Biosequestration of copper by bacteria isolated from an abandoned mine by using microbially induced calcite precipitation

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Abandoned mine sites are frequently polluted with high concentrations of heavy metals. In this study, 25 calcite-forming bacteria were newly isolated from the soil of an abandoned metal mine in Korea. Based on their urease activity, calcite production, and resistance to copper toxicity, four isolates were selected and further identified by 16S rRNA gene sequencing. Among the isolates, Sporosarcina soli B-22 was selected for subsequent copper biosequestration studies, using the sand impermeability test by production of calcite and extracellular polymeric substance. High removal rates (61.8%) of copper were obtained when the sand samples were analyzed using an inductively coupled plasma-optical emission spectrometer following 72 h of incubation. Scanning electron microscopy showed that the copper carbonate precipitates had a diameter of approximately 5–10 μm. X-ray diffraction further confirmed the presence of copper carbonate and calcium carbonate crystals.

Key Words: biosequestration; copper; copper carbonate; isolation; urease

Introduction

Decontamination of soils with heavy metals is challenging because of the strong adsorption of many heavy metals to the surface of the soil particles. The accumulation of toxic metals in humans has several consequences, such as growth and developmental abnormalities, carcinogenesis, mental retardation, and a wide range of other illnesses (Thiele, 1995). Copper is a naturally occurring trace element present in all types of environments, including soil, sediments, air, and water. It is used extensively in agriculture to control plant diseases and in many other industrial processes. Agricultural and industrial drains are discharged into the water and soil, thus causing copper pollution.

The conventional methodologies for the removal of toxic heavy metals include phytoremediation, on site chemical leaching of contaminants, and bioremediation by heavy metal-tolerant bacterial species (Achal et al., 2012; Kang et al., 2015); all these treatment methods are expensive and not successful in the long term. Although rapid in effect, some other methods such as the addition of cement or chemical fixatives are expensive ($30–$300/m3) and destroy the future productivity of the soil (Cunningham et al., 1995). Microbially induced calcite precipitation (MICP) has been considered a novel solution for this problem, and several bacterial species have been already utilized for MICP (Kang et al., 2016; Rivadeneyra et al., 1993). 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 NH4+, carbonate ions are formed, which precipitate as calcite (CaCO3) in the presence of Ca2+ (Hammes and Verstraete, 2002). Moreover, when these reactions occur in sand, crystals are formed between sand particles and they hold the sand particles together. The ability to assay urease activity is essential for understanding whether urea, which is either transported into the cell (Stocks-fischer et al., 1999) or produced internally through the catabolism of amino acids or purines, is assimilated into the microbial biomass.

In this study, in search for efficient bacterial strains for...
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Table 1. Characteristics of soil samples.

| Parameters                  | Value          |
|-----------------------------|----------------|
| Texture percentage          |                |
| Sand                        | 81.87          |
| Silt                        | 5.50           |
| Clay                        | 12.63          |
| pH (H₂O)                    | 3.18           |
| Electric conductivity (μS/cm)| 249.50        |
| Cation exchange capacity (emol/kg) | 6.84       |
| Organic matter content (%)  | 0.62           |
| Available phosphorous (mg/kg)| 2.91          |
| As (mg/kg)                  | 0.33           |
| Cr (mg/kg)                  | 0.03           |
| Fe (mg/kg)                  | 427.25         |
| Mn (mg/kg)                  | 1.11           |
| Pb (mg/kg)                  | 0.07           |
| Zn (mg/kg)                  | 2.79           |
| Cu (mg/kg)                  | 0.09           |

copper bioremediation by MICP, we first isolated 25 new copper-resistant bacterial strains from abandoned mine sites and selected a strain with high MICP activity. Copper sequestration by MICP was confirmed using a scanning electron microscope, X-ray diffraction (XRD) analyses, and an inductively coupled plasma-optical emission spectrometer (ICP-OES).

