

Physiological and Genetic Response Characteristics of *Stenotrophomonas Rhizophila* JC1 Under Heavy Metal Stress

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Abstract

In this study, the Cu^{2+} (120 mg/L) and Cr^{6+} (80 mg/L) removal rate of *S. rhizophila* JC1 reached at 79.9% and 89.3%, respectively. Scanning electron microscopy showed that Pb^{2+} and Zn^{2+} had no obvious effect on cell structure, but the cells became smaller and brighter under Cu^{2+} stress, and many nanoparticles formed on the cell surface under Cr^{6+} stress. The physiological response analyses demonstrated that moderate change of membrane permeability was necessary for adsorption. FHR and EDS analyses showed that exopolysaccharides (EPS) and the replacement of basic elements (ie., C, O) might be the main means of heavy metals adsorption by strain. In addition, 323 transport proteins were predicted in the genome of *S. rhizophila* JC1. Among them, two, six and five proteins of the cation diffusion facilitator, resistance-nodulation-division efflux and P-type ATPase families were respectively predicted. The expression of genes showed that the synergistic action of transport proteins played an important role in the process of adsorption. The comparative genomics analysis revealed that *S. rhizophila* JC1 has long-distance evolutionary relationships with other strains, but the efflux system of *S. rhizophila* JC1 contained the same types of metal transport proteins as other metal-resistant bacteria.

Introduction

Precipitating, chelating, or altering the oxidation state of various heavy metals by using microorganisms has become a hotspot of research and a topic of industrial application in the field of environmental remediation (Kang and So. 2016). To ensure normal growth and metabolism under heavy-metal stress, microorganisms have developed homeostatic systems by controlling the processes of intake, transport, and efflux of heavy metals (Ma et al.2009; Valencia et al. 2013).

Efflux is a main approach applied by bacteria to command metal ions transport to reduce intracellular metal concentrations. There are three recognized efflux systems that play crucial roles in the heavy metal detoxification by bacteria. The cation diffusion facilitator (CDF) family is ubiquitous in bacteria, archaea and eukaryotes (Paulsen and Saier 1997), and it transports heavy metals, including Co^{2+} , Cd^{2+} , Zn^{2+} and possibly Ni^{2+} , Cu^{2+} and Hg^{2+} (Kambe 2012). Lots of members of the CDF family consist of 300–550 amino acids, which include six transmembrane domains, the regulatory cytoplasmic C-terminal domain and a histidine-rich region that may be a potential metal-binding site (Barber et al. 2016). The P-type ATPase, located on the plasma membrane, promotes the metal efflux system through hydrolysis (Arguello et al. 2011). It transfers substrates from the outside of the cell or the peripheral cytoplasm to the cytoplasm, as well as from the cytoplasm to the extracellular or peripheral cytoplasm through P-type ATPases (Hiraizumi et al. 2019). Simultaneously, owing to the stimulating effects of sulfhydryl compounds on the metal efflux activity, CPx-type ATPases may also use glutathione to expel metals from the cytoplasm (Meng et al. 2015). The transporter-dependent resistance-nodulation-division (RND) efflux system has been considered to play a crucial role in heavy-metal resistance of bacteria (Saier et al. 1994). In most cases, the gene adjacent to the encoded protein is the member of membrane fusion protein (MFP) family (Nagakubo et al. 2002). Along with the outer membrane factors (OMFs)(Maseda et al. 2002), three of these protein families form an excretion protein complex, which can excrete the matrix from the cytoplasm, cell membrane or periplasmic space through the outer membrane (Kim et al. 2011; Yang et al. 2014).

In this paper, the adsorption ability of *S. rhizophila* JC1 (CPO50062) for Cr^{6+} , Cu^{2+} , Pb^{2+} and Zn^{2+} of different concentration was validated. Furthermore, the morphological feature of strain JC1 stressed by different heavy metals was observed through scanning electron microscopy (SEM), the element composition of heavy metals adsorbed on bacterial surface was analyzed by energy-dispersive X-ray spectroscopy (EDS) and the functional groups were analyzed by Fourier Transform Infrared Spectrometry (FT-IR). We also analyze the changes of membrane permeability and macromolecular before and after heavy metal adsorption to reveal the physiological response mechanism of adsorption. Moreover, to better understand the molecular mechanism of adsorption, heavy-metal transporters were predicted by using the TCDB database, and representative genes were verified by qPCR.

Materials And Methods

Cultivation of strain JC1

The strain JC1 was respectively cultured in LB, LB+ Cu^{2+} , LB+ Cr^{6+} , LB+ Pb^{2+} and LB+ Zn^{2+} liquid medium with the final concentration of metal ions were 0, 40, 80, 120, 160 and 200 mg/L. Each treatment was repeated three times.

Metal ions removal efficiency evaluation

The growth status of strain JC1 was evaluated by measuring the OD_{600} value after culturing for 24 hours. The removal efficiency was evaluated by measuring the metal ions residues in the culture medium with atomic absorption spectrometry. The removal efficiency (%) was calculated as follows:

$$\text{Removal efficiency (\%)} = (C_0 - C_1) / C_0$$

Where C_0 is the initial concentration of metal ions before adsorption, and C_1 is the final concentration of metal ions after adsorption.

Morphology, functional groups and element composition analyses

The strain JC1 was respectively stressed with 80 mg/L of Pb^{2+} , Cr^{6+} , Cu^{2+} and Zn^{2+} for 24 hours and centrifugated at 8 000 g for 10 minutes. The cell sediments were fixed in 2.5% glutaraldehyde for 2 hours and washed three times with deionized water. Then washed with alcohol and tertiary butanol in turn, the concentration of alcohol was 30, 50, 70, 80, 90, 100%, respectively. After that, the cells were re-suspended in tertiary butanol with the cell concentration was 1×10^5 CFU/mL. Finally, the samples were dried before observed with scanning electron microscope (SEM, JSM-6701F) (Wang et al. 2021).

The strain JC1 stressed at 80mg/L of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ were also collected for functional groups and element composition analysis. Cell sediments were collected by centrifugation at 10 000 g for 10 minutes and dried at 45 °C. After that, the infrared absorption spectra in the range of 4000-400 cm⁻¹ was measured by Fourier Transform Infrared Spectrometry (FT-IR) and screened by 325 meshes for Energy Dispersive X-ray spectroscopy (EDS) examination [Wang et al. 2020].

The physiological response of *S. rhizophila* JC1 under different metal ions stress

To reveal the physiological response mechanism of strain JC1 after heavy metal ions stress, membrane permeability and intracellular macromolecular (i.e., protein, phospholipid, alkaline phosphatase) substances were analyzed. Each treatment was repeated three times.

The physiological response of membrane permeability

The cell outer membrane (OM) permeability of *S. rhizophila* JC1 under different metal ions stress was analyzed by the method described by Loh (Loh et al. 1984). The strain JC1 was respectively stressed with 0, 40, 80, 120mg/L concentration of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ for 24h. After that, N-phenyl-1-naphthylamine (NPN) solution with the final concentration was 10 µmol/L was added, then slowly oscillate at 37 °C about 3 minutes. Finally, the fluorescence value was measured at excitation wavelengths was 350nm and emission wavelengths was 440 nm with fluorescence spectrophotometer. Three repeated experiments were performed.

