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Repeated use of *Bacillus subtilis* cell walls for copper binding

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Purified cell walls from *Bacillus subtilis* were repeatedly suspended in 5 mM $CuCl_2$ and, after removing unbound Cu, were suspended in 1% (v/v) HNO_3 to release bound Cu. The walls were then regenerated by washing in H_2O. After five cycles, copper binding actually increased slightly, probably due to enhanced exposure of binding sites in the walls. Thus bacterial walls may be used repeatedly for metal removal during bioremediation of heavy metal pollution.

Key words: Bacillus subtilis, bioremediation, copper, Gram-positive walls.

The anionic nature of bacterial surfaces enables them to bind metal cations through electrostatic interactions (McLean & Beveridge 1990). Because of their thickness and anionic character, due mainly to peptidoglycan, teichoic and teichuronic acids, the cell walls of Gram-positive bacteria have a high capacity for metal binding (Beveridge 1989). This has led to several investigations into their use for bioremediation of heavy metal pollution (Mullen *et al.* 1989; Walker *et al.* 1989; Flemming *et al.* 1990). As copper is often a pollutant in several environments, several studies have been conducted to establish the feasibility of copper bioremediation (Mullen *et al.* 1989; Walker *et al.* 1989; Flemming *et al.* 1990). One aspect that has not been previously addressed is the potential for repeated use of bacterial walls as biosorbants. This would have significant economic benefits during the industrial scale-up of any bioremediation process. This point was addressed in the present study.

Materials and Methods

Bacterial Growth and Wall Purification

Bacillus subtilis strain 168 (Beveridge & Murray 1976) was the kind gift of T.J. Beveridge, Department of Microbiology, University of Guelph, Guelph, Ontario, Canada. It was grown and its cell walls purified as described previously (Beveridge & Murray 1976). Briefly, this involved growing the organisms in broth, harvesting by centrifugation, disrupting the cells with a French pressure cell, treating the cell wall fragments with enzymes to remove contaminating nucleic acids and proteins, washing with SDS to remove contaminating membrane fragments, washing in de-ionized ultrapure water (McLean *et al.* 1990a), and lyophilizing. The lyophilized walls were then stored at room temperature until use.

Materials

Polycarbonate tubes and bottles were used during metal binding. Prior to use, they were rinsed in NHO_3 as described by McLean *et al.* (1990a). Ultrapure, membrane-filtered water was used at all stages during metal binding (McLean *et al.* 1990a). $CuCl_2$ solution, 5mM, was prepared immediately before use with analytical grade material. The 1% (v/v) HNO_3 used to release bound Cu from *B. subtilis* walls was also prepared from analytical grade NHO_3. Background Cu concentrations in this acid were measured and subtracted during inductively coupled plasma/mass spectrometry (ICP-MS) sample analysis. Typically this contamination was less than 1% of the total Cu present. For ICP-MS calibration, four standard solutions of 0, 20, 100 and 200 μg Cu/l in 1% (v/v) high purity HNO_3 were prepared from 1000 mg/l stock solutions and ultrapure water.

Metal Binding Procedure

The metal binding protocol is outlined in Figure 1. For metal uptake experiments, *B. subtilis* walls were suspended in unbuffered 5 mM $CuCl_2$. Centrifugation was for 30 min at 18,000 × *g*. Walls were suspended in 1% (v/v) HNO_3 to release bound Cu. A total of 10 replicates was done for each experiment. The dry weight of bacterial walls was obtained before and after use to see if any appreciable disintegration and weight loss had occurred.

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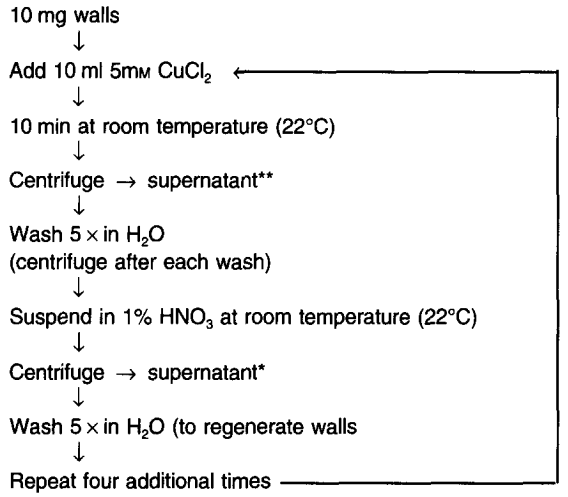

Figure 1. Protocol for Cu binding by *Bacillus subtilis* walls. Samples were taken for analysis for bound (*) and unbound (**) Cu by inductively coupled plasma-mass spectrometry.

ICP-MS Analysis

Cu concentrations were measured on the basis of $^{63}Cu^{+}$ concentrations using a modified Perkin Elmer Sciex ELAN 500 ICP-MS instrument. ICP-MS operating conditions were similar to those described previously (McLean *et al.* 1990a).

