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Extracellular polymeric substances from copper-tolerance *Sinorhizobium meliloti* immobilize Cu^{2+}

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HIGHLIGHTS

- EPS produced by *Sinorhizobium meliloti* CCNWSX0020 restricts uptake of Cu^{2+}.
- We focused on the EPS, which is divided into three main parts.
- LB-EPS played a more important role than S-EPS and TB-EPS in Cu^{2+} immobilization.
- Proteins and carbohydrates were the main extracellular compounds which had functional groups such as carboxyl (—COOH), hydroxyl (—OH), and amide (N—H), primarily involved in metal ion binding.

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ABSTRACT

The copper tolerance gene of wild-type heavy metal-tolerance *Sinorhizobium meliloti* CCNWSX0020 was mutated by transposon Tn5-a. The mutant was sensitive up to 1.4 mM Cu^{2+}. Production, components, surface morphology, and functional groups of extracellular polymeric substances (EPS) of the wild-type strains were compared with sensitive mutant in immobilization of Cu^{2+}. EPS produced by *S. meliloti* CCNWSX0020 restricts uptake of Cu^{2+}. The cell wall EPS were categorized based on the compactness and fastness: soluble EPS (S-EPS), loosely bound EPS (LB-EPS), and tightly bound EPS (TB-EPS). LB-EPS played a more important role than S-EPS and TB-EPS in Cu^{2+} immobilization. Scanning electron microscopy (SEM) analysis LB-EPS had rough surface and many honeycomb pores, making them conducive to copper entry; therefore, they may play a role as a microbial protective barrier. Fourier transform-infrared (FT-IR) analysis further confirm that proteins and carbohydrates were the main extracellular compounds which had functional groups such as carboxyl (—COOH), hydroxyl (—OH), and amide (N—H), primarily involved in metal ion binding.

1. Introduction

Wastewater from anthropogenic sources contains many heavy metal ions. Various chemical, physical, and biological methods have been developed to remove heavy metals from wastewater. During the last decade, research has focused on biological methods, which are more economical and efficient. Biosorption is a cost-effective technology for the treatment of high volumes of wastewater [1,2].

Certain types of microbial biomass secrete high molecular mass polymers, which are released into the surrounding environment or remain attached to cell surfaces (capsular polysaccharides) [3]. A number of studies show that extracellular polymeric substances (EPS) are effective absorbents for removing heavy metals and organic pollutants such as dyes and pesticides [4–7]. EPS are metabolic products that accumulate on the microbial cell surface, causing the cells to aggregate and providing protection by reducing chemical exposure, stabilizing the membrane against the harsh external environment, and serving as carbon and energy reserves during starvation [8]. This protective mechanism has been observed in comparisons of cells embedded in EPS and free cells without EPS. Cellular tolerance is partially due to binding and/or reaction of EPS components to/with heavy metal ions [9]. EPS are heterogeneous gel-like matrices of polymers composed of polysaccharides, proteins, nucleic acids, and lipids. Heavy metal ions are embedded through electrostatic and hydrophobic interactions. EPS are classified as soluble (S-EPS) and bound (B-EPS). S-EPS are dissolved in solution; B-EPS are bound to cells and are generally subdivided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [10,11]. LB-EPS may function as the primary surface for cell

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attachment and flocculation. However, most experimental work on EPS has not specifically addressed the role of the three types EPS in biosorption in solution. Thus, little information is available to distinguish various compositions of the three types of EPS and their effects on surface properties and biosorption.

Sinorhizobium meliloti CCNWSX0020 is a symbiotic nitrogen-fixing bacteria tolerance to 1.4 mM copper; it was isolated from *Medicago lupulina* growing in mine tailings [12]. To the best of our knowledge, the relative contributions of S-EPS, LB-EPS, and TB-EPS to copper immobilization have not been investigated. Therefore, the goal of this study was: (1) to investigate the main compositions of three types of EPS from the wild-type strain and sensitive mutant strain (2) and to identify the adsorption mechanisms, determine the individual effects of the three types EPS on biosorption by scanning electron microscopy (SEM) and Fourier transform-infrared (FT-IR) spectroscopy spectra analysis. We believe that a better understanding of the immobilization mechanisms of copper occurring within EPS and the factors that affect those mechanisms may contribute to the development of more efficient biosorption treatment processes.

2. Materials and methods

2.1. Bacterial culture and mutation

S. meliloti CCNWSX0020 was cultivated in TY medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ per liter) for 72 h at 28 °C with shaking at 150 rpm. Mutation was constructed using plasmid pRL1063a, which harbors a Tn5 transposon with promoterless *luxAB* genes and a kanamycin resistance marker [13]. Tri-parental mating was carried out as described by Chu [14]. Briefly, cells of the donor strain *E. coli* DH5a, harboring the suicide plasmid pRL1063a or the helper plasmid pRK2013, and the recipient strain, *S. meliloti* CCNWSX0020, were grown in LB supplemented with kanamycin and TY medium, respectively. The transposon Tn5 was transferred into wild-type *S. meliloti* CCNWSX0020, and the mutant strain was screened on plates containing 1.4 mM Cu^{2+}.