The cell inner membrane (IM) permeability of *S. rhizophila* JC1 stressed by different metal ions was analyzed by measuring the β-galactosidase activity (Liu et al. 2004). The logarithmic phase cells that cultured in LB medium containing 2% lactose were collected and washed twice with 0.85% NaCl solution, then respectively stressed with 0, 40, 80, 120mg/L concentration of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ for 24h. Thereafter, O-Nitrophenyl-β-D-Galactopyranoside (ONPG) solution with the final concentration was 1.5 mM was added, then slowly oscillate at 37 °C about 3 minutes. Finally, the absorption value was measured at 415 nm with Spectrophotometer. Three repeated experiments were performed.

The physiological response of intracellular macromolecular

The strain JC1 treated with 0, 40, 80, 120mg/L concentration of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ was cultured to the logarithmic growth stage, then the cell culture medium was collected. The protein content was determined by the Bradford method, and measured with BSA as standard (Panja et al. 2008, Wang et al. 2021).

The strain JC1 treated with 0, 40, 80, 120mg/L concentration of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺ was also used for phospholipid analyses. 1, 6-Diphenyl-1,3,5-Hexatriene (DPH) with the final concentration was 0.5 mM was added into the cell culture medium, then diluted the mixture 15 times by 100 mM CaCl₂, incubated away from light for 40 minutes. Finally, the fluorescence value was measured at excitation wavelengths was 350nm and emission wavelengths was 440 nm with fluorescence spectrophotometer. (Waschuk et al. 2001).

The chromogenic substrate p-nitrophenylphosphate (pNPP) was used to investigate alkaline phosphatase enzyme activity (Schlesinger 1989). The strain JC1 was initially cultured in tris-glucose medium to the logarithmic growth stage. Then washed three times with phosphorus-free tris-glucose before cultured in phosphorus-free tris-glucose solution at 37 °C for 40 minutes. After that, the cell sediments were re-suspended in deionized water after washed, then treated with treated with 0, 40, 80, 120mg/L concentration of Pb²⁺, Cr⁶⁺, Cu²⁺ and Zn²⁺. Finally, the mixture involving 1.0 mL metal-laden bacteria and 2.0 mL pNPP of 200 mM were incubated at 37°C about 15 minutes. The absorbance at 420 nm was measured.

Three repeated experiments were performed of above experiments.

Genomes

The genomic protein sequences and 16S rRNA of *S. rhizophila* JC1 were obtained from whole-genome sequencing results. Corresponding sequences of other strains were obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>).

Transport protein classification and comparison

Diamond software was used to compare the amino acid sequence of the target species by TCDB database and match genes of the target species with their corresponding functional annotation information. SWISSPROT and TrEMBL databases were used for function analyses. The phylogenetic relationship of 15 comparative strains was obtained by 16S rRNA sequences analysis (Rozycki and Nies 2009).

Functional validation of predicted genes by qPCR

To identify the function of transport proteins of *S. rhizophila* JC1 under four heavy metal ions stress, four genes (i.e., *czcD*, *czcB*, *ZntA*, Cu²⁺-exporting ATPase) were examined by quantitative real-time PCR (qPCR). The reverse-transcribed was carried out according to the instructions of Super RT Kit (TaKaRa). Each qPCR procedure was carried out according to the description by Novinscak and Fillion (Novinscak and Fillion 2011). The 16S rRNA and β-actin gene were used as an internal standard. Three rounds of independent qPCR reactions were used to verify the expression of each gene and the data were showed as arithmetic means ± the standard deviation. The P-value less than 0.05 was considered to be statistically significant (Wang et al. 2020).

Results And Discussion

Metal ions removal efficiency

The toleration and adsorption to different metal ions of strain JC1 showed great difference. For instance, during *S. rhizophila* JC1 was respectively cultured in LB+Cu²⁺, LB+Cr⁶⁺, LB+Zn²⁺ that the concentration of metal ions was 40~120 mg/L, 40~200 mg/L, 40 mg/L for 24 hours, there were no significant difference in OD₆₀₀ value. However, during the concentration of Zn²⁺ and Pb²⁺ were increased to 160 mg/L, the strain JC1 hardly grew. The adsorption efficiency was 79.8% when the concentration of Cu²⁺ was 120 mg/L⁻¹, while it reduced to 26.1% when the concentration reached to 160 mg/L. More interesting was that with the concentration was 40~200 mg·L⁻¹ of Cr⁶⁺, there had no significant suppression on the growth of bacteria, but only showed maximum adsorption efficiency of 89.3% when the concentration was 80 mg/L. In terms of Pb²⁺ and Zn²⁺, the strain JC1 hardly showed adsorption ability (Fig. 1). Hence, this phenomenon indicated that the adsorption of metal ions by bacteria was selective, which was also suggested by Ye (Ye et al.2014)

Analysis of cells structure under metal stress by SEM

In this study, the morphological feature of strain JC1 comparison between metal ions stressed-cells and unstressed-cells was performed with SEM analysis. In different metal ions stress condition, different cell morphology was observed (Fig. 2). For unstressed-cells, the cell structure was intact, showed rod-shaped (Fig. 2a). However, the rod-shaped of JC1 after Cr⁶⁺ stress was almost impossible observed, there were crowds of particles gathered on the surface and the cells were wrapped in the membrane (Fig. 2b). In the case of Cu²⁺, cells showed shorter and brighter (Fig. 2c). The change caused by Pb²⁺ and Zn²⁺ was similar. In brief, the surface of the JC1 became rougher, but the difference was that the cells condensed into clusters after Zn²⁺ stress (Fig. 4d&e).

The change of cell morphology was the result of bacteria response to heavy metal stress. The decrease in cell size may be due to dehydration of the cells under Cu²⁺ stressed and also may be explained as a negative response of bacteria against further uptake of metal by decreasing the area of contact with the Cu²⁺ (Mohite et al. 2018). The rough of cell surface might be interpreted as a strategy to accumulate more metal ions on the surface of cells as in case Pb²⁺ and Zn²⁺. For Cr⁶⁺, we speculated that due to the change of membrane permeability, a large amount of particles, formed through extracellular polysaccharides (EPS) binding with Cr⁶⁺, attached to the cell surface.

Analysis of chemical structure under metal stress by FT-IR

Studies had confirmed that the adsorption of heavy metals by bacteria is energy independent and can be mediated by functional groups (Zhang et al. 2017). In this study, the functional groups of strain JC1 for adsorption with four heavy metals was analyzed by Fourier transform infrared spectroscopy (FT-IR). Obviously, among four metal-laden samples, significant shifts of C-O-C and C=O were observed after the treatment of Cr⁶⁺, significant shifts of O-H was observed after the treatment of Cu²⁺ and Zn²⁺, significant shifts of C-O was observed after the treatment of Pb²⁺(Fig. 2f). According to the FT-IR analysis, we suggested that the binding by EPS was one of the important way for heavy metal detoxification. In addition, the detoxification that depends on the functional groups of the bacteria and the valence state of metal ions is selective (Ye et al. 2014, Zhang et al. 2017).

