GENETIC ENGINEERING OF S-LAYER OF CAULOBACTER CRESCENTUS FOR BIOREMEDIATION OF HEAVY METALS

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ABSTRACT

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The recombinant strain JS4022/p723–6H (Patel, et al., 2009), expressing RsaA-6His fusion protein was examined for its ability to sequester Cd(II) from the bacterial growth medium. When mixed with 1 ppm CdCl$_2$, JS4022/p723–6H removed 94.3~99.9% of the Cd(II), whereas the control strain removed only 11.4~37.0%, depending on experimental conditions. The effective contact time of the cells and Cd(II) was as short as 15 min. When higher concentrations of CdCl$_2$ were tested, JS4022/p723–6H consistently demonstrated enhanced binding capacity over the control strain. At 15 ppm of Cd(II), each gram of JS4022/p723–6H dry cells retrieved 16.0 mg of Cd(II), comparing to 11.6 mg g$^{-1}$ achieved by the control strain. The morphology of the biofilms formed by Caulobacter crescentus recombinant strain JS4022/p723-6H, which expresses hexahistidine peptides anchored to its surface layer protein RsaA, was examined. The density of the biofilm reached a maximum after 48 h of incubation and was not affected by exposure to cadmium. When treated with 0.4 ppm Cd(II), biofilms formed by the engineered strain removed 76.9% of the total metal, whereas a control strain only removed 13.5%. This work provides a potential cost-effective solution toward bioremediation of heavy metals from aqueous systems, and effective means of constructing large scale remedial bioreactors in a cost effective manner.
Dedicated to my family and friends.
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INTRODUCTION

Heavy Metal Pollution

Heavy metal pollution is a huge risk to humans and the environment, and thus its concentration in drinking water is strictly regulated. Contamination by heavy metals is of continuing medical and political concern. Heavy metals can be accumulated in the food chain and exposure to them, even at low levels, can be harmful and may eventually cause adverse health problems, such as nerve damage and cancer. The ability of wildlife to accumulate and concentrate toxic metals increases the threat of toxicity to those further up the food chain. Increased use of heavy metals in everyday life has greatly enhanced the risk for human and animal health (Mishra, 2009).

Heavy metal exposure may induce autoimmunity as well as immunotoxicity. Autoimmune diseases are those in which an individual’s own immune system attacks one or more tissues or organs resulting in functional impairment, inflammation and sometimes-permanent tissue damage. Research indicates links between heavy metals (particularly cadmium and chromium) and multiple sclerosis and other demyelinating syndromes (Ingalls, 1989). Studies also suggest that chronic exposure to low levels of heavy metals such as nickel, arsenic, and chromium increase cancer incidence among affected individuals (Virag, et al., 2009), and may result in abnormal child birth (Huo, et al., 2008).

Heavy metals do not degrade to harmless products, and so their presence is harmful. The presence of heavy metals can be detrimental as unlike organic pollutants heavy metals do not degrade to harmless products. Therefore, considering their toxicity, treatment of ground and wastewater containing heavy metals is extremely important for public health. Thus, there is demand for safe and effective ways to remove metal contaminants from the environment.
Traditional Remediation Technologies

Traditional technologies available to remove heavy metals from industrial wastewater are as follows: precipitation, adsorption, ion exchange, reverse osmosis, evaporation, electrolysis, and cementation (Lee, et al., 1997). The three most widely used techniques: precipitation, adsorption, and ion exchange are discussed below.

Precipitation is the preferred technique to remove toxic heavy metal wastes from electroplating waters. Most heavy metals associated with electroplating wastes form relatively stable metal ions; this property of heavy metals is used in the precipitation technique. Heavy metals are converted to metal sulfides or metal hydroxides by hydroxide, lime and/or sulfide treatments. Although 75% of electroplating facilities treating aqueous metal-bearing wastes use precipitation there are certain disadvantages of the process. Some heavy metals like chromium cannot form a stable metal ion so it is difficult to remove it using precipitation. Solubility constraints of the metal hydroxides for variable waste water matrices results in limitation of metal removal by precipitation (Grosse, 1986).

Adsorption is a separation process which concentrates metals at a contacting surface without requiring any chemical reactions. Adsorption systems are generally used to polish effluents from waste streams containing organic compounds. Activated carbon is used in adsorption systems to remove cadmium wastes and activated alumina bed is used to remove arsenic wastes. In general adsorption systems have limited applications in treating higher strength hazardous metal bearing wastes (Grosse, 1986).

Ion exchange is a separation technology that helps remove various species of ions by interchanging reversible ions between the aqueous wastes and the exchanger (e.g. resin).
Exchangers may be comprised of synthetic resins, insoluble salts, molecular sieves or liquid membranes. In liquid ion exchange organic polar molecules are used. The problem with ion exchange is the recovery of metals after removal and the cost of maintaining and establishing the system (Grosse, 1986).

These methods are either costly or ineffective to treat large solution volumes containing a low concentration of heavy metal contaminants, for example at sub-parts-per-million (sub-ppm) levels. Thus recent studies have given much importance to biological methods to eliminate the toxic heavy metals.

