, however, the mechanisms for these are not yet established. For example, a fast-growing fern *Pteris vittata* that rapidly takes up arsenic (As) has been reported, while the factors underlying As hyperaccumulation remain to be fully understood [7]. Elucidating the biological factors which are involved in metal accumulation or biosorption in naturally occurring species, such as transcriptional changes or the production of proteins and lipids, present a significant technical challenge. However, understanding these natural mechanisms is essential for the effective remediation and detoxification of toxic metal environmental contaminants [3,8,9].

A common feature of microorganisms is rapid evolution, acquiring resistances to toxic contaminants via rapid adaptive evolution [10]. Various protective mechanisms are developed by microbes to tolerate toxic metals [3], such as the immobilization of toxic metals in a physiologically inaccessible form [11]. Metal-binding proteins such as Metallothioneins (MTs), are able to sequester and immobilize toxic metals inside microbial cells, thereby making them innocuous [11,12]. It is well established that metal-binding proteins are produced as a consequence of elevated metal concentrations within the organism [13]. However, microbial metalloproteomes remain largely uncharacterized to date, partially due to the limitations of current

* Corresponding author.
E-mail address: lghu@rcees.ac.cn (L. Hu).
[1] These authors contributed equally to this work.

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methods [14]. The identification of novel metalloproteins provides valuable insights into microbial resistance mechanisms to toxic metals [15]. Furthermore, it provides an important baseline for the development of effective remediation approaches for toxic metals, using naturally metal resistant microbes and novel microbial metalloproteins [16].

Pb is a mutagenic and teratogenic toxin and environmental contamination levels have increased more than 1000-fold over the past three centuries due to anthropogenic activities [17,18]. Although a large variety of Pb tolerant microbial strains have been isolated from various environmental niches [18], microbial Pb-binding proteomes have not been fully characterized. For instance, Pb-binding proteins expressed by the mammals, which are rich in aspartic and glutamic acids, have been found to largely attenuate Pb toxicity [19], however, these proteins have not yet been found in microbial proteomes. MT production in rats could be induced by Cd and Zn to a greater degree than Pb [20], with the overall biosorption efficiency of MTs-engineered *E. coli* also greater for Cd and Zn than Pb [21]. To date, the lack of knowledge regarding microbial Pb-binding proteins has hindered our understanding of microbial Pb tolerance and biosorption mechanisms.

The objective of this study was to construct a pipeline method to identify the biological factors responsible for microbial metal biosorption. This allows utilization of microbes that have naturally evolved to be resistant to the stress of toxic metal exposure, with Pb contamination selected as an example case study.

2. Materials and methods

2.1. Chemicals

Lead nitrate ($Pb(NO_3)_2$, ≥99%) and sodium chloride (NaCl, ≥99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tryptic Soy Broth (TSB) and Luria-Bertani (LB) Broth were purchased from Sangon Biotech (Shanghai, China) for bacterial incubation. Phosphate Buffered Saline (PBS, pH 7.4) was provided by Thermo Fisher Scientific Inc. (MA, USA). High-purity water was supplied by a Milli-Q water system (Millipore, Eschborn, Germany). Stock solutions of $Pb(NO_3)_2$ were prepared by dissolving $Pb(NO_3)_2$ in high-purity water to form a final concentration of 1.0 M, purified by filtering through a 0.22-μm membrane and stored in the dark at 4 °C prior to use.

2.2. Isolation of Pb resistant bacteria

All Pb-resistant strains were isolated from highly Pb-contaminated soils in a Pb-Zn mining area in Baoji, Shaanxi province (ca. 106°36′15″ E and 33°52′35″ N), as shown in Fig. S3 (supplementary information (SI)). The sampling area had a temperate mountainous climate, with an annual mean temperature of 11.3 °C and annual rainfall level of 428.6–972.4 mm. A total of 112 soil samples were collected from 20 sampling sites in arable land, grassland and woodland within the distance of 2 km apart from the mining areas in November 2012. In brief, soil samples were taken from the depth of 0 to 20 cm at each sampling site using shovel, and were immediately kept in Ziploc plastic bags for delivering back to the laboratory. Half of each sample was stored at 4 °C to determine soil bio-properties and the rest was air-dried for the subsequent analysis of physicochemical properties. The soil samples were dispersed and passed through a 2 mm sieve, and pH, organic matter content and the concentrations of total nitrogen (N) and phosphorus (P) were measured using the procedures described in the literature [22]. The basic properties of sampled soils were measured: pH (1:2 w/v soils to water) 7.51–8.69; organic matter concentration of 15.0-35.0 g kg^{-1}; total N concentration of 0.39-2.89 g kg^{-1}; and total P concentrations of 3.10-14.22 g kg^{-1}. Total Pb content in soil samples were analyzed using a flame atomic absorption spectrometer (VARIAN-GTA120AA240FS) after digestion with three acids (HNO_3, HCl, and $HClO_4$). Pb concentration in the soils was in the range of 13–2500 mg kg^{-1}. Soil samples with Pb concentrations being higher than 2000 mg kg^{-1} were chosen to isolate Pb tolerant bacterial strains. Details of the screening method used to identify Pb-tolerant strains are described in the SI. A red bacterial colony (Se1998) surviving on TSB agar containing 40 mM Pb was isolated and sub-cultured for further assay.

