Metal Biosorption by Surface-Layer Proteins from Bacillus Species

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Received: September 30, 2010 / Revised: October 27, 2010 / Accepted: November 11, 2010

Bacillus species have been involved in metal association as biosorbents, but there is not a clear understanding of this chelating property. In order to evaluate this metal chelating capacity, cultures and spores from Gram-positive bacteria of species either able or unable to produce surface layer proteins (S-layers) were analyzed for their capacity of copper biosorption. Only those endowed of S-layers, like Bacillus sphaericus and B. thuringiensis, showed a significant biosorption capacity. This capacity (nearly 50%) was retained after heating of cultures, thus supporting that structural elements of the envelopes are responsible for such activity. Purified S-layers from two Bacillus sphaericus strains had the ability to biosorb copper. Copper biosorption parameters were determined for strain B. sphaericus 2362, and after analyses by means of the Langmuir model, the affinity and capacity were shown to be comparable to other bacterial biosorbents. A competitive effect of Ca$^{2+}$ and Zn$^{2+}$, but not of Cd$^{2+}$, was also observed, thus indicating that other cations may be biosorbed by this protein. Spores that have been shown to be proficient for copper biosorption were further analyzed for the presence of S-layer content. The retention of S-layers by these spores was clearly observed, and after extensive treatment to eliminate the S-layers, the biosorption capacity of these spores was significantly reduced. For the first time, a direct correlation between S-layer protein content and metal biosorption capacity is shown. This capacity is linked to the retention of S-layer proteins attached to Bacillus spores and cells.

Keywords: Metal biosorption, S-layer, Bacillus, spores

Metals are essential elements, but at high concentrations they become toxic to living organisms, including soil microorganisms. Bioremediation is an important tool for environmental remediation of heavy metals [1]. Biological methods to remove metals from liquid effluents present many potential advantages. The use of bacterial and fungal biomass as biosorbents should be of special interest to industries in undeveloped countries, where pollution generators cannot afford to install costly high-performance treatment facilities. These biosorbents are an alternative to conventional chemical methods [9].

The members of the family Bacillaceae present potential characteristics for metal biosorption owing to their multiple additional envelopes and composition of their spores (coat or exosporium). Their cell wall may be overlaid by a number of surface structures that can interact with metal ions, such as the exopolysaccharide (EPS) and the paracrystalline surface layer (S-layer). Regarding the EPS, its biosorption capacity has been established for Paenibacillus polymyxa P13 [22], whereas the S-layer is present in species of Archaea and Bacteria and constitutes the outermost structure of the cell. The latter is essentially composed of proteins arranged in a crystalline multilayer array formed by the self-assembly of monomer subunits on the surfaces of the cells. The S-layer covers the whole surface of the cell and could represent 15% of total proteins [25]. These proteins are amenable to introduce surface motifs as metal-binding domains and immunological motifs [20, 16]. Some S-layer proteins are glycosylated and/or phosphorylated. Amine, carboxyl, and hydroxyl groups are also aligned on the surface and maintained by hydrophobic, electrostatic, and Van der Waals forces. The S-layer is not only a protective coat, but is also involved in cell adhesion, surface recognition, and pathogenicity [7, 14, 23]. In fact, a Bacillus sphaericus strain (JG-A12) isolated from an uranium mining waste pile is able to accumulate high amounts of toxic metals such as U, Cu, Pb, Al, and Cd as well as precious metals; this capacity has been attributed to the highly ordered paracrystalline proteinaceous surface layer that envelopes the cell [21]. In addition, the genus Bacillus has the ability to form spores.

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that resist chemical and physical constraints and survive in harsh places. Most of the resistance properties of these spores have been in part attributable to the multiple-layer structure (the coat) surrounding the spore in a protective and flexible shield. Basic biochemical features of the coat have shown that it is composed largely of proteins with smaller amounts of carbohydrates and lipids. This multilayered structure is composed of up to 25 proteins, often highly cross-linked, which grants the spore resistance to UV light, treatments with solvents such as chloroform, or access to lysozyme. Coat proteins are especially rich in the amino acids tyrosine and cysteine [6], which have a great potential capacity for metal biosorption, owing to the presence of sulfide bonds. Moreover, the CotA protein displays similarities with multicopper oxidases [12] since it contains, in particular, four copper-binding sites. Selenska-Pobell et al. [26] reported that spores from several Bacillus isolates (B. megaterium, B. sphaericus, B. cereus) biosorb U, Cu, Pb, U, and Al. On the other hand, Chung et al. [11] reported that spores of the marine Bacillus sp. SG-1 are capable of oxidizing Mn(II) and Co(II) and exhibit a high affinity for Cu(II). These authors argued that in Bacillus sp. SG-1, the surface charge is primarily associated with the outermost layer of the spore, the exosporium, and that this would be involved in the metal association. Several reports have indicated that enzyme-binding metals are present in this structure [5, 8]. To date there is no clear understanding of how this metal interaction has been obtained. In our laboratory, we have studied the S-layer proteins of Bacillus species to try to solve the lack of information concerning metal–bacteria interaction.