Biological Methods

Biological methods have received increased attention in recent years because of their potential for providing a cost-effective technology for heavy metal remediation, especially at dilute concentrations.

The use of plants to remove heavy metals has gained popularity in recent years. Scientists discovered that the fern *Pteris vittata* (brake fern) is extremely efficient in extracting arsenic from soils and translocating it into its above-ground biomass (Ma, et al., 2001). Brake fern is mesophytic and is widely cultivated and naturalized in many areas with a mild climate. In another study it was discovered that for biosorption of cadmium by pectin-rich fruit materials, citrus peels are most suitable and can remove absorb cadmium effectively (Schiewer, et al., 2008). Fungi like *Penicillium purpurogenum* and *Agaricus macrosporus* are also quite effective in removal of heavy metals. Both the fungi can remove heavy metals from aqueous solutions, but the process is pH dependent and takes about 5 hours to reach equilibrium in case of *Penicillium purpurogenum* (Melgar, et al., 2007; Say, et al., 2003).
The efficacy of using bacterial biomass for sequestering heavy metals is well documented. The selective adsorption of Pb, Cu, and Cd with fixed bed columns containing immobilized bacterial biomass was 25%-30% more efficient than columns packed with a chemical matrix (Chang, et al., 1998). In another example, it was discovered that *Pseudomonas aeruginosa* can accumulate nickel as its phosphide and carbide crystal in the cell envelope region (Sar, et al., 2001). It has been discovered that a filamentous fungus *Rhizopus arrhizus* can be used for biosorption of Pb ions from storage battery industry waste waters, and it can remove 2.643 mg Pb(II)/g microorganism (Bahadir, et al., 2007). Animal biomass is also used to remove heavy metals. For example, crab shell particles can be used in biosorption of heavy metals from aqueous solutions (Lee, et al., 1997).

All the above discussed approaches for removal of heavy metals do not have any kind of mechanism for specific heavy metal adsorption. The effectiveness of these methods might be compromised if the aqueous solution contains chemicals that interfere with the biomass. This lack of specificity led to the quest of seeking to design ways to eliminate heavy metals from aqueous systems with more specificity.

**Heavy metal-binding peptides**

Researchers have over-expressed metal-binding peptides, also known as affinity peptides to achieve specific adsorption to heavy metals. Metal-binding peptides are peptides which can bind specifically to heavy metals, and thus help in their removal. Some organisms have these peptides in their cell walls and/or cells and it is also possible to genetically engineer some organisms to express these peptides. Affinity peptides such as hexahistidines, glutathione S-transferase, metallothionein (Kang, et al., 2007; Kille, et al., 1991), maltose-binding proteins and synthetic phytochelatins have been expressed in microbial cells to aid in removal of heavy
metals. The main drawbacks of this strategy are limited uptake of the metals through the cell membrane and the fact that high concentrations of metals can be toxic to the cell itself.

As a solution to these issues, scientists tried the approach of expressing affinity peptides like polyhistidines (Patel, et al., 2009; Xu & Lee, 1999), and synthetic phytochelatin (Bae, et al., 2000; Bae, et al., 2001) on bacterial cell surfaces through genetic manipulations. Microorganisms constructed by this approach are then used as whole cell adsorbents to remove heavy metals from aqueous systems. The ratio of surface area to volume of most bacterial cells is greater as compared to plant and animal tissues. This suggests that use of such strategy may enhance the heavy metal adsorption capacity. The metal-binding peptides are expressed on the surface of the cell which minimizes the uptake of heavy metals by the cells and thus lowers the risk of cell toxicity.

The metal-binding capacity of cells can be improved by genetic methods but the retrieval of cells after treatment is challenging and is a technical barrier in employing these cells for practical use. One solution to this problem is immobilizing the cells on columns packed with chemical matrices (Chang, et al., 1998; Katircioğlu, et al., 2008). The cost of packing materials and nonspecific binding to column materials are disadvantageous. Thus for bioremediation of heavy metals using microbial cells, the simple and reliable immobilization of cells is very important. The bacterium *Caulobacter crescentus* may provide a unique solution to this problem.

*Caulobacter crescentus*

*Caulobacter* spp. is harmless to human beings and environment, and is ubiquitously distributed. *Caulobacter* spp. is found in freshwater (Poindexter, 1964), seawater (Anast, et al., 1988), ground water (Mannisto, et al., 1999), wastewater (MacRae, et al., 1991), drinking water (Dott, et al., 1985; Gonzalez, et al., 1987; Jungfer, et al., 2007), soil (Smith, et al., 2005), deep-
sea sediment (Marchesi, et al., 2001), a gold mine (Inagaki, et al., 2003), and even ancient ice dated back thousands of years (D'Elia, et al., 2008). Cells of *Caulobacter* are able to survive in nutrient-poor habitats. They have evolved complex metabolic pathways to utilize aromatic compounds (Nierman, et al., 2001) in order to survive under limiting nutrient conditions. *Caulobacter* spp. have genes to protect themselves from heavy metals (Hu, et al., 2005), especially cadmium (Braz, et al., 2005) making them ideal for use in cadmium bioremediation.