Results and Discussion

Results for bound and unbound Cu are shown in Figure 2A and 2B, respectively. Cu binding (Figure 2A) increased slightly through repeated use of the walls. At the same time, unbound Cu decreased (Figure 2B). The most probable explanation for this is that partial wall hydrolysis occurred during the metal binding or suspension in 1% (v/v) HNO_3. Significant hydrolysis did not occur since the average dry weight loss from 10 mg of *B. subtilis* walls during Cu binding was < 0.02 mg.

Cu binding turned the walls green, possibly due to one or more copper oxide or hydroxide minerals (McLean *et al.* 1990a). The concentration of HNO_3 (1%) used to liberate bound Cu was chosen since it represented the minimum concentration of acid that removed this discoloration. To avoid increased wall hydrolysis, higher concentrations of acid were not used. In preliminary experiments (R.J.C. McLean, unpublished work), we noted that metal binding equilibrium is achieved with *B. subtilis* walls after 1 to 2 min exposure at room temperature (22°C). The 10-min binding time was chosen to ensure equilibrium. The use of additional chemicals, such as buffers, was avoided since we have found them to influence bacterial metal binding and mineral formation (McLean *et al.* 1990b, 1991).

In *B. subtilis*, the major wall component responsible for metal binding is peptidoglycan (Beveridge & Murray 1980).

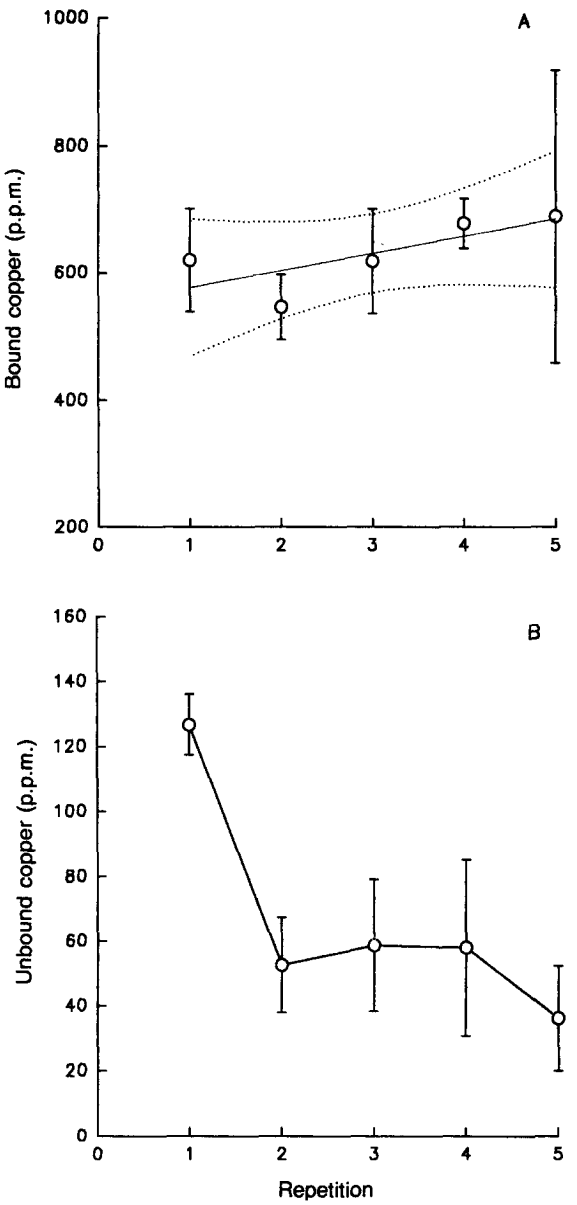

Figure 2. Copper bound (per mg wall) (A) and unbound copper remaining in solution (B) during repeated use of *Bacillus subtilis* walls for Cu binding. Graph and statistics were calculated using Sigma Plot (version 5.0) and Sigma Stat (version 1.01) software (Jandel Scientific, San Rafael, CA, USA). Error bars represent standard deviations. In (A): —— —linear regression of binding data; - - - -—95% confidence interval for Cu binding.

Teichoic and teichuronic acids contribute less to this function. Maintenance of *B. subtilis* wall metal binding therefore should correlate with maintenance of peptidoglycan. In contrast, *B. licheniformis* metal binding is predominately due to teichuronic acid (Beveridge *et al.* 1982). While the walls of both organisms have a high affinity for metals, the susceptibility of teichoic and teichuroic acids to hydrolysis by acidic and basic solutions would indicate that the *B. licheniformis* walls are a less suitable candidate for repeated use as a heavy metal biosorbant.

The high capacity of bacterial surface polymers for metal binding [reviewed in Beveridge (1989) and McLean & Beveridge (1990)] has led to research into their use in a number of areas, including bioremediation of heavy metal pollution and precious metal recovery during industrial and mining operations. We have shown these polymers to maintain their capacity for Cu binding upon repeated use. Re-use of biosorbants, such as *B. subtilis* cell walls, has the potential for lowering the costs associated with bioremediation of heavy metal pollution.

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