Analysis of element composition under metal stress by EDS

Generally, non-metallic elements such O, C, Si and non-toxic metal elements such as Ca²⁺, Fe²⁺/Fe³⁺ were the basic elements to maintain normal growth and metabolism of microorganism. However, some toxic metals such as Cr⁶⁺ or Pb²⁺ will exist in the cells through sites replacement due to atomic radius, cell structure. The result of element composition analysis after metal ions stress was shown in Fig. 3. Obviously, the corresponding metal elements were increased in the bacteria when they were stressed by different metals. Moreover, the metal content is consistent with the adsorption efficiency. Further analysis we found that, Cr⁶⁺ and Cu²⁺ mainly replaced the O element, while the Pb²⁺ and Zn²⁺ mainly replaced the C element, this may be related to the radius of the metal.

Analysis of physiological response of *S. rhizophila* JC1 under different metal ions stress

Determination of cell membrane permeability

The change of OM permeability under different heavy metal stress was shown in Fig. 4. Distinctly, the OM permeability of strain JC1 was significantly affected by the type and concentration of metal ions. What is more, its variation trend was positively correlated with the adsorption capacity of Cr⁶⁺ and Cu²⁺ (Fig. 1&4). However, it was interesting to note that the change induced by Pb²⁺ and Zn²⁺ were higher than Cr⁶⁺ and Cu²⁺, but the cell survival rate and adsorption capacity were contrary to this. Secondly, the higher the concentration of Pb²⁺ and Zn²⁺, the higher the change of OM permeability, but the cell survival rate and adsorption capacity were also contrary to this(Fig. 1&4). We speculated that: 1): detoxification factors for Cr⁶⁺ and Cu²⁺ were more abundant compared with Pb²⁺ and Zn²⁺ in cells of strain JC1, 2): cells will die when the membrane permeability changes exceeds its self-repair ability.

Similar to the OM permeability, the change of IM permeability were also affected by the type and concentration of metal ions. But it did not mean that the higher of the concentration of metal ions, the greater the change of membrane permeability, nor the stronger of the adsorption capacity. In summary, the change of membrane permeability was the key factor for bacteria to adsorb heavy metals, but when degree of change exceeded its self-repair capacity, it became non-resistant or low-resistant.

Analysis of changes in bioactive macromolecules

The excessive release of macromolecules such as proteins (Pr), phospholipid (PL), alkaline phosphatase (ALP) will definitely affect the normal function of cells. Without a doubt, with the change of membrane permeability, macromolecules will release to the outside of the cells. In terms of proteins and phospholipid, the variation trend was consistent with the change of membrane permeability (Fig. 4&5), which was also the reason that why cells hardly grow under the stress of Pb²⁺ and Zn²⁺. On the contrary, the release trend of alkaline phosphatase was consistent with the heavy metal adsorption capacity of

strain JC1. We speculated that alkaline phosphatase participates in the hydrolysis reaction of microorganisms and generates phosphate ions ($-\text{PO}_4^{3-}$) and free hydroxyl groups ($-\text{OH}$), then the $-\text{PO}_4^{3-}$ and $-\text{OH}$ complexed with the positive metal ions such as Cr^{6+} and Cu^{2+} , also with Pb^{2+} and Zn^{2+} .

Transport proteome of *S. rhizophila* JC1

In total, 323 transport proteins belonging to nine major transport protein classes were predicted in the genome of *S. rhizophila* JC1 (4.28 Mb) (Table S1). Most of them were primary active transporters (TC#3), electrochemical potential-driven transporters (TC#2) and channels/pores (TC#1). They represented only 9% of the total number of predicted proteins. In addition, only 33%–50% of superior heavy metal-resistant bacteria which genome larger than 4 Mb (Rozycki and Nies 2009). The distribution of transport proteins was similar to these bacteria. Thus, the adsorption and resistance of *S. rhizophila* JC1 to heavy metals may be independent of the number of transporters, the types of transporter may be the key factor.

Heavy-metal transport proteins

Because cells do not contain the NADPH level required for reductase activities and there is a lack of methylation or other covalent modification mechanisms, some divalent metal ions cannot be discharged from cells independently. However, the three efflux systems (CDF, RND and P-type ATPases), containing almost all of the metal transport proteins, actively mediate the intracellular to extracellular excretion of divalent metal ions (Rozycki and Nies 2009). Therefore, the numbers and functions of transport proteins involved in the three efflux systems were analyzed in *S. rhizophila* JC1.

The cation diffusion facilitator (CDF) system

The CDF system members are chemiosmotic ion/proton exchangers that are involved in the efflux of divalent metal cations (Nies 2003) and the transport of metals from the cytoplasm across the cytoplasmic membrane into the periplasmic space (Higuchi et al. 2009) As shown in Table 1, two predicted proteins constituted the CDF system in *S. rhizophila* JC1. The *czcD/zitB* (JC1_GM001116) was predicted as $\text{Co}^{2+}/\text{Zn}^{2+}/\text{Cd}^{2+}$ efflux system protein that included cation efflux, cation transporter ATPase C terminal as well as zinc transporter dimerization domains. Furthermore, its homolog was confirmed to bind Cu^{2+} and Ni^{2+} in *Ralstonia metallidurans* (Anton et al. 2004). The other CDF system member (TC#2.A.4.4.7) (JC1_GM001883) was predicted to be an integral membrane protein, which was confirmed as a lead (Pb^{2+}) efflux transporter (PbtF) in *Achromobacter xylosoxidans* (Hložková et al. 2013), and it may provide sites for the targeted utilization of proteins. The sequences of these two proteins were provided in Additional file 1.

The resistance-nodulation-division (RND) efflux system

On the basis of transport substrate by bacteria, the RND was subdivided into six sub-groups: RND1 (Zn^{2+} , Co^{2+} and Cd^{2+}), RND2 (Co^{2+} and Ni^{2+}), RND3a (divalent cations), RND3b (monovalent cations), RND4 (Cu^+ / Ag^+) and RND5 (Ni^{2+}) (Nies et al. 2003). Generally, these proteins are trimeric proteins that generate protein complexes named outer membrane factor (OMF) (Koronakis et al. 2000) and a periplasmic adapter protein of membrane fusion protein (MFP) family (Akama et al. 2004).

Six heavy-metal efflux proteins were predicted in *S. rhizophila* JC1 (Table 1). Among them, two types of *czcCBA* operons (TC#2.A.6.1.15 and TC#2.A.6.1.16) form a complete cobalt–zinc–cadmium resistance system homologous to that in *Escherichia coli* (Tam et al. 2019). *czcA* is the RND protein, having at least one transmembrane domain and a membrane-spanning helical or beta-stranded domain that embeds in the membrane. *czcC* is a member of the OMP family that forms a trimeric channel and a long helical barrel that allows the export of a variety of substrates in Gram-negative bacteria. In *S. rhizophila* JC1, the *czcCBA* operon may transport Cd^{2+} and Zn^{2+} , as well as Co^{2+} , because there is a gene downstream of *czcA* that encodes a $\text{Cd}^{2+}/\text{Zn}^{2+}$ -exporting ATPase (Fig. 6).

