WRRI Annual Report FY2008

Bioremediation of heavy metals using genetically engineered bacterium *Caulobacter crescentus*

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1. Problem and Research Objectives

Every year, around 2.4 million tons of metal wastes from industrial sources and 2 million tons from agriculture are generated in the US (http://es.epa.gov/ncer/publications/meetings/8-18-04/ppt/wilfred_chen_aug_20.ppt). Pollution caused by heavy metals poses a great danger to humans and the environment which has led to stringent regulations over the allowable limits of heavy metals in drinking water. Heavy metals such as Pb^{2+}, Hg^{2+}, and Cd^{2+} are currently ranked second, third, and seventh, respectively, on the EPA's priority list of metals that are of major environmental concern. Because heavy metals bioaccumulate in the food chain, exposure to them even at low levels is harmful and may eventually cause adverse health problems, such as nerve damage and cancer. A recent study has also suggested links between heavy metals (particularly cadmium) and some autoimmune diseases, such as multiple sclerosis and scleraderma. Because of their extreme toxicity, there are growing needs for safe and effective ways to remediate metals released to the environment.

Lake Erie provides drinking water for about eleven million people (http://www.epa.gov/glnpo/lakeerie/), including several million Ohio residents living within its watershed. However, four Ohio coastal areas have been designated in the Great Lakes Water Quality Agreement as Areas of Concern , at least in part due to elevated levels of heavy metals, which contributes to impaired beneficial use of these areas, including "restrictions on drinking water consumption, or taste and odor" (http://www.epa.gov/greatlakes/aoc/).

Although contaminant levels in the Great Lakes are declining in general in recent years, health concerns from environmental heavy metal exposures in the Lake Erie basin remain (http://www.great-lakes.net/humanhealth/drink/index.html). As a result, reducing the loading of persistent bioaccumulative toxics (such as heavy metals) to Lake Erie remains a priority on Ohio's Lake Erie Action List. Although more stringent regulations will help to reduce the loading of heavy metals to the Lake, what has been released previously can only be retrieved by remediation. According to the Ohio Coastal Management Program, "It is the policy of the State of Oho to coordinate the development and implementation of Remedial Action Plans for Ohio's four Lake Erie Basin Areas of Concern as identified in the International Joint Commission's reports on Great Lakes water quality".

The objectives of this study included the following:

– Surface-expression of heavy metal-binding peptides on Caulobacter

Heavy metal-binding peptides were fused to the surface layer protein RsaA of *Caulobacter* by gene manipulation techniques. Due to the intrinsic ability of *Caulobacter* to process RsaA to the cell exterior, metal-binding peptides tagged to RsaA are pre-coded to be transported to the cell surface, ready to directly interact with the external environment.

– Bioaccumulation of heavy metal ions by engineered Caulobacter cells

Engineered *Caulobacter* strains were evaluated for their ability to sequester dissolved cadmium ions. Bacterial cells were cultivated and harvested according to standard procedures. Drinking water spiked with Cd^{2+} metal ions as well as environmental samples from Lake Erie were tested with the engineered *Caulobacter* strains (Fig 1).

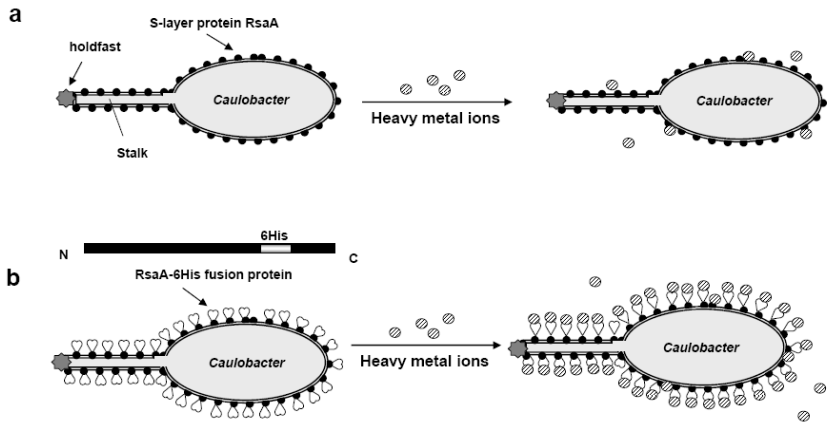

Fig. 1. *Scheme showing sequestration of heavy metals using surface expressed foreign peptides (not to scale). (a), wild type* Caulobacter *strains adsorb low levels of heavy metal ions through non-specific ionic attractions. (b), engineered* Caulobacter *strains retrieve heavy metals by both non-specific binding and specific affinity between hexa-histidine peptides and heavy metal ions.*