In order to evaluate the metal chelating capacity, using copper as a model, we analyzed Gram-positive bacteria, spores, or cultures of species either able or unable to produce S-layers. To address this fact, different treatments of cells and spores were tested, including purification of S-layer proteins. We describe here the first report for a direct correlation between S-layer content and metal biosorption capacity.

**Materials and Methods**

**Microorganisms and Media**

All the Bacillus strains used belong to the collection of our laboratory. *B. subtilis*, *B. sphaericus*, and *B. thuringiensis* (listed in Table 1) were provided originally by the Pasteur Institute. The CP1 strain is a crystal negative ErmR mutant obtained by introducing the pTV1ts plasmid containing the transposon Tn917::ermR [24] in the *B. thuringiensis var. israelensis* 1884 strain. Spores were obtained after culturing the different Bacillus strains for 2–3 days at 32°C on solid Schaeffer or Yousten media. Plates were scrapped with 1 M NaCl, washed four times in bi-distilled water, and freeze-stored at −20°C [4].

*Lactobacillus acidophilus* ATCC4356 belongs to the Collection of our laboratory and was provided originally by the CERELA-CONICET Institute (Tucumán, Argentina). The *L. acidophilus* strain was cultured in MRS medium at 32°C.

**S-Layer Preparations**

For S-layer preparations, *Bacillus sphaericus* 2362 and Kellen Q strains were grown in LB medium in order to avoid sporulation. *Lactobacillus acidophilus* was cultured in MRS medium. Cells from 100-ml exponential cultures were harvested and washed once with PBS. The S-layer proteins were extracted by using 6 M LiCl, followed by centrifugation (15,000 ×g for 15 min). The pellets were extracted again with 10 ml of the same reagent and incubated for 30 min at room temperature. The supernatants were collected and dialyzed against distilled water overnight at 4°C, allowing the S-layer proteins to precipitate. After centrifugation (10,000 ×g for 20 min.), the pellets containing the S-layers were suspended in dionized water and stored at −20°C.

**Copper Absorption Experiments**

Analytical-grade CuSO$_4$·5H$_2$O (Sigma) was prepared in dionized water as 1,000 ppm stock solutions.

Freeze-dried spores were weighed, and aliquots of 0.1 to 1 mg were suspended in dionized water containing the metal solutions (Cu$^{2+}$ at 100 or 200 ppm) at optimized conditions of pH and temperature (pH 5 and 25°C) [22]. After biosorption reached equilibrium, spores

### Table 1. Copper absorption experiments with spores and cultures from different Bacillus strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Biosorption capacity of Spores (ppm of Cu$^{2+}$)</th>
<th>Biosorption capacity of Cultures (ppm of Cu$^{2+}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> YB886</td>
<td>8.9±0.1</td>
<td>9.8±4.8</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> 168</td>
<td>10.3±1.1</td>
<td>9.7±0.7</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em> 2362</td>
<td>12.5±0.8</td>
<td>36.3±1.7</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em> 1593</td>
<td>22.7±1.7</td>
<td>7.4±2.2</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em> var.</td>
<td>15.8±1.5</td>
<td>22.9±1.3</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em> var.</td>
<td>18.8±0.2</td>
<td>31.9±2.7</td>
</tr>
</tbody>
</table>

*Experiments were performed with either spores or whole cells as described in Materials and Methods. Assays were performed with 1 mg of spores and 100 ppm of copper for 2 h.*

*For cultures (whole cells), 200 ppm of copper was used and incubated with 1 mg of whole cells for 16 h. The ppm of Cu$^{2+}$ biosorbed was calculated and the results shown are the average of four independent experiments.*