No regulatory gene was predicted near the two types of *czcCBA* operon, which was in contrast to the *cnr* in genome of *R. metallidurans* CH34 (*cnrYXHCBA*), *czc* in genome of *R. metallidurans* and *Alcaligenes eutrophus* (*czcCBADRS/czcNICBADRS*) and *ncc* in genome of *Achromobacter xylosoxidans* 31A (*nccYXHCBAN*) (Grass et al. 2000, Grosse et al. 2004). The amino acid alignment showed these paralogs share very low overall identity levels, as follows (Figure 6): JC1_001887 and JC1_003695 (*czcC*, OMF), 23.37% identity, JC1_001888 and JC1_003694 (*czcB*, MFP), 19.71% identity, and JC1_001889 and JC1_003693 (RND protein), 47.45% identity. Moreover, two additional genes (JC1_001886 and JC1_001890) were contained in the *czc* locus. JC1_001891, located downstream of the *czcA* gene, encodes the $\text{Cd}^{2+}/\text{Zn}^{2+}$ -exporting ATPase (*ZntA*) that provides energy for Cd^{2+} , Zn^{2+} and Co^{2+} efflux through hydrolysis. The low similarity level of these proteins suggested that they have bifurcated considerably in the process of evolution, they may have obtained unique way in protecting strain JC1 from being damaged by heavy metals (Valencia et al. 2013). The $\text{Ni}^{2+}/\text{Co}^{2+}$ -uptake system was not predicted in the genome of strain JC1, but fortunately the $\text{Mg}^{2+}/\text{Co}^{2+}$ -uptake system was predicted. It may be that *S. rhizophila* JC1 decreased its $\text{Ni}^{2+}/\text{Co}^{2+}$ -detoxification function, but to maintaining homeostasis through controlling the efflux of outer membrane (Rozycki and Nies 2009). This finding meant the stepwise evolutionary from the ancestor of *S. rhizophila* had already able to deal with heavy metals (Nies 2003, Rozycki and Nies 2009).

One possible $\text{Cu(I)}/\text{Ag(I)}$ -exporting RND4 protein (TC#2.A.6.1.8) was predicted in *S. rhizophila* JC1. This protein, as part of the copper resistance operon cluster, was encoded by chromosomal DNA and induced by Cu^{2+} (Moraleda et al. 2010). According to Nies (2006) classification of HME-RND, no specific RND3a (Zn^{2+}) or RND3b (Co^{2+}) transporters were predicted in *S. rhizophila* JC1. We speculated that Zn^{2+} and Co^{2+} detoxification by strain JC1 may depend on the chelation of related anionic groups.

P-type ATPase

S. rhizophila JC1 contains a relatively low number (5) of predicted P-type ATPases (Table 1). After concerned, *copA* involved a polypeptide domain of approximately 50 amino acid residues with two cysteines. Owing to the presence of this domain that the *copA* may be related to the detoxification of Cu^+/Ag^+ ,

but ineffective to divalent ions that in contrast to copB. However, in *Bradyrhizobium liaoningense* by Liang (2016), copA is responsible for resistance to Cu⁺, Zn²⁺ and Cd²⁺. Moreover, copA induced gold (Au²⁺) detoxification due to a cytoplasmic metal-binding protein (Pontel et al. 2007).

The *ZntA*(JC1_GM001891) predicted in *S. rhizophila* JC1 was the P5-type ATPase, having plasma membrane C-terminal and N-terminal auto inhibitory domains. As described in *Cupriavidus metallidurans*, *ZntA* was the downstream gene of *czcCBA* operon that encoded the Cd²⁺/Zn²⁺-exporting ATPase, and its side chains, containing Met254, Cys476 and His807 contributed to Cd²⁺, Co²⁺ and Zn²⁺ binding and transporting, respectively(Smith et al. 2017).

Mercury detoxification

Three mercury detoxification proteins, MerP/T/R (JC1_GM001926–JC1_GM001928), were predicted in *S. rhizophila* JC1. MerP contributed to binding the highly toxic Hg²⁺, MerT was responsible for importing Hg²⁺ into cytoplasm, MerR was the regulator of *mer* operon (Silver and Phung 1996). Unlike in *C. metallidurans* CH34 (Rozycki and Nies 2009), there were no MerA and *merRPT* fragments predicted in *S. rhizophila* JC1. However, two copper efflux regulators (JC1_GM002046 and JC1_GM002150) and a redox-sensitive transcriptional activator SoxR (JC1_GM000920) were predicted.

In summary, the efflux system of *S. rhizophila* JC1 contained the same types of metal transport proteins as many other metal-resistant bacteria. Its low number of transport proteins may be the result of evolution.

Comparative genomics analysis

Molecular evolutionary analysis

A molecular evolutionary analysis is a valuable measure to evaluate similarities or differences, and to match functional gene information, between model organisms and uncharacterized newly sequenced genomes(Gabaldón and Koonin 2013).

The evolutionary relationships of the 15 investigated bacteria were shown in Fig. S1. *S. rhizophila* JC1 had long-distance evolutionary relationships with other strains, which indicated that there may be great differences in gene expression levels. In addition, the lower number of transporters in genome of *S. rhizophila* JC1 compared with the other strains was the result of evolution.

Numbers of paralogs and transporters in the compared bacteria

For more information about the origin of metal-resistance gene of *S. rhizophila* JC1, the paralogs of predictive gene products of 15 investigated bacteria were analyzed by BLAST comparisons. On the whole, the frequency level of paralogs among compared bacteria was approximately 8% (Table 2). The relatively closest relationship with *Salmonella enterica* P-stx-12, has 30% paralogs, indicating that most of its increased genome size may be due to gene replication events during the bacterial species formation process.

In terms of most investigated strains, the frequency of paralogs of transporters was close to the 7% (Table 2). High percentages of paralogous protein-encoding genes were located on plasmids in *S. rhizophila* JC1 (28%), unlike the plasmid in *Enterococcus hirae* NCTC12204 (5%). When only considered the plasmid encoding paralogs of transporters, 29% of the *S. rhizophila* JC1 proteins represented paralogs. Therefore, duplicating genes encoding transport proteins, relocating them to plasmids and altering the substrate range and their products' expression patterns may have been important evolutionary processes in the evolution of *S. rhizophila* JC1.

Comparison of transition-metal transporters

A higher number of heavy metal-specific transport proteins were predicted of strain JC1 through comparing with 15 investigated strains, especially proteins belonging to three efflux system (Table 3). For instance, the number of P-Type ATPases that contribute to Cd²⁺/Zn²⁺ even to Cu⁺/Cu²⁺ was nearly twice of other compared strains. Regrettably, the number of zinc transporters and mercuric transporters were relatively lower. The Mg²⁺/Co²⁺-transport protein (TC[#]9.A.40.1.2, JC1_GM001315) that belong to the HlyC/CorC family was predicted in *S. rhizophila* JC1. We speculated that it may have great detoxification ability to divalent cations due to its approximately 440 amino acids with an N-terminal four TMS domain. Here, we further speculated that the metal resistance gene of *S. rhizophila* JC1 appeared to have evolved by horizontal acquisition or duplication (Rozycki and Nies 2009). The detoxification ability to heavy metal was perfect, even though the number of detoxification system had decreased.