Materials and Methods

Soil sample collection and characterization. Calcite-producing bacteria were isolated from Imgi, an area comprising abandoned mine sites, in Busan, Korea. The location of the soil samples collection are 35°18′54.43″ N (latitude) and 129°00′79.12″ E (longitude). The samples were collected in sterile Pyrex media bottles and stored on ice. The significant characteristics of the soil samples are listed in Table 1. The total heavy metal in the soil was dissolved using HCl. The digested liquid was filtered (Whatman 125 mm, 0.45 μm filter) and the filtrate (discarding initial 25 mL of volume) was analyzed for heavy metal content using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Ultima 2C, Horiba, Japan). The total content of organic matter was analyzed by the Tyurin method (NAAS, 2006). Available phosphorous was determined photometrically using the Bray method (Bray, 1954).

Isolation and identification of ureolytic bacteria. The media containing the soil samples were serially diluted and spread onto plates containing 3 g/L beef extract, 5 g/L peptone, 20 g/L urea, 10 g/L micro agar containing 100 μg/mL cycloheximide (BPU agar; pH 7). The plates were then incubated at 30°C for 3 days. Subsequently, the colonies were visually picked for determination of urease activity and calcite production. After the pure culture procedure, the isolated strains were stored at −20°C in 20 g/L yeast extract with 10 g/L ammonium sulfate (YA broth; pH 7) and containing 25% (v/v) glycerol; the strains were grown at 30°C and 200 rpm.

The selected colonies were isolated in pure cultures and identified by 16S rRNA sequencing. BLAST analysis was performed to compare the sequences with available DNA sequences in the GenBank database of the National center for biotechnology information (NCBI). The sequences were aligned using the PHYDIT (http://plaza.snu.ac.kr/~jchun/phydix/) program, the alignment was manually corrected, and a phylogenetic tree was constructed using the neighbor-joining method implemented in the MEGA 5.0 software (Tamura et al., 2011).

Measurement of urease activity and calcite production. Urease activity was determined using the phenol-hypochlorite assay (Natarajan, 1995). The isolates were cultured in YA broth for 18 h and sub-cultured into YA broth at 30°C for 8 h with continuous aeration at 200 rpm. The bacterial strains grown in YA broth were harvested (2,400 × g, 10 min), twice washed with phosphate buffer (PBS; NaH₂PO₄ 0.1 g/L, Na₂HPO₄ 26.6 g/L, 1 mM EDTA), and resuspended in PBS to a final OD₆₀₀ of 0.1. The bacterial suspension (250 mL) was added to 250 mL of PBS (0.1 M) containing 500 mL of urea solution (3 M). The mixture was incubated at 37°C for 72 h. Subsequently, 2 mL of phenol nitroprusside solution was added to an alkaline hypochlorite solution and then incubated at 50°C for 40 min. After incubation, the absorbance was measured at 626 nm in a spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Ammonium chloride (0–10 μM) was used as a standard. One unit of urease activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μM urea per minute.

For calcite production, the isolates were cultured in YA broth for 24 h and sub-cultured into BPU broth at 30°C for 72 h with continuous aeration at 200 rpm. 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. The precipitate was dried for 48 h at 50°C prior to being weighed.

Effect of pH and minimal inhibitory concentration (MIC). To determine the optimum pH, the pH of the YA broth was adjusted using 0.1 M HCl and 0.1 M NaOH to obtain aliquots with pHs varying from 3.0 to 9.0. The MIC test was done in order to determine the concentration of copper chloride, which prevents the growth of bacteria. Isolated strains cultured for 24 h were diluted till they reached an OD₆₀₀ of 1.0 in YA broth, and 100 μL (10% v/v) of the diluted culture was inoculated into each aliquot of YA broth with different concentrations of copper (0.5; 1.0; 1.5; 2.0; 2.5; 3.0; and 5.0 mM). After 24 h of incubation, each culture broth was spread over a sterile YA plate, incubated at 30°C for 24 h, and bacterial growth was assessed by colony counting.