Like many other prokaryotic organisms *Caulobacter* cells possess surface layers (S-layers). Microbial S-layers provide the first line of defense against phages, lytic enzymes, and host immune systems (Fernandez, et al., 2000). Electron microscopy studies of S-layer of *Caulobacter* prove that the cells have a uniform S-layer attached to the surface of the outer membrane. S-layer in *Caulobacter* is composed of almost 40,000 identical subunits of protein RsaA assembled into lattice array with hexagonal symmetry. S-layer proteins are present covering the entire cell surface during all stages of cell growth and division (Smit, et al., 1992). Location and high copy number of S-layer proteins makes them ideal carriers to display foreign peptides on the cell surface.

The S-layer secretion apparatus has been exploited in order to express heterologous proteins with the yields of resulting protein products secreted from *C. crescentus* ranging up to 250 mg/liter (Nomellini, et al., 2007). The S-layer of *Caulobacter crescentus* has been used to display foreign peptides. It can effectively display foreign peptides at specific sites without any damage to the protein and normal cell function (Bingle, et al., 1997). This presentation system suggests many potential applications, such as the development of whole-cell vaccines, tumor suppressors, cellular adsorbents, and peptide display libraries and the screening of antibody libraries (Nomellini, et al., 2007). Small fragments of antigens of pathogenic bacteria and viruses
were fused with RsaA and were able to boost immunological responses in animal hosts (Bingle, et al., 1997; Umelo-Njaka, et al., 2001). *Caulobacter crescentus* S-layer protein (RsaA) secretion apparatus is promising for the generation of particulate enzymes, for example, β-1,4-glucanase (Duncan, et al., 2005). All of these considerations have implications for the development of biotechnological applications based on the expression of foreign proteins using the *Caulobacter* S-layer protein.

*Caulobacter* has a dimorphic life cycle. Each new cell possesses a single polar flagellum and is called “swarmer cell” (Fig. 1a). Swarmer cells are motile and non-reproductive. After the obligatory swarmer phase, the cell differentiates into a stalked cell by initiating DNA replication, releasing the flagellum, and synthesizing a stalk, a thin cylindrical extension of the cell membrane (Poindexter, 1964). A mature *Caulobacter* cell possessing the stalk is called “stalked cell”, and is reproductive. The stalk has an adhesive holdfast, a polysaccharide adhesion, at the distant end. A mature *Caulobacter* cell attaches itself to a surface with the help of the holdfast so that water currents will not dislodge the cell from its substrate. The force with which a single cell can attach itself to a borosilicate surface has been measured and it is in micronewton range. It is the strongest ever measured for any biological adhesive, and is stronger than some commercial superglues (Bodenmiller, et al., 2004; Li, et al., 2005; Tsang, et al., 2006). Using this distinct adhesion, *Caulobacter* cells are able to overlay a solid surface with monolayer biofilms (Smit, et al., 2000). The high density biofilms resist starvation, recover rapidly from stress, and self-seal from mechanical abrasion (Smit, et al., 2000).

These features of *Caulobacter crescentus*, the ability to express foreign peptides on S-layer and self immobilization can be used to design an ideal bioremediation system reactor to clean up contaminants such as heavy metals (Fig. 1).
Genetic engineering of *Caulobacter crescentus*

A S-layer negative strain of *Caulobacter crescentus* (JS4022) was genetically engineered by inserting 6His polypeptide to a permissive site of the S-layer protein RsaA in the plasmid p4ArsA(723Δ)GSCCΔ (chl^r^). The recombinant strains were named JS4022/p4ArsA(723Δ)GSCCΔ (contains s-layer but no polyhistidine insert) and JS4022/ p723-6H. The sequence of the rsaA-6His fusion gene was verified by DNA sequencing. The expression of protein was checked by extracting the S-layer proteins from recombinant and performing a western blot to detect 6His (This work was done by Dr. Qiong Zhang). It is known that polyhistidine peptides can remove heavy metals effectively (Xu & Lee, 1999).

**Purpose of the study and research questions**

The present study aims to check the ability of the recombinant *Caulobacter* strain to remove Cadmium from aqueous solution, and to investigate the biofilms formed by the recombinant strain on a borosilicate surface and evaluate their potential for removing heavy metals from water samples.

The two main aims of this study were:

1) To determine optimum conditions for heavy metal removal using free cells.

2) To determine optimum conditions for biofilm formation and heavy metal removal using immobilized cells.
Figure 1. Schematic drawings of (a) life cycle of a *C. crescentus* cell and (b) bioremediation of heavy metals using *Caulobacter* cells self-immobilized on a solid surface through their holdfasts. Not to scale. (Drawing by Dr. Zhaohui Xu)
Figure 2. Scheme showing sequestration of heavy metals using surface expressed foreign peptides (not to scale). (a), wild type *Caulobacter* strains adsorb low levels of heavy metal ions through non-specific ionic attractions. (b), engineered *Caulobacter* strains retrieve heavy metals by both non-specific binding and specific affinity between hexa-histidine peptides and heavy metal ions. (Drawing by Dr. Zhaohui Xu)
MATERIALS AND METHODS

Bacterial strains and their cultivation

Recombinant *Caulobacter crescentus* strain JS4022/p723-6H expressing RsaA-6His fusion proteins was tested for Cd(II) removal (Patel, et al., 2009). Strain JS4022/p4ArsaA(723Δ)GSCCΔ expressing RsaA with a cloning linker at its 723 amino acid position (Nomellini, et al., 2007) was used as a control for cadmium binding assays. *C. crescentus* strains were grown at 30 °C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% CaCl$_2$·2H$_2$O, and 0.02% MgSO$_4$·7H$_2$O) supplemented with 2 μg mL$^{-1}$ of chloramphenicol. For plates, 1.5% (w/v) agar was added. Cell growth in liquid was monitored by measuring the optical density of cell cultures at 600 nm (OD$_{600\text{nm}}$).