Validation of predicted genes by qPCR

The expression level of 4 genes that respectively represents three efflux systems were identified by qPCR. Each gene to be validated was successfully amplified and produced a single band. *CzcD*, *czcB* and *ZntA* were up regulated after treated with Cr⁶⁺, Pb²⁺ and Zn²⁺ as compared to the CK. Cu²⁺-exporting ATPase and *ZntA* were up regulated in after treated with Cu²⁺ as compared to the CK. Among them, *CzcD*, *czcB* and *ZntA* exhibited higher expression levels in *S. rhizophila* JC1 treated with Cr⁶⁺ compared to samples treated with Pb²⁺ and Zn²⁺. The expression level of each gene used for identification showed significantly difference by P-value evaluation. The expression trend of 4 selected genes was consistent with the metal ions-removal efficiency and predicted results, which further confirmed that transport proteins play important roles in process of heavy metal adsorption.

Conclusion

The *S. rhizophila* JC1 showed great adsorption capacity for heavy metals, especially for Cu²⁺ and Cr⁶⁺. The moderate change of membrane permeability and replacement of essential element sites may be the key factors for the adsorption of heavy metals by bacteria, and the exopolysaccharides (EPS) is the main

means of heavy metals adsorption by bacteria. In addition, transport proteins play the important roles in the adsorption and detoxification of heavy metals. We hope that our research can provide relevant genetic resources for heavy metal detoxification, and then make a certain contribution to the remediation of heavy metal contaminated soil.

Declarations

Authors contributions Design: Jixiang Chen, Yonggang Wang, Performance: Shangchen Sun, Kexin Zhang, Yamiao Wu, Data analysis: Shangchen Sun, Yonggang Wang, Feifan Leng, Writing-original draft preparation: Shangchen Sun, Kexin Zhang, Writing-review and editing: Jixiang Chen, Feifan Leng, Funding acquisition: Jixiang Chen, Yonggang Wang. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

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

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Tables

Table 1 Heavy metal-specific transport proteins in *S. rhizophila* JC1

Predicted protein	Transporter	TC number	TMSs
CDF proteins			
slm:BIZ42_15070	czcD, zitB	2.A.4.1.1	6
stek:AXG53_19310		2.A.4.4.7	6
RND proteins			
stek:AXG53_08210	czcA	2.A.6.1.15	12
stek:AXG53_19335	czcC		0
psd:DSC_03900	czcB		0
slm:BIZ42_09005	czcA	2.A.6.1.16	12
srh:BAY15_0023	czcB		0
slm:BIZ42_09015	czcC		1
sdf:ACG33_13555	cusA, silA	2.A.6.1.8	12
slm:BIZ42_11620	TC.CPA1	2.A.36.3.1	13
smt:SmaI_3140	TC.KEF	2.A.37.1.5	13
xpe:BJD13_00190	pcoB,copB	1.B.76.1.1	1
P-type ATPase			
stek:AXG53_08205	zntA	3.A.3.6.27	5
xga:BI317_24540	copA,	3.A.3.5.39	8
stek:AXG53_00105	copB	3.A.3.5.15	8
sdf:ACG33_13605	copB	3.A.3.5.38	8
spe:Spro_2413	copA,	3.A.3.5.20	8
Mer proteins			
sdf:ACG33_13625	merT	1.A.72.3.1	3

Table 2 Numbers of paralogs of proteins and transport proteins in the compared bacteria

Bacterial strain	Replicons	All proteins		Transport proteins	
		Total number	Paralogs (%)	Total number	Paralogs (%)
<i>S. rhizophila</i> JC1	All	3717	448 (12%)	323	32 (10%)
	plasmids	276	77 (28%)	21	6 (29%)
<i>A. xylooxidans</i> MN001	All	5227	418 (8%)	665	53 (8%)
	plasmids	1023	153 (15%)	26	4 (15%)
<i>A. endophyticus</i> AER10	All	6187	557 (9%)	983	108 (11%)
	plasmids	485	63 (13%)	34	5 (15%)
<i>A. eutrophus</i> H16	All	6573	564 (8%)	1053	84 (8%)
	plasmids	416	130 (31%)	45	13 (28%)
<i>B. subtilis</i> 168	All	6367	446 (7%)	1018	92 (9%)
	plasmids	638	128 (20%)	40	12 (30%)
<i>C. albicans</i>	All	5428	271 (5%)	784	63 (8%)
	plasmids	386	97 (25%)	36	11 (31%)
<i>C. metallidurans</i> CH34	All	6351	643 (10%)	1002	93 (9%)
	plasmids	416	141 (34%)	40	22 (52%)
<i>E. hirae</i> NCTC12204	All	2574	201 (8%)	209	21 (10%)
	plasmids	87	35 (4%)	8	0
<i>M. xanthus</i> NBRC 13542	All	5983	658 (11%)	957	77 (8%)
	plasmids	1026	308 (30%)	36	10 (28%)
<i>P. putida</i> DSM3601	All	4419	663 (15%)	718	43 (6%)
	plasmids	379	68 (18%)	28	51 (18%)
<i>P. stutzeri</i> DSM4166	All	4212	787 (19%)	802	72 (9%)
	plasmids	722	101 (14%)	26	3 (12%)
<i>S. enterica</i> P-stx-12	All	4690	1407 (30%)	962	88 (9%)
	plasmids	998	250 (25%)	31	4 (13%)
<i>S. maltophilia</i> D457R	All	5626	563 (10%)	839	92 (11%)
	plasmids	1124	135 (12%)	28	5 (18%)
<i>S. pneumoniae</i> MGAS315	All	2029	162 (8%)	243	19 (8%)
	plasmids	89	8 (9%)	5	0

^a All the predicted proteins from respective bacteria were compared using BLASTP-based searches.

Table 3 Heavy metal-specific transport proteins in fifteen bacteria

TC number	TC family	<i>S. rhizophila</i> JC1		Orthologs ^c					
		Abs ^a #	Paral ^b #	<i>A.xylosoxidans</i> MN001	<i>A.endophyticus</i> AER10	<i>A.eutrophus</i> H16	<i>B.subtilis</i> 168	<i>C.albicans</i>	<i>C.me</i>
2.A.6	HME-RND ^d	18	3	3	2	3	6	3	2
8.A.1	MFP	12	2	5	4	2	5	4	3
1.B.7	OMF	9	2	7	4	5	2	1	2
3.A.3	P-Type ATPase	13	5	6	6	8	6	7	5
2.A.4	CDF	2	2	2	2	2	1	2	0
9.A.2	MerT	1	1	1	1	0	0	0	2
2.A.5	ZUP	2	0	0	0	0	0	0	0
9.A.40	MgCoT	1	0	0	0	0	0	0	0
TC number	TC family	Orthologs							
		<i>P.putida</i> DSM3601	<i>P.stutzeri</i> DSM4166	<i>S.enterica</i> P-stx-12	<i>S.maltophilia</i> D457R	<i>S.pneumoniae</i> CP2215	<i>M. xanthus</i> NBRC13542	<i>S. hire</i> NCTC2204	
2.A.6.1	HME-RND	5	5	4	1	2	2	1	
8.A.1	MFP	6	5	2	4	3	4	3	
1.B.7	OMF	4	3	1	5	2	3	1	
3.A.3	P-Type ATPase	4	8	5	6	8	7	7	
2.A.4	CDF	1	2	2	1	3	2	1	
9.A.2	MerT	0	1	0	0	0	0	0	
2.A.5	ZuP	0	0	0	0	0	0	0	
9.A.40	MgCoT	0	0	0	0	0	0	0	

^aThe absolute number.