2. Methodology

2.1. *Construction of genetically engineered Caulobacter crescentus with surface-expressed heavy metal-binding peptides.*

A DNA fragment encoding a heavy metal-binding peptide, hexa-histidine 6H or synthetic phytochelatin ECn, flanked by *Bgl*II and *Pst*I sites were obtained by polymerase chain reaction (PCR) and were introduced to the corresponding sites inside of the *rsaA* gene sequence (Fig 2). The p4ArsaAGSCC vector was digested with *Bgl*II and *Pst*I and mixed with the PCR fragment pre-digested also with the same enzymes. The two fragments were joined by DNA ligase, generating a recombinant plasmid which carries the synthetic sequence "sandwiched" in *rsaA* gene. Finally, the recombinant plasmids, coding for the S-layer (RsaA) fusion proteins, were introduced to a *Caulobacter* host strain and selected by a solid agar medium.

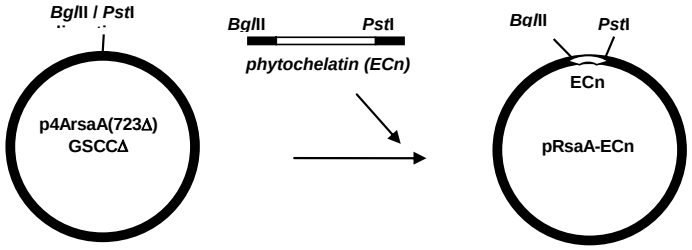

Fig. 2. *Construction of the recombinant plasmid expressing RsaA-ECn (not to scale).*

2.2. Bioaccumulation of heavy metal ions using engineered *Caulobacter* cells

Stationary phase *Caulobacter* cell cultures grown in PYE were diluted with the same medium to cell densities ranging from 0.1 OD_{600nm} to 1.0 OD_{600nm}. 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 6500 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Thermo Electron Corporation) or 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. 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. One liter of cell culture at 1.0 OD_{600nm} provided 300 mg of CDW.

3. Principal Findings

3.1 Expression of RsaA-6His protein in *Caulobacter* JS4022.

S-layer proteins were extracted from recombinant *Caulobacter* strains JS4022/ p4ArsaA(723Δ)GSCCΔ and JS4022/ p723-6H using a low-pH method. The $RsaA^-$ strain JS4022 was used as the negative control. As based on SDS-PAGE analysis, a strong band of ~ 110 kDa

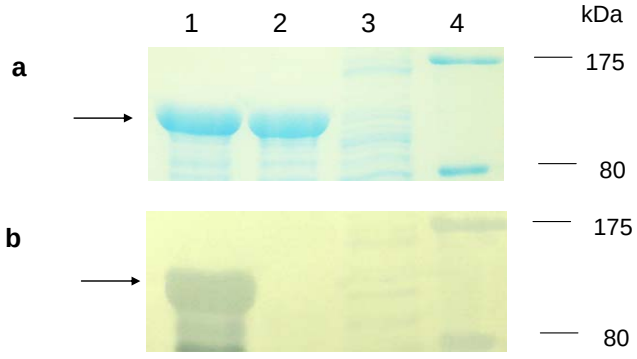

Fig. 3. *Scheme showing sequestration of heavy metals using surface expressed foreign peptides (not to scale). (a), wild type Caulobacter strains adsorb low levels of heavy metal ions through non-specific ionic attractions. (b), engineered Caulobacter strains retrieve heavy metals by both non-specific binding and specific affinity between hexa-histidine peptides and heavy metal ions.*

was detected in both recombinant strains but was missing from the host strain JS4022 (Fig. 3a). The size of the bands correlates well with the deduced 108 kDa molecular weight of the two proteins. The correct insertion of the 6His fragment was confirmed by Western blotting (Fig. 3b) with a positive band corresponding to the size of RsaA proteins detected from the JS4022/ p723-6H strain only. These results demonstrate that RsaA-6His was successfully expressed in strain JS4022, and its expression level was as high as RsaA(723).