Physiological characteristic studies

The integrity of cell envelopes of the recombinant strains was tested by exposure of the cells to 0.1% of sodium dodecyl sulfate (SDS) and 2 mM of ethylene diaminetetraacetic acid (EDTA) as previously described (Xu, et al., 1999). The effect of SDS and EDTA on bacterial cultures was measured by monitoring the optical density of cell cultures at 600 nm (OD$_{600\text{nm}}$) every 25 min for 100 min.

Removal of cadmium from solutions

Stationary phase *Caulobacter* cell cultures grown in PYE were diluted with the same medium to cell densities ranging from 0.1 OD$_{600\text{nm}}$ to 1.0 OD$_{600\text{nm}}$. Various amounts of 1000 ppm CdCl$_2$ were added to the cell cultures. The mixtures were incubated at 30°C at 250 rpm for 15 -120 min followed by centrifugation at 3300 g at 4°C. The cadmium content in the supernatant was measured with an iCAP 6000 Induced Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Thermo Electron Corporation) or A Analyst 100 Atomic Absorption
Spectrometer (AAS) (Perkin Elmer Corporation) at a wavelength of 228.8 nm and was compared with the input concentration of cadmium.

Determination of cell dry weight

Cell dry weight (CDW) was determined by filtering cell cultures through 0.22 μm GV Durapore membrane filter cups (Fisher Scientific) and drying overnight at 77°C. The dry weight of cells was measured the next day. One liter of cell culture at 1.0 OD$_{600nm}$ provided 300 mg of CDW.

Cultivation of biofilms

The procedure for cultivation of biofilms was adapted from Smit et al. (Smit, et al., 2000). Briefly, circular glass cover slips with a diameter of 12 mm (Ted Pella Inc., Bedding, CA,) were cleaned and sterilized by flaming with ethanol and placed in the wells of a 24-well, sterile, NUNCLON™ tissue culture plate containing 1.5 ml of fresh PYE medium. Twenty microliters of overnight culture of C. crescentus was added to each well and incubated at 30°C with gentle shaking at 100 rpm. The medium was replaced every 24 h. Individual coverslips were removed periodically for morphological analyses or Cd(II) removal assays.

Biofilm imaging

For phase contrast imaging, one side of the coverslips coated with C. crescentus biofilms was cleaned with a cotton ball and ethanol and air dried. The coverslips were then mounted to a glass slide with the polished side facing up and examined with a Carl Zeiss Axiophot light microscope fitted with a 100× oil-immersion objective. Images were captured with a RS Princeton Instruments digital camera (Trenton, NJ) using Metamorph software (Universal Imaging Co., Westchester, PA).
For ultrastructural studies, the biofilms were fixed with 2% glutaraldehyde for 30 min, washed twice with 0.2 M sodium phosphate buffer (pH 7.2) for 20 min, dehydrated through a graded ethanol series to 100% ethanol, and dried by hexamethyl di-silizane treatment for 30 min. After being coated with gold palladium, biofilms were examined under a Hitachi S-2700 scanning electron microscope (SEM).

**Determination of biofilm cell density**

To determine cell density of biofilms five areas in size of $100\mu m^2$ were selected from each SEM image using Image J (http://rsbweb.nih.gov/ij/), and the cells presented in each area were counted manually.

**Cd(II) removal assay using immobilized cells**

After the biofilms were cultivated for up to 7 days, liquid culture medium was withdrawn from each well and discarded. The biofilms were washed in the same wells 3 times with 1.5 ml Millipore purified water, each time for 2 min. The same volume of PYE medium, spiked with CdCl$_2$ ranging from 0.4 to 1.0 ppm, was added to each well. After 30 min of incubation at 30°C with gentle shaking at 100 rpm, the remaining cadmium content in the liquid was measured using an Analyst 100 Atomic Absorption Spectrometer (AAS; Perkin Elmer Corporation) at a wavelength of 228.8 nm and was compared with the input concentration of cadmium.