^bThe number of proteins that are paralogs to proteins in strain JC1.

^cOrthologs in the indicated bacterial strains.

^dHeavy-metal efflux proteins of the RND superfamily. Names and TC classifications from <http://www.tcdb.org/>.

Figures

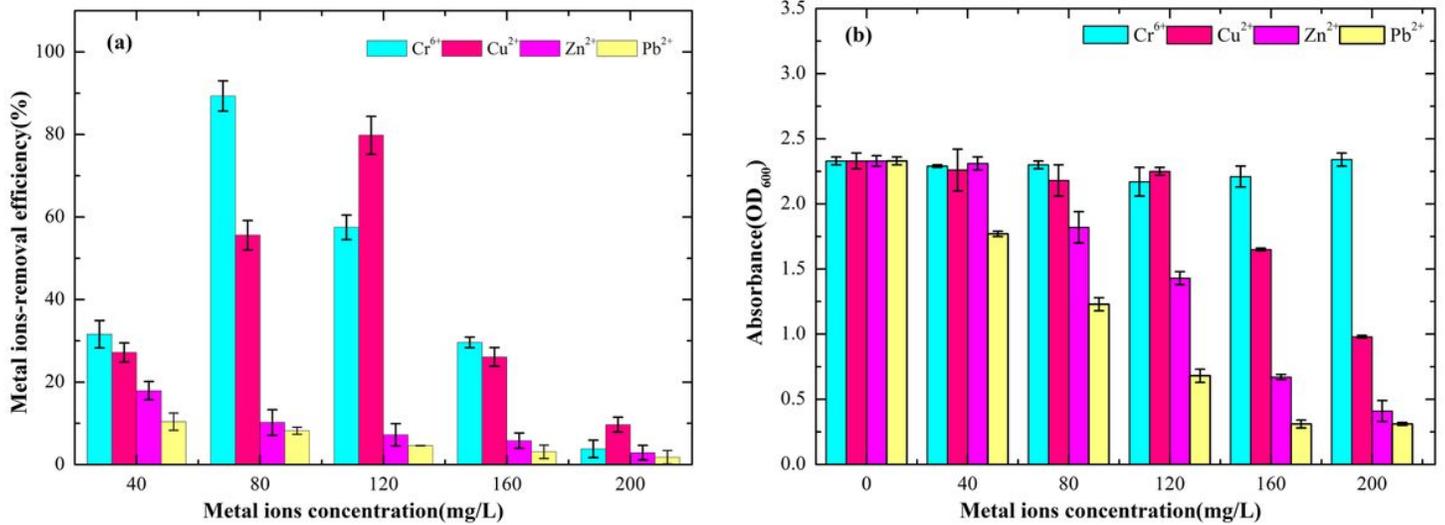


Figure 1

The toleration and adsorption to different metal ions of strain JC1 showed great difference. For instance, during *S. rhizophila* JC1 was respectively cultured in LB+Cu²⁺, LB+Cr⁶⁺, LB+Zn²⁺ that the concentration of metal ions was 40~120 mg/L, 40~200 mg/L, 40 mg/L for 24 hours, there were no significant difference in OD₆₀₀ value. However, during the concentration of Zn²⁺ and Pb²⁺ were increased to 160 mg/L, the strain JC1 hardly grew. The adsorption efficiency was 79.8% when the concentration of Cu²⁺ was 120 mg/L, while it reduced to 26.1% when the concentration reached to 160 mg/L. More interesting was that with the concentration was 40~200 mg/L of Cr⁶⁺, there had no significant suppression on the growth of bacteria, but only showed maximum adsorption efficiency of 89.3% when the concentration was 80 mg/L. In terms of Pb²⁺ and Zn²⁺, the strain JC1 hardly showed adsorption ability

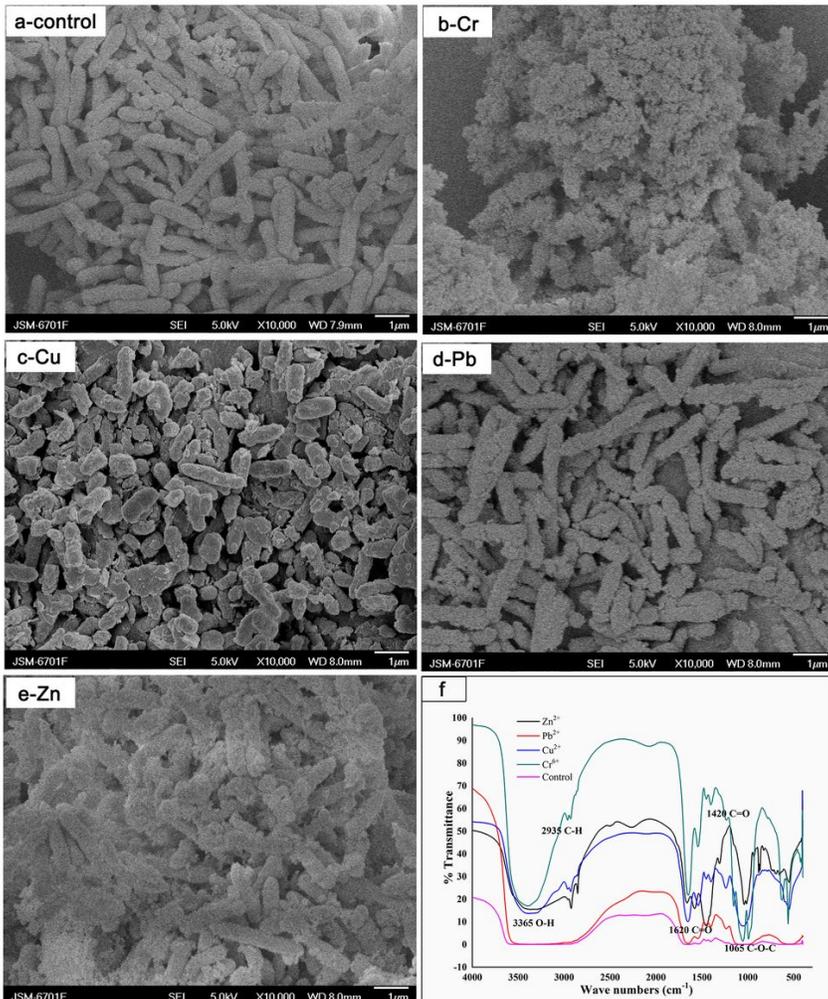


Figure 2

The morphological feature of strain JC1 comparison between metal ions stressed-cells and unstressed-cells was performed with SEM analysis. In different metal ions stress condition, different cell morphology was observed