Impermeability test. The cells grown overnight in YA broth were harvested (6,000 × g, 5 min), twice washed with 0.9% sodium chloride solution, and resuspended in 0.9% sodium chloride solution to a final OD₆₀₀ of 1.0. Sterile silica sand (200 g, 0.45–0.7 mm, Joomoonjin Sand Co. Ltd., Gangneung, Korea) was mixed with 10 mL urea (40 g/L) and calcium chloride dihydrate solution (25 g/L). Sand slurry (40 g) was packed into a 25 mL plastic column (Corning Co. Ltd., Corning, NY, USA). After drying for 48 h at 50°C, the columns were run once by gravity with 10 mL of cell suspension. The columns were stored for 48 h to allow for calcite crystal growth. Then, 2 mL of...
crystal violet (CV) was pipetted into a packed sand column. The degree of impermeability was determined by measuring the migration distance of CV (Kang et al., 2014).

**Quantification of extracellular polymeric substance.** Extracellular polymeric substances (EPS) produced by the isolate (*Viridibacillus arenosi* B-21 and *Sporosarcina soli* B-22) were purified according to the method described by Prado Acosta et al. (2005) with some modification. The culture medium was boiled for 10 min to remove the attached EPS and centrifuged at 10,000 × g for 30 min to remove the cells. Trichloroacetic acid (TCA) was added to achieve 10% (v/v) final concentration to precipitate the proteins, followed by centrifugation at 10,000 × g for 30 min. After centrifugation, the supernatant was filtered through a 0.22 μm filter membrane. Two volumes of cold ethanol (4°C) were mixed thoroughly with the supernatant and incubated in the refrigerator (4°C) overnight to precipitate the crude EPS. The crude EPS was centrifuged at 10,000 × g for 30 min and the precipitate was resuspended in 1/10th of original volume in sterile distilled water. The purified EPS samples were freeze-dried by lyophilization at –18°C for further analyses. The dry weights of the cells and EPS were expressed in g/L.

**SEM and XRD analyses.** XRD analysis was used to construct a crystal structure model for copper carbonate and calcium carbonate. The suspension was placed on a glass cover slip and dried in an oven at 50°C. The samples were then analyzed using a DMAX-2500 system (Rigaku, Tokyo, Japan). A modified scanning electron microscopy (SEM) method was used to observe the produced copper carbonate and calcium carbonate crystals. Previously harvested crystal samples were suspended in water, covered with a glass plate, and dried in an oven at 50°C. After the samples had completely dried, they were coated with platinum in an ion sputter. A field emission scanning electron microscope (S-4300; Hitachi, Tokyo, Japan) was used to visualize the morphological features of the crystals.

**Removal of copper.** After 12 h of growth, 5 μL of the culture medium was inoculated into 45 mL of YA broth in 100 mL conical flasks containing 0.22 μm filter sterilized, 5 mM urea, and 25 mM CaCl₂ supplemented with 2 mM copper. The flasks were incubated in a shaking incubator (200 rpm) at 30°C for 48 h. In all experiments, sets of control without any added bacterial cells were also included. The cultures were centrifuged at 8,000 × g for 15 min. The copper biosequestration by selected strains was calculated as the difference in percentage between the initial and final concentrations of copper in the supernatants. Concentrations of copper were determined by an OPTIMA-7300DV (Perkin Elmer Inc., Waltham, MA, USA) inductively coupled plasma-optical spectrometer (ICP-OES).

**Results and Discussion**

**Characterization of soil in abandoned mines**

The significant characteristics of the soil samples in the abandoned mine at Imgi are listed in Table 1. The soil analysis showed that it contained 0.62% total organic matter and 2.91 mg/kg available phosphorus. The soil profile consisted of sand, silt, and clay, 81.87%, 5.50%, and 12.63%, respectively. The pH of the water mixture (soil to water ratio of 1:1.5) was 3.18. The heavy metals in the soil were primarily originated from the contamination of the exhausted industrial mine (Haynes et al., 2009).

