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Heavy metal and antibiotic resistance of ureolytic bacteria and their immobilization of heavy metals

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ABSTRACT

Environmental pollution by toxic heavy metals is spreading worldwide along with industrial progress. The isolation and characterization of microorganisms capable of resisting elevated concentrations of heavy metals as well as multiple types of antibiotics are critical to the development of an effective bioremediation strategy for polluted sites. In this study, we first investigated the interplay between heavy metals and the antibiotic resistance of ureolytic bacteria. The antibiotic resistance patterns revealed that the heavy metal resistance of these isolates was closely associated with their resistance to antibiotics. In addition, we examined the immobilization of heavy metals by these isolates, based on microbially induced calcite precipitation (MICP). The unconfined compressive strength of a cylinder specimen injected once with the selected bacterial culture showed a 3.7-fold increase relative to an untreated specimen. In addition, it was found that the heavy metals were highly immobilized in the bacteria-treated cylinder samples.

1. Introduction

A worldwide environmental problem has developed over the past few decades owing to the rapid increase in industrialization and urbanization. Elevated concentrations of heavy metals are introduced into the environment through metalliferous mining, metal smelting, activities of metallurgical industries, waste disposal, and corrosion of metals in use (Bachate et al., 2013; Fan et al., 2014; Prithviraj et al., 2014; Kang et al., 2015). Several methods for treating environmental contaminants have been developed over the past decades, such as applying physical, chemical, and biological processes. Although physical and chemical approaches are capable of removing a broad spectrum of contaminants, the main disadvantages of these methods lie in their increased energy consumption and need for additional chemicals (Máthé et al., 2012).

The use of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals (Rittle et al., 1995) through reduction, accumulation, mobilization, and immobilization (Lovley, 1994; Avery, 1995; Valentine et al., 1996; Kang et al., 2014) has been studied in several countries. During bioremediation, the metabolic activity of microorganisms is involved in the breakdown of contaminants into non-toxic compounds. This technique is cost-effective, is applicable over large areas, and can lead to the complete breakdown of the organic contaminants, potentially ending in their mineralization (Sarkar et al., 2004; Trindade et al., 2004).

Microbially induced calcite precipitation (MICP)-based degradation of urea occurs through the ureolytic pathway, which produces ammonium ions as an energy source and leads to the alkalization of the surrounding environment (Whiffin et al., 2007). In addition to NH_4^+, carbonate ions are formed, which precipitate as calcite ($CaCO_3$) in the presence of Ca^{2+} (Hammes and Verstraete, 2002). Moreover, when these reactions occur in sand, crystals are formed between the sand particles and they hold the sand particles together. Carbonate precipitation is an important aspect of biomineralization and has been investigated extensively owing to its wide range of technological applications (Ivanov and Chu, 2008; Anbu et al., 2016). The hydroxide ions result in an increase in pH, which in turn can shift the bicarbonate equilibrium, resulting in the formation of carbonate ions. This shift can then precipitate heavy metal ions in wastewater or soil (Hoffman and Deccho, 1999).

The introduction of certain concentrations of heavy metals into the environment kills the majority of the microflora, thereby selecting for a few cells that have evolved resistance mechanisms to the heavy metals. The resistance mechanisms used by microorganisms to tolerate heavy metal stress include permeability barriers, intra- and extracellular sequestration, efflux pumps, enzymatic detoxification, and reduction (Nies, 1999). In some cases, resistance to metal ions has been reported to be plasmid-mediated and

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observed to be encoded by genes in close proximity to antibiotic resistance genes (Alonso et al., 2000; Spain, 2003). A correlation between heavy metal tolerance/resistance and antibiotic resistance in *Escherichia coli* (Spain, 2003) and *Staphylococcus* sp. (Groves and Young, 2016) has been reported. Alonso et al. (2000) implicated a cluster of genes to be involved in the antibiotic and heavy metal resistance of a clinical isolate of the gram-negative bacterium *Stenotrophomonas* (*Xanthomonas*) *maltophilia*. Therefore, heavy metal and antibiotic resistance may sometimes be transferred together in the environment. Furthermore, as several bioremediative pathways are also located on mobile genetic elements (e.g., plasmids), a long-term exposure to heavy metals and/or antibiotics may be linked to the widespread distribution of bioremediative capabilities (Roy et al., 2002).

In this study, we determined the heavy metal resistance patterns of isolated ureolytic bacteria that showed resistance to elevated concentrations of multiple types of heavy metals. Additionally, the isolated strains were screened for resistance to multiple types of antibiotics. The organisms with a combination of heavy metal and antibiotic resistance would be useful for bioremediation of environments polluted with heavy metals and would also help to overcome metabolic bottlenecks still existing in the bioremediation processes by applying them in processes such as the immobilization of heavy metals.