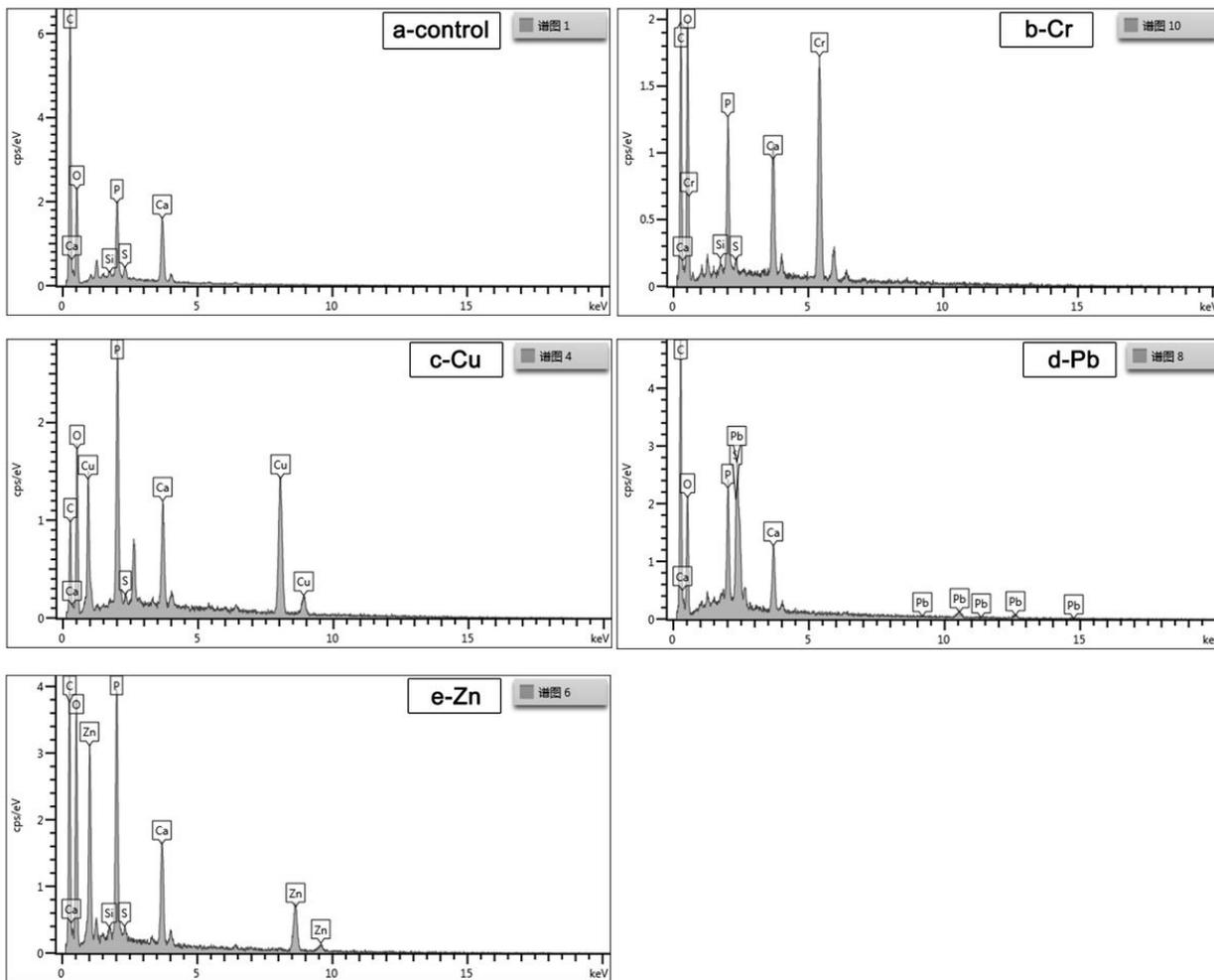


Figure 3

Generally, non-metallic elements such O, C, Si and non-toxic metal elements such as Ca^{2+} , $\text{Fe}^{2+}/\text{Fe}^{3+}$ were the basic elements to maintain normal growth and metabolism of microorganism. However, some toxic metals such as Cr^{6+} or Pb^{2+} will exist in the cells through sites replacement due to atomic radius, cell structure. The result of element composition analysis after metal ions stress was shown in Fig. 3.

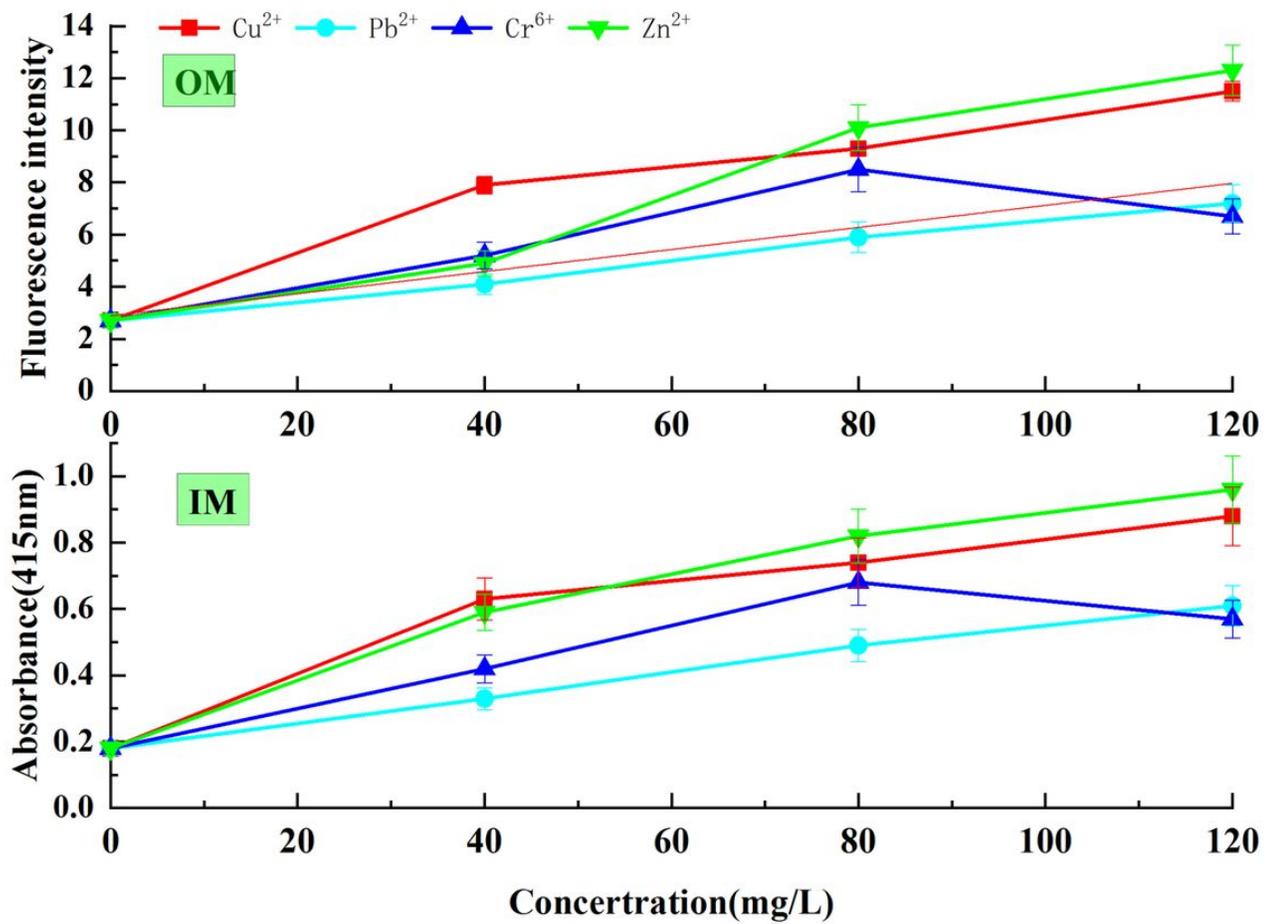


Figure 4

The change of OM permeability under different heavy metal stress.