**Isolation and identification**

The isolates from the copper mining area effectively removed copper from the contaminated sites. About 25 morphologically different colonies were isolated from the spreads on BPU (containing cycloheximide at 100 μg/mL) plates. From these, four strains, designated B-21, B-22, B-24, and B-25, were selected based on their high urease activity and calcite production. The four isolates selected from the initial screening were identified based on their 16S rRNA gene sequences. Figure 1 showed that the 16S rRNA sequences of the isolates B-21 and B-25 were highly similar (99%) to that of *Viridibacillus arenosi*. Other isolates B-22 and B-24 were phylogenetically closely (99%) related to *Sporosarcina soli* and *Sporosarcina sp.*, respectively. The 16S rRNA gene sequences of identified strains were deposited in the GenBank of NCBI under accession numbers KJ671467 (B-21), KJ485701 (B-22), KJ485702 (B-24), and KJ671468 (B-25).

**Urease activity and calcite production**

Urease is a key enzyme that leads to calcite precipitation in microorganisms, and its activity is significant in media containing urea and sources of calcium (Muynck et al., 2010). All the isolates produced significant amounts of urease (Fig. 2). The strain B-25 showed the highest urease activity (6.85 U), followed by B-21 (6.35 U), B-22 (5.00 U), and B-24 (4.13 U). In addition, the highest calcite production was observed with B-24 (19.93 mg/mL), followed by B-22 (17.03 mg/mL), B-25 (15.83 mg/mL), and B-21 (12.50 mg/mL).

**Effect of pH and minimal inhibitory concentration (MIC)**

The control and optimization of the bioremediation proc-
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esses is complicated due to many factors. These factors include the existence of a microbial population capable of degrading the pollutants, the availability of contaminants to the microbial population, and environmental factors (type of soil, temperature, pH, etc.) (Vidali, 2001) Although microorganisms have been isolated in extreme conditions, most of them grow optimally over a narrow range of conditions; therefore, it is important to achieve optimal conditions for their successful growth. The strains B-21 and B-22 grew in a pH range from 3.0 to 9.0, whereas optimal growth in other isolated strains was in a pH range from 6.0 to 9.0. A pH range of 6.5–8.5 was selected to cover the pH range of 7–8 in which most environmental strains of denitrifying bacteria showed their optimum growth (Wang et al., 1995).

Of the four bacterial isolates, two strains (B-21, B-22) exhibited the highest MIC at a copper concentration of 5.0 mM (Fig. 3). Wei et al. (2009) reported that Agrobacterium tumefaciens CCNWR33-2 grew in 2.0 mM copper in TY liquid medium. This growth at different Cu concentrations is explained by different resistance levels of microorganisms to various pollutants, which they developed in order to survive when exposed to different toxic pollutants in different environments (Hideomi et al., 1977).

**Impermeability test**

In this study, the distance of CV migration decreased for strains B-21 and B-22 (Fig. 4). This decrease in retention time can be attributed to the calcium carbonate crystals that were deposited between sand particles and were acting as plugs. Of the four isolated strains, the strain designated as B-21 was selected for further analyses based on its MIC and the impermeability test results.

**Production of EPS**

It has been well documented that EPS contains many negatively charged functional groups, proteins, and polysaccharides. When microorganisms come close to heavy metals or other pollutants, the EPS outside the cell wall makes initial contact (Pulsawat et al., 2003). The results show that EPS production by B-22 increased in the presence of heavy metals. The production of EPS by B-22 in the presence of copper chloride is shown in Fig. 5. The growth of the strain B-22 decreased the concentration of copper chloride in the medium, because the toxic metal ions were removed from the solution. The removal of metal ions from the solution might be due to the bioremediation capability of the EPS produced by the bacterium. Among the various reactive components associated with bacterial cell walls, bacterial EPS is of particular importance be-
cause it affects biofilm formation and cell adhesion to solid substrates (Gebauer et al., 2010). EPS exhibits a greater binding ability to complex heavy metals, and mechanisms such as proton exchange, global electric field or micro-precipitation of metals may occur in metal adsorption by EPS (Rodriguez-Navarro et al., 2007).