**Statistical analysis**

Data were subject to one-way analysis of variance (ANOVA) followed by a Tukey Honestly Significance Difference (HSD) test using VassarStats: Web Site for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html). Comparison of Cd removal by JS4022/p723-6H and its corresponding control strain was made using a one-tailed $t$-test for independent samples (Statistical Analysis was performed by Dr. Robert Michael L. McKay).
RESULTS

Physiological studies of the recombinant strain JS4022/p723-6H

Recombinant and non-recombinant strains were compared to determine if genetic manipulation affected the growth rate of recombinant strain. The growth rate of JS4022/p723-6H was comparable to that of the control strains JS4022 and JS4022/p4ArsA(723Δ)GSCCΔ proving that expression of polyhistidine in S-layer did not impair the ability of strain to grow (Fig. 3a). The sensitivities of JS4022/p723-6H to detergents and chelators were monitored, to appraise its suitability to serve as a whole-cell adsorbent. Exposure to 0.1% (w/v) SDS resulted in widespread cell lysis with a 65-75% decrease in culture turbidity (one-way ANOVA; P < 0.0005) within 25 min of exposure (Fig. 3b). Initially each strain was affected to a similar degree by SDS exposure; however, following 100 min, control strain JS4022 proved most sensitive with >90% decrease in culture turbidity (one-way ANOVA; P < 0.001), likely due to the lack of a S-layer, which provides reasonable protection to the integrity of cell envelopes. Strains JS4022/p723-6H and JS4022/p4ArsA(723Δ)GSCCΔ showed similar, although limited, resistance to SDS treatment (Tukey HSD Test).

Exposure to 2 mM EDTA also resulted in cell lysis although the effect was less severe than that of SDS with a 20-35% decrease in culture turbidity (one-way ANOVA; P < 0.05) within 25 min of exposure (Fig. 3c). Similar to SDS exposure, there was no difference in the degree to which each strain was affected by EDTA through 75 min incubation. Following 100 min exposure, control strain JS4022/p4ArsA(723Δ)GSCCΔ proved most resistant to the chelator showing only 53% decrease in culture turbidity compared to 75-80% declines for JS4022 and JS4022/p723-6H (one-way ANOVA; P < 0.01).
Figure 3. Physiological characteristics of JS4022/p723-6H. The growth rates of recombinant and control strains are compared in (a), as indicated by the optical density of cell cultures at 600 nm. The sensitivities of the strains to 0.1% (w/v) SDS and 2 mM EDTA are summarized in (b) and
(c), respectively. Percentages of absorbance at 600 nm prior to addition of the test agent are presented. Data in (b) and (c) represent results from three independent experiments.

Optimization of contact time for removal of cadmium

Recombinant strain JS4022/p723-6H was tested for its metal-binding efficiency in solution by using free cells. Within 15 min, JS4022/p723-6H removed 94.3% of cadmium from the aqueous phase, whereas, the control strain JS4022/p4ArsA(723∆)GSCC∆ removed only 13.2% (Fig. 4). Extending the incubation time to 120 min increased the cadmium removal only marginally to 97.7% and 18.7% by JS4022/p723-6H and JS4022/p4ArsA(723∆)GSCC∆, respectively. These results suggest that JS4022/p723-6H can sequester heavy metals in just 15 min. The contact time of 30 min was used for further studies for convenience of handling multiple samples.

Effect of the amount of biomass on sequestration of cadmium

At cell density of 0.03 g L\(^{-1}\) (CDW), non-specific binding of Cd(II) was dominant, and JS4022/p723-6H removed just 3% more cadmium than the control strain (Fig. 5). With increase in cell density the specific binding of metal ions by RsaA-6His also increased, and as the cell density was increased from 0.03 g L\(^{-1}\) to 0.21 g L\(^{-1}\), the Cd(II) removed by JS4022/p723-6H improved step-wise from 15.2% to 99.9%. In contrast, the non-specific binding of cadmium by the control strain varied at lower levels, ranging from 11.4% to 18.4%. Further increases in cell density to 0.30 g L\(^{-1}\) had little effect on binding of Cd(II) by JS4022/p723-6H but increased the non-specific binding of JS4022/p4ArsA(723∆)GSCC∆ to 37%.
Figure 4. The percentages of cadmium removed by recombinant strains as a function of time. JS4022/p723-6H (indicated as RsaA-6His, square) and JS4022/p4ArsaA(723Δ)GSCCΔ (indicated as RsaA(723), diamond). Total input of Cd(II) was 1 ppm.
Figure 5. The percentages of cadmium removed by recombinant strains at various cell densities.

Solid bars, JS4022/p723-6H, shown as RsaA-6His. Crossed bars, JS4022/p4ArsA(723Δ)GSCCA, shown as RsaA(723). Total input of Cd(II) was 1 ppm.
Removal of cadmium at different initial concentrations