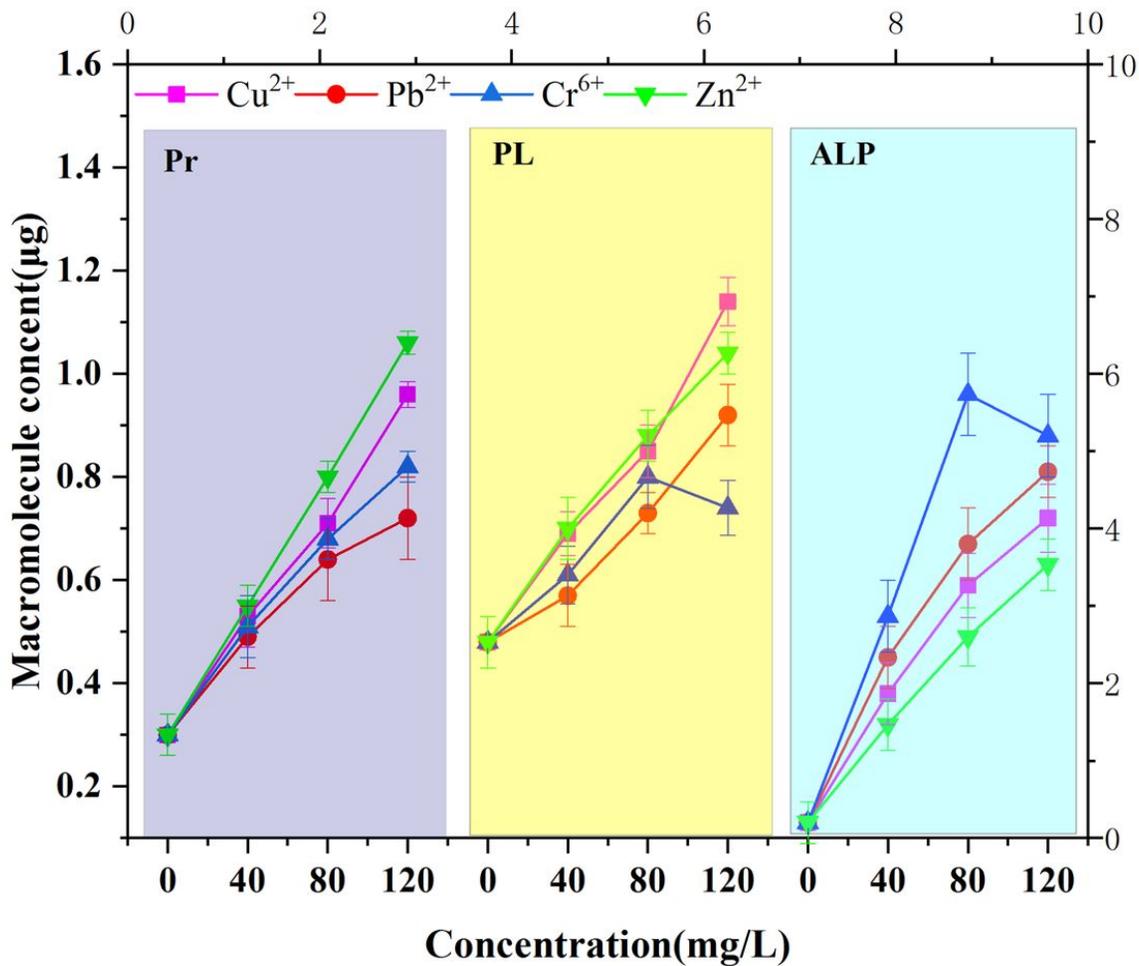


Figure 5
 The excessive release of macromolecules such as proteins (Pr), phospholipid (PL), alkaline phosphatase (ALP) will definitely affect the normal function of cells. Without a doubt, with the change of membrane permeability, macromolecules will release to the outside of the cells. In terms of proteins and phospholipid, the variation trend was consistent with the change of membrane permeability

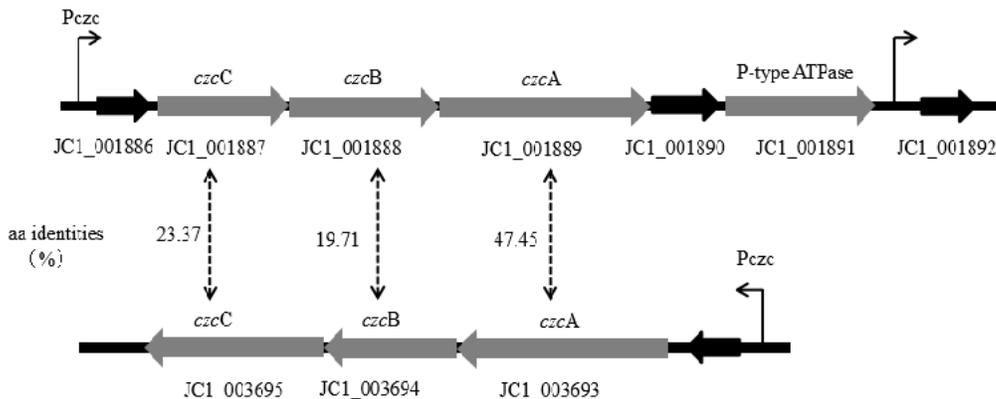


Figure 6
 Six heavy-metal efflux proteins were predicted in *S. rhizophila* JC1 (Table 1). Among them, two types of *czcCBA* operons (TC#2.A.6.1.15 and TC#2.A.6.1.16) form a complete cobalt–zinc–cadmium resistance system homologous to that in *Escherichia coli* (Tam et al. 2019). *czcA* is the RND protein, having at least one transmembrane domain and a membrane-spanning helical or beta-stranded domain that embeds in the membrane. *czcC* is a member of the OMP family

that forms a trimeric channel and a long helical barrel that allows the export of a variety of substrates in Gram-negative bacteria. In *S. rhizophila* JC1, the *czcCBA* operon may transport Cd²⁺ and Zn²⁺, as well as Co²⁺, because there is a gene downstream of *czcA* that encodes a Cd²⁺/Zn²⁺-exporting ATPase

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