**SEM and XRD analysis**

The SEM image showed the formation of copper carbonate and calcium carbonate crystals by *Sporosarcina soli* B-22 in media containing calcium chloride (Fig. 6A). The calcium chloride precipitates produced during bacterial ureolysis were roughly rhombohedral and spherical in shape, generally 5–10 μm in size, whereas the copper carbonate compounds in Fig. 6B are mostly spherical in shape. In addition, Li et al. (2013) calculated the average grain size of copper compound samples to be 5–10 μm.

The XRD analyses confirmed the presence of azurite, malachite, and calcium carbonate crystals (Fig. 7). Copper carbonate (copper hydroxy carbonate) is naturally produced as azurite, 2CuCO₃·Cu(OH)₂, or malachite, CuCO₃·Cu(OH), whereas calcium carbonate crystals are produced from calcium chloride. Previous study (Gorospe et al., 2013) used calcium chloride as a source of Ca²⁺ for precipitation of rhombohedral calcium carbonate. Calcium carbonate forms three anhydrous polymorphs: calcite, aragonite, and vaterite (Gebauer et al., 2010). Among them, calcite and vaterite are the most common bacterial calcium carbonate polymorphs (Rodriguez-Navarro et al., 2007), but other polymorphs such as monohydrocalcite and aragonite were also reported (Sánchez-Navas et al., 2009). Many studies investigated the species-specific precipitation of carbonate biominerals by various bacteria (Dhami et al., 2013).

**Removal of copper**

The pH of the media significantly increased with the increase in the growth of bacterial isolates (Fig. 8). Ini-
tially, the pH of the media with B-22 was 6.38, and in 72 h it increased to its maximum level of 9.64. Continuous increase in pH follows the reaction of carbonate precipitation with calcium and copper in the form of calcite and copper carbonate, respectively, catalyzed by urease. This change in pH can be explained by ureolysis that leads to an increase in pH and a shift in bicarbonate equilibrium. Urea hydrolyzing bacteria can promote calcium carbonate precipitation by hydrolyzing urea and producing ammonium and bicarbonate ions, thereby increasing the pH (Fujita et al., 2008). The generation of NH₄⁺ increases local pH, and the reaction continues spontaneously to form calcium carbonate in the presence of calcium ions and the availability of nucleation sites (Rodriguez-Navarro et al., 2003).

Our results showed that the isolate removed 61.8% of copper at 2 M concentration in 72 h in YA media. Several studies have indicated that more than 30–40% of heavy metals can be removed from a solution with copper concentration greater than 300 mg/L (Andreazza et al., 2011). These results indicate that biominalerization of heavy metals into calcite occurs as a competitive co-precipitation reaction in which suitable divalent cations are incorporated into the calcite lattice. Recently Kurmaç (2009) reported on the impact of varying concentrations of different metals such as Pb, Cd, Cr, Zn, Cu, and Ni in MICP technology and on the important role of metal toxicity in microbial substrate degradation.

Conclusion

Recently, microbially induced biominerals have been used extensively in various fields particularly for the removal of heavy metals. For an effective bioremediation, microorganisms must enzymatically attack the pollutants and convert them to harmless products. In this study, we demonstrated that heavy metal contaminants such as copper could be precipitated by bacterial urease. This can prove to be a good strategy for an effective, efficient, and economic method for Cu bioremediation, and the introduction of these indigenous bacteria could provide a potential bioremediation process in environments with high copper contamination.

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