The capacity of JS4022/p723-6H to remove metal was tested at different concentrations of cadmium: 1, 5, 10, and 15 ppm. Cell density of 0.30 g L\(^{-1}\) (CDW) was used for this test because results (Fig. 5) suggests that more biomass would be needed to adsorb higher levels of Cd(II). At each concentration tested, strain JS4022/p723-6H was more efficient at removing cadmium compared to control strain JS4022/p4ArsA(723\(\Delta\))GSCC\(\Delta\) (one-tailed \(t\)-test) (Fig. 6). The largest difference in Cd removal efficiency was observed at 1 ppm Cd where JS4022/p723-6H removed 2.7 mg of Cd(II) per gram CDW, which was 154\% higher than the 1.1 mg g\(^{-1}\) removed by control strain JS4022/p4ArsA(723\(\Delta\))GSCC\(\Delta\) (one-tailed \(t\)-test; \(P < 0.0001\)). As total Cd concentration increased, the difference in Cd removal efficiency between strains became less distinct. At 15 ppm Cd, strain JS4022/p723-6H removed 16.0 mg g\(^{-1}\) (or 37\% of the added) of the metal compared to 11.6 mg g\(^{-1}\) (or 27\% of the added) for the control strain (one-tailed \(t\)-test; \(P < 0.05\)). Adsorption with more concentrated Cd(II) was not tested. Removal efficiency is likely to improve with an increase of biomass.
Figure 6. Total amount of cadmium removed by recombinant strains at various Cd(II) concentrations. Solid bars, JS4022/p723-6H, shown as RsaA-6His. Crossed bars, JS4022/p4ArsA(723Δ)GSCDΔ, shown as RsaA(723). Cell density of 0.30 g L$^{-1}$ (dry weight) was used for both strains. Results of five independent tests.
Morphology of biofilms

Cell cultures of JS4022/ p723-6H or JS4022/ p4ArzaA(723∆)GSCC∆ were cultivated on sterile coverslips for up to 7 days in PYE medium and were examined for formation of biofilms by light and electron microscopy. *Caulobacter* cells appear to form monolayer biofilms coating the surface of the cover slips (Fig. 7) with no distinguishable differences observed between the two strains, which is not astonishing since the two strains possess the same type of holdfast and stalk. Monolayer biofilms had already formed after 24 h of cultivation, and this basic morphology remained the same for at least 7 days. The SEM photos reveal that the cells constituting the biofilms are either stalked cells or predivisional cells (Figs. 8 & 9); no swarmer cells were identified. The attachment of the cells to the glass cover slips is presumably through their holdfast, because when the biofilm was scratched by the tips of forceps, bacterial cells were extricated, but their holdfasts remained; cross bands that are characteristic to *Caulobacter* stalks were clearly visible (Fig. 8b). The edge of coverslip was broken to form a rough surface, and check the ability of the *Caulobacter* cells to form a biofilm on rough surface. The ability to form biofilm was not affected by change in texture of the surface (Fig. 9).

Biofilms incubated with 1 ppm Cd for 30 min showed no perceivable morphological damage (Fig. 9). Cell counting suggests that there was no change in the cell density of biofilm after Cd treatment (Fig. 10; two-tailed *t*-test, *P*>0.05, df = 5). However, the cell density changed with the age of biofilm. The cell density was highest for 2-day biofilms (61 cells 100 μm⁻²) and it declined for 5- and 7-day biofilms (45 and 40 cells 100 μm⁻², respectively; Fig. 10; *P*<0.001, one-way analysis of variance followed by Tukey honestly significance difference test). It was also noticed that the cell density fluctuated markedly among different samples of the same age; meanwhile, by day 5, the number of abnormal filamentous cells started to go up (Fig. 11).
Therefore, further investigations were conducted with biofilms cultivated for two days. Based on the size of the coverslips and density of biofilms formed by JS4022/p723-6H, each coverslip can host up to $1.38 \times 10^8$ cells with both sides.

Figure 7. Phase contrast light micrograph (a) and scanning electronic micrograph (b) of the biofilm formed by *C. crescentus* JS4022/p723-6H at day 5. Scale bars, 20 µm. (The scanning electron micrograph was taken by Gregory Wilson)
Figure 8. SEM micrographs of a biofilm formed by JS4022/p723-6H at day 2 with magnification of $2500 \times$ (a) and $130000 \times$ (b). Notice the mechanical abrasion to the biofilm caused by forceps in “a” and the scattered holdfasts in “b”. The boxed area in a represents the field of view shown
in “b”. Arrows in “b” point to division marks that are characteristic of *Caulobacter* stalks.

(Photos by Gregory Wilson)
Figure 9. SEM micrographs of a 2-day biofilm formed by JS4022/p723-6H after exposure to 1 ppm CdCl₂ for 30 min. a, overhead view of the cover slip; b, view showing cells adhered on the broken, rough surface of the coverslip; c, enlarged view of the cells on the broken, rough surface.
Figure 10. Cell density of the biofilm formed by JS4022/p723-6H cells at different age. Data of 2-3 independent experiments.
Figure 11. SEM micrograph of a biofilm formed by JS4022/p723-6H at day 5 with magnification 3000 X; Arrows show abnormally elongated cells.
Cd(II) removal by immobilized cells at different initial concentrations