2.2. Copper accumulation

Cu^{2+} solution (1.4 mM) was prepared by dissolving $CuCl_2$ in distilled water. The solution was sterilized by filtering with a 0.2 μm pore size filter [15]. Experiments were carried out using 100 mL TY medium in 150-mL Erlenmeyer flasks. The wild-type and mutant were cultured in Cu^{2+} medium at 28 °C for 72 h with shaking at 150 rpm. The cells were harvested from early stationary cultures (OD_{600} = 1.2–1.5) by centrifugation at 5000 rpm for 10 min. Residual Cu^{2+} in the medium was measured by an atomic absorption spectrophotometer – AAS every 6 h. A blank experiment was performed with Cu-free TY medium. Cu^{2+} tolerance was evaluated by comparison with the control. The removal rate of Cu^{2+} was evaluated as follows:

$$\text{Removal rate}\,(\%) = \frac{(C_0 - C_e)}{C_0} \times 100$$

where C_0 was the initial metal ion concentration ($mg\,L^{-1}$), C_e was the residual metal concentration in solution ($mg\,L^{-1}$).

2.3. Isolation, extraction, and characterization of EPS

After 3 days of pure cultivation in TY medium, cells were harvested at room temperature by centrifugation at 5000 rpm for 10 min. The supernatant was dialyzed against Milli-Q water using regenerated cellulose (RC) membranes (8000 MWCO from Spectrum) to remove low-molecular-weight impurities. Milli-Q water was changed every 3 h for 24 h dialysis [16]. S-EPS solution was freeze-dried and reserved. To separate LB-EPS, cells were dissolved in Milli-Q water and sonicated at 40 w for 1 min. The suspension was centrifuged at 7000 rpm for 20 min. Dialysis yielded the LB-EPS solution, which was freeze-dried and reserved. Cells were dissolved in Milli-Q water and heated at 80 °C for 10 min; the suspension was centrifuged at 15,000 rpm for 20 min. Dialysis of the supernatant yielded TB-EPS solution [17].

The EPS carbohydrate content was measured by the phenol-sulfuric acid method with glucose standard [18]. Protein content was determined by the Bradford method with bovine albumin serum standard [19] and the nucleic acid content was determined by the diphenylamine method [20].

Biosorption experiments were carried out in order to investigate Cu^{2+} removal by 10 mg EPS were placed in dialysis bags (8000 MWCO) in the solution (at 28 °C, pH 7 and 1.4 M Cu^{2+}).

2.4. Scanning electron microscopy (SEM)

The S-EPS, LB-EPS, TB-EPS of the wild strain and mutant surface structure and morphology were observed by SEM (JSM-6360 LV; JEOL, Peabody, MA, USA). All EPS were dried in a critical-point dryer using CO_2 (K850; EMITECH, East Grinstead, UK). Metal-loaded samples were mounted on a stainless steel slab with double-sided adhesive tape and a thin layer of gold in a high vacuum. Specimens were examined by SEM.

2.5. Fourier transform-infrared spectroscopy (FT-IR)

Infrared analysis was performed with a 330 FT-IR (Fourier transform-infrared spectrometer, NICOLET AVATAR, USA Nicolet Co.) at a range of 4000–400 cm^{-1}. Powdered EPS (1 mg) was mixed and ground with 100 mg KBr (Spectrum pure) in an agate mortar to investigate the functional groups relevant to Cu^{2+} biosorption.

2.6. Experimental design and statistical analysis

All experiments were performed in triplicate and the mean values are presented. Statistical analysis was performed with SPSS 13.0 Bivariate Correlation Analysis. The Pearson rank order coefficient was determined to compare Cu^{2+} tolerance between EPS types and the removal of Cu^{2+} by the wild-type and mutant strains. Distinct differences were detected by Dunnett and Tukey grouping tests; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Cu^{2+} accumulation

The wild-type strain grew well in medium supplemented with 1.4 mM Cu^{2+}, while the sensitive mutant strain grew extremely slowly. The wild-type and mutant exhibited different Cu^{2+} removal abilities. At the end of the 3rd day, the sensitive mutant strain and wild-type adsorbed 9.8 and 20.6% of Cu^{2+} onto the cell surface and accumulated 6.1 and 3.9% of the Cu^{2+} into their intracellular spaces (Fig. 1). The wild-type absorbed more Cu^{2+} than the sensitive mutant strain. Bacteria, fungi, yeast, and algae are thought of as microbial biosorbents for removal of heavy metals. Previous studies have shown that EPS has important roles in metal ion biosorption by *Shewanella* species [21]. In order to cope with heavy metal exposure, strains have evolved several metal tolerance strategies. Intracellular metal tolerance mechanisms include efflux, complexation, or reduction of metal ions to a non-toxic form; chelation of metal ions by certain extracellular polymeric substances provide extracellular protection [22]. These observations are consistent with the chemical nature of the released extracellular compounds, which contain large amounts of siderophores and