2. Materials and methods

2.1. Microbial strains and culture conditions

Six bacterial strains, isolated from soil from an abandoned mine, were used in this study because of their abilities to induce urease activity, produce calcite, and resist heavy metals (Kang et al., 2014, 2015). These strains were *Viridibacillus arenosi* B-21 (B-21), *Sporosarcina soli* B-22 (B-22), *Enterobacter cloacae* KJ-46 (KJ-46), *Enterobacter cloacae* KJ-47 (KJ-47), *Lysinibacillus sphaericus* KJ-64 (KJ-64), and *Sporosarcina pasteurii* WJ-2 (WJ-2). The 16S rRNA gene sequences determined in this study were deposited in the NCBI GenBank database under the accession numbers KJ671467 (B-21), KJ485701 (B-22), KF598853 (KJ-46), KF598854 (KJ-47), KF598850 (KJ-64), and KC211294 (WJ-2). The cultures were routinely grown at 30 °C and 200 rpm in YA broth (20 g/L yeast extract and 10 g/L ammonium sulfate, pH 7).

2.2. Biochemical and physiological characterization

Biochemical characterization was carried out to detect the presence of enzymes, such as gelatinase, oxidase, galactosidase, and urease, and the utilization of glucose, sucrose, arabinose, among others. Other tests included the indole, Voges–Proskauer, citrate utilization, and H_2S production tests.

To select heavy-metal-resistant bacteria, it is necessary to standardize the cultural and physiological conditions of the isolated strains. Among the physicochemical conditions to consider, acid (pH), temperature, and starvation are of great importance to bacterial growth. The isolated strains were grown at different temperatures (5, 20, 30, 40, and 50 °C) in YA broth, with incubation carried out at 200 rpm for 24 h. The strains were also grown in YA broth of different pH values (from 4.0 to 10.0), where incubation was carried out at 30 °C and 200 rpm for 24 h. The growth was measured in terms of optimal density (OD) at 600 nm using a UV/visible spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech Inc., NJ, USA). For the starvation test, bacterial cells (1×10^8 CFU/mL) suspended in YA broth were starved at 30 °C for 35 days. The cell viability after the 35 days was determined by the spread plate technique, using triplicate samples and YA agar plates.

2.3. Measurement of calcite production and urease activity

One milliliter of a culture of isolated bacteria was grown overnight in BPU broth at 30 °C and 200 rpm for 48 h. The bacterial suspension (500 μL) was added to 500 μL of calcium chloride dihydrate solution (350 mM). The mixture was centrifuged at 16,179 × *g* for 5 min at 25 °C to collect the precipitate, which was then dried for 24 h at 50 °C and weighed.

Urease activity was determined using the phenol-hypochlorite assay (Natarajan, 1995). The bacterial suspension (250 μL) was added to 250 μL of sodium phosphate buffer (0.1 M) containing 500 μL of urea solution (3 M). The mixture was incubated at 37 °C

Table 1
Biochemical characteristics of isolated strains.

Biochemical Tests	Isolated strains					
	B-21	B-22	KJ-46	KJ-47	KJ-64	W-2
Gram reaction	+	+	–	–	+	+
2-nitrophenyl-βD-galactopyranoside (ONPG)	+	–	+	+	+	+
L-arginine (ADH)	–	–	+	+	–	–
L-lysine (LDC)	–	–	–	–	+	–
L-ornithine (ODC)	–	–	+	+	–	–
Trisodium citrate (CIT)	–	–	+	+	+	–
Sodium thiosulfate (H_2S)	–	–	–	–	–	–
Urea (URE)	+	+	+	+	+	+
L-tryptophane (TDA)	–	–	–	–	–	–
L-tryptophane (IND)	–	–	–	–	–	–
Sodium pyruvate (VP)	+	–	+	+	+	–
Gelatin (GEL)	–	–	–	–	+	+
D-glucose (GLU)	–	–	+	+	–	–
D-mannitol (MAN)	–	–	+	+	–	–
Inositol (INO)	–	–	–	–	–	–
D-sorbitol (SOR)	–	–	+	+	–	–
L-rhamnose (RHA)	–	–	+	+	–	–
D-sucrose (SAC)	–	–	+	+	–	–
D-melibiose (MEL)	–	–	+	+	–	–
Amygdalin (AMY)	–	–	+	+	–	–
L-arabinise (ARA)	–	–	+	+	–	–
Identification	*Viridibacillus arenosi*	*Sporosarcina soli*	*Enterobacter cloacae*	*Enterobacter cloacae*	*Lysinibacillus sphaericus*	*Sporosarcina pasteurii*
GenBank Accession number	KJ671467	KJ485701	KF598853	KF598854	KF598850	KC211294