Biofilms formed by JS4022/ p723-6H or JS4022/ p4ArsA(723Δ)GSCCΔ were exposed to different concentrations of Cd(II) to determine their metal removal abilities. At each concentration tested, strain JS4022/p723-6H removed cadmium more effectively as compared to the control strain (Fig. 12; one-tailed t-test, P<0.001). At the concentration of 0.4 ppm Cd, strain JS4022/p723-6H removed 76.9% of the Cd(II), whereas the control strain only achieved 13.5% removal (Fig. 12a). The removal efficiency of JS4022/p723-6H decreased with the increase in Cd(II) concentration (P<0.001, one-way analysis of variance followed by Tukey honestly significant difference test), whereas that of the control strain remained constant (Fig. 12a). If the absolute amount of metal removed by the Caulobacter biofilms is taken into account, it was evident that the removal capacity of strain JS4022/p723-6H had become saturated at the 0.4 ppm Cd(II) level with a total binding capacity of ~ 0.5 µg (Fig. 12b). In contrast, the nonspecific binding by the control strain was heightened with increasing Cd(II) and reached ~ 0.2 µg when exposed to 1 ppm Cd(II). Attempts to conduct the assays at metal levels lower than 0.4 ppm were not successful due to the detection limit of the analytical equipment.
Figure 12. The removal of cadmium by 2-day-old biofilms of JS4022/p723-6H and JS4022/p4ArsaA(723Δ) GSCCΔ depicted as percentages (a) and absolute mass amount (b) of removal. RsaA-6His represents strain JS4022/p723-6H, and RsaA(723) refers to the control strain JS4022/p4ArsaA(723Δ) GSCCΔ. Results of three independent tests; duplicate samples for each test.
DISCUSSION

This study shows that expression of RsaA-6His fusion proteins sustains normal growth of host cells, and provides some protection to the host from the destructive effects of detergents and chelators (Fig. 3). The lipidic structure of the cell outer membrane is disrupted by detergents and chelators weakening the interactions between lipopolysaccharide (LPS) molecules found in outer membranes. LPS molecules interact with each other by crosslinking of divalent cations, particularly calcium ions, for a stable leaflet of outer membrane (Nikaido, 2003). Studies also suggest that RsaA attaches to a smooth LPS possibly via calcium bridging (Smit, et al., 1992; Walker, et al., 1994). Structure of the lipidic membrane, including the LPS molecules should not be altered by insertion of hexa-histidine peptide into RsaA. Thus, JS4022/p723-6H strain and JS4022/p4ArsA(723Δ)GSCCΔ shows similar resistance to SDS. JS4022/p723-6H is slightly more sensitive to EDTA and it could be due to the competition for calcium ions between LPS, RsaA and 6His, as 6His binds to divalent cations. This competition should not interfere with the binding of Cd(II) at later remediation stage since 6His has a higher affinity to heavy metal ions.

The strain JS4022/p723-6H was useful as a whole cell adsorbent to sequester Cd(II) from aqueous solutions. It demonstrated noticeable affinity to the heavy metal, especially at sub-ppm levels, where 94.3 ~ 99.9% of the Cd(II) could be removed from the growth medium depending on experimental conditions. Control strain JS4022/p4ArsA(723Δ)GSCCΔ, which expresses RsaA(723), was able to remove only 11.4% ~ 37.0% of the added Cd(II). Time required to sequester heavy metal can be as short as 15 min. When concentration was increased to 15 ppm Cd(II), the total Cd(II) bound to JS4022/p723-6H was still consistently higher compared to JS4022/p4ArsA(723Δ)GSCCΔ. The solution used to conduct binding assays (PYE) contains
700 µM Ca(II) and 800 µM Mg(II). Meanwhile, the Cd(II) concentrations ranged from 9 to 134 µM (1–15 ppm) in this study. This suggests that JS4022/p723-6H has specificity towards Cd(II) as it sequestered Cd(II) even in the presence of excessive amount of Ca(II) and Mg(II) ions.

The uptake of Cd(II) was rapid and showed high efficiency. Ninety percent (90%) removal efficiency of cadmium was reported with dead cells of the cyanobacterium *Microcystis aeruginosa* immobilized in a column containing alginate beads and 11 ppm Cd(II) (Chen et al. 2005). Lactic acid bacteria were also competent to remove up to 99% of the added cadmium with initial concentrations of 0.1 and 1 ppm Cd(II) (Halttunen, et al., 2007).

JS4022/p723-6H was able to remove 16 mg g⁻¹ CDW of Cd(II), which is also comparable to the findings from other studies. Engineered *E. coli* strains, when tested with 2–25 ppm Cd(II), reportedly showed up to 3.6 mg g⁻¹ (equals to 32 µmole g⁻¹) removal (Kang, et al., 2007; Kotrba, et al., 1999; Xu & Lee, 1999). Bacterial cells are negatively charged and thus might account for some nonspecific binding of divalent metal ions. In this study, when the biomass to metal ratio became low significant non-specific binding of Cd was observed. The reason probably lies in the two-dimensional lattice assembly of S-layers. Structural analysis suggested that the S-layer of *Caulobacter crescentus* is an array of ring structures, each composed of six RsaA molecules, creating an open pore 2.5 to 3.5 nm in diameter; this uniform porosity allows the S-layer to exclude molecules larger than 17 kDa (Smit, et al., 1992). Smaller molecules, such as mineral ions, simple carbohydrates, and short DNA and peptide chains, however, are free to pass the open-mesh frame of the S-layer and exchange in and out of the cells. Cd(II) is one of the small molecules that can easily cross the S-layer. At high biomass to metal ratio the high affinity between 6His and Cd(II) allows most of the metal ions to be specifically associated with the engineered S-layer of the strain JS4022/p723-6H, which is composed of RsaA-6His subunits. If
the biomass to metal ratio drops, RsaA-6His molecules become limited and can become saturated with Cd(II). Increased concentration of heavy metal in the solution also reduces the minerals to metal ratio. The excessive metal ions then pass through the S-layer and associate with the vast open space of the negatively charged outer membrane beneath, resulting in rapid growth of non-specific binding.