The bacterial strain Se1998, was incubated in LB medium (5 g of Yeast extracts, 10 g of Peptone and 10 g of NaCl in1 L distilled water) on a rotary shaker at 150 rpm, at 28 ± 1 °C. Cells were harvested during the late exponential growth phase (about 24 h) by centrifugation at 9000 rpm for 10 min at 4 °C, then washed twice with 8.5 g L^{-1} NaCl. Cell pellets were re-suspended in 8.5 g L^{-1} NaCl solution, to obtain a cell suspension with an optical density of 1.0 at 600 nm according to Colony-Forming Unit (CFU) counting (1.84×10^9, determined by counting CFU on LB agar plates). To obtain bacterial growth curves, 100 μL aliquots of bacterial cultures (7.36×10^6 cells) were inoculated into 200 mL of LB medium. Flasks were capped using plastic filter membranes (0.2 μm pore size) allowing gaseous exchange with the ambient environment. Bacteria were incubated on a rotary shaker at 150 rpm and 28 ± 1 °C. Bacterial growth curves were measured by monitoring the optical density of cultures at 600 nm.

2.3. Identification of bacterial isolate by 16S rRNA gene sequence

The Se1998 strain was identified by analyzing 16S rRNA gene sequences. In brief, DNA was extracted using UNIQ-10 Column kit for bacterial genomes (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.). The universal bacterial 16S rRNA gene primers (50 forward, AACACATGCAAGTCGAACG; 1492 reverse, GGTTACCTTGTTACGACTT) were used for PCR amplification. PCR reactions were performed in a 25-μL solution containing a 0.5 μL dNTP mixture (10 mM), 2.5 μL 10 × PCR buffer, 0.5 μL of each primer (10 μM), 0.2 μL Taq polymerase (5 U/μL) (TaKaRa Biotechnology, China) and 1 μL DNA template. The thermal cycling conditions for gene amplification were as follows: initial denaturation at 94 °C for 5 min, followed by 40 denaturation cycles at 94 °C for 30 s, annealing at 55 °C for 35 s and elongation at 72 °C for 60 s. Final extension was performed at 72 °C for 8 min. PCR products were isolated by electrophoresis on 3% agarose gel and purified using a UNIQ-10 Column Gel Purification kit. The 16S rRNA gene amplicon was subjected to sequencing analysis and then 16S rRNA gene sequences were compared to blasted against the database of the NCBI.

2.4. Assay of Serratia Se1998 Pb resistance

Bacterial cultures at the exponential growth stage (50 μL) were inoculated into 5 mL of LB medium supplemented with serial dilution concentrations of Pb from 0.5 to 14 mM. Considering that obvious inhibitory effect was observed between 8 and 12 mM, Pb concentration was set to be denser in this range. The inoculation cell number of Se1998 was 8.61×10^6. Bacteria were incubated on a rotary shaker at 150 rpm, 28 ± 1 °C for 24 h. The optical density of bacterial culture was measured at 600 nm A bacterium-free medium containing serial dilution Pb concentrations from 0.5 to 14 mM were used as a blank and bacterial cultures devoid of Pb were used as controls. Bacterial cultures were serially diluted with NaCl solution (8.5 g L^{-1}) and spread on LB agar plates for counting CFU.

2.5. Cell lysis

Cells were harvested by centrifugation at 4500 *g* for 6 min, then washed with cold Phosphate Buffered Saline (PBS) three times. Cell pellets were re-suspended in 10 mL of 10 mM Tris buffer (pH = 7.4) containing 0.1 M NaCl and 0.1% Triton X-100, then lysed via sonication. Cell lysis solutions were then centrifuged at 15,000 *g* for 30 min and supernatants were collected for subsequent analysis.