This study confirms that JS4022 recombinant strains form monolayer biofilms on borosilicate surfaces which is consistent with an earlier study using *Caulobacter CB2A/Rif* (Smit, et al., 2000). Both JS4022 and CB2A/Rif were derived from strain CB2A. The uniform monolayer biofilms formed by CB2A strains differ significantly from diphasic biofilms containing densely packed, mushroom-shaped structures scattered on the lawn of monolayer biofilms formed by strain CB15 (Entcheva-Dimitrov, et al., 2004). For CB15, many areas of the coverslip stay devoid of the monolayer even after 4 days of incubation, and mushroom shaped structures start showing up at around 72 hours (Entcheva-Dimitrov, et al., 2004). CB2A derivatives form a uniform monolayer on the surface of the coverslip after 24 h, although the absolute number of attached cells was low (Smit, et al., 2000). Cell density of CB2A biofilms reached saturation at day 2 and decreased thereafter. Nonetheless, mushroom-shaped structures were not observed. Along with the genotypic differences between CB15 and CB2A, different biofilm cultivation conditions may also have contributed to the different morphologies exhibited by the two strains. In both cases glass coverslips were used to support the formation of biofilms. The CB2A strains were kept in the wells of tissue culture plates with the PYE medium, whereas the CB15 strain was incubated in a flow chamber with a defined medium containing 2 mM xylose (Entcheva-Dimitrov, et al., 2004).
Recent studies correlate optimal attachment of *Caulobacter* cells to a solid surface with the synthesis of the holdfast, as swarmer cells differentiate to stalked cells (Bodenmiller, et al., 2004; Levi, et al., 2006). The attachment becomes irreversible once established due to the unusually strong adhesive force of the holdfast, which is consistent with results presented here. On application of physical abrasion the bacterial body gets separated from the stalk but the stalk remains attached to the borosilicate surface. Attachment was unaffected upon exposure to Cd(II) at 1 ppm, sodium azide at 0.05% (wt/vol) (Bodenmiller, et al., 2004), or formaldehyde at 1.7% (wt/vol) (Bodenmiller, et al., 2004). These features are highly desirable for development of remedial bioreactors. The resistance to sodium azide and formaldehyde indicates that growth inhibition or cell death does not compromise the integrity of the biofilm, and the resistance to Cd(II) suggests that the biofilm is suitable for heavy metal treatment. Release of swarmer cells from immobilized stalked cells should not be a concern for the intended applications as the doubling time of *Caulobacter* is about 2h and the time required to sequester most heavy metals from the solution is just 15min (Patel, et al., 2009).

The miniature bioreactors used in this work are composed of a glass coverslip of 12 mm in diameter in a plastic well of 16 mm in diameter and 6 mm in height – the height of the wet line from 1.5 ml liquid shaken at 100 rpm. The highest cadmium binding efficiency (76.9% removal) and absorption capacity (0.5 µg per biofilm) were observed with JS4022/p723-6H exposed to 0.4 ppm Cd(II). When exposed to higher concentrations of cadmium only the nonspecific binding of the control strain was enhanced. Similar trends have been noticed with free cell suspensions. The percentage removal as well as the total amount of captured metals can be increased if the ratio of biomass to metal is increased. It has been established that *Caulobacter* cells can also bind to plastic surfaces (Bodenmiller, et al., 2004). In this study, cells attached to the wells of a tissue
culture plate also remove Cd(II). It was observed that if biofilm-coated coverslips were transferred to a fresh well for the metal binding assay, the total removal of Cd(II) dropped about \(~0.25\mu g\). data not shown). This again reiterates the importance of increasing immobilized biomass. If bioreactors with rich internal surface areas are constructed it should increase metal removal efficiency.

CONCLUSIONS

In conclusion, the construct strain JS4022/p723-6H presents a powerful bioremediation agent that is likely to be useful in applications where low levels of heavy metals contaminate a system such as natural water bodies, sediments, and industrial wastewater or sewage sludge that are in need of a secondary remediation process. Although only Cd(II) was tested in this study, the constructed strain is expected to be effective in removing other divalent heavy metal ions too, such as nickel, lead, copper, and zinc. The binding capacity and specificity of the *Caulobacter* recombinant strains can be improved by incorporating different types of heavy metal-binding peptides such as, metallothionines, phytochelatins, or desired sequences screened from peptide libraries, in RsaA. This work demonstrates a complete separation of heavy metal ions from aqueous phase in a single step through the integration of two useful techniques (cell surface display and self-immobilization). Biofilms can be cultivated inside the reactor and can be used readily. No downstream processing is required once the cells are cultivated. Once the waste water is treated the bound metal ions should be washed off by small volumes of acids or chelators and be recycled or disposed properly. The bioreactor prototype developed in this study would also be useful for biosensing, bioconversion, biocatalysis, and related applications.
References