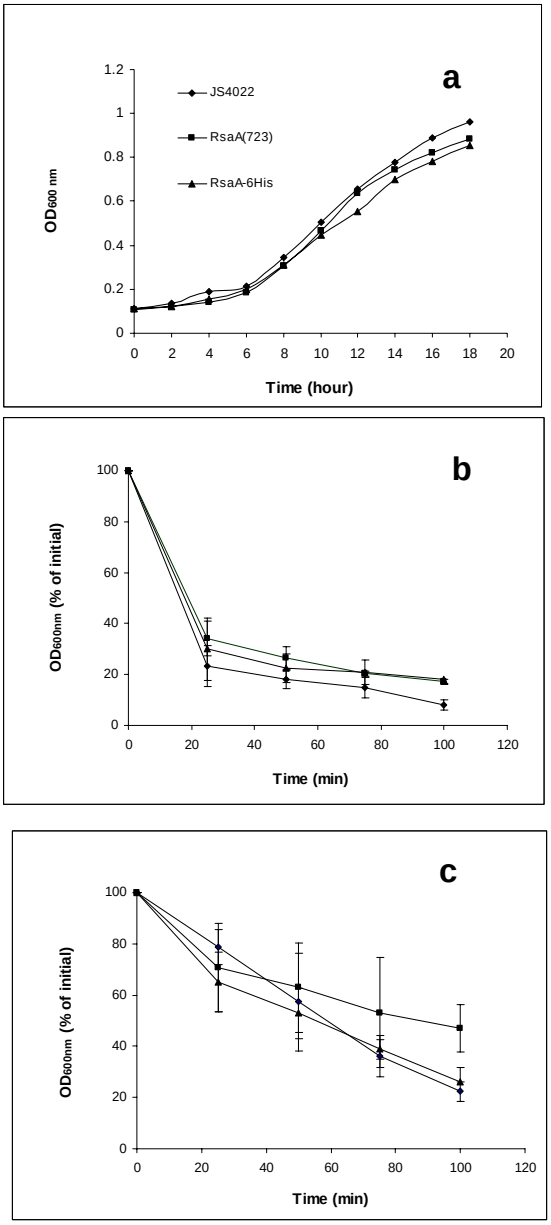

Fig. 4. *Physiological characteristics of JS4022/p723-6H. The growth rates of recombinant and control strains are compared in (a), as indicated by the optical density of cell cultures at 600 nm. The sensitivities of the strains to 0.1% (w/v) SDS and 2 mM EDTA are summarized in (b) and (c), respectively. 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.*

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

The expression of RsaA-6His S-layer proteins did not inhibit the growth of host cells as the growth rate of JS4022/p723-6H was comparable to that of the control strains JS4022 and JS4022/p4ArsaA(723Δ)GSCCΔ (Fig. 4a). To evaluate its suitability to serve as a whole-cell adsorbent, the sensitivities of JS4022/p723-6H to detergents and chelators were monitored. 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. 4b). Initially each strain was effected to a similar degree by SDS exposure; however, following 100 min, control strain JS4022 proved most susceptible with >90% decrease in culture turbidity (one-way ANOVA; $P < 0.001$), likely due to the lack of a S-layer, which provides modest protection to the integrity of cell envelopes. Strains JS4022/p723-6H and JS4022/p4ArsaA(723Δ)GSCCΔ offered similar, albeit 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. 4c). 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/p4ArsaA(723Δ)GSCCΔ proved most resistant to the chelator EDTA showing only 53% decrease in culture turbidity compared to 75-80% declines for JS4022 and JS4022/p723-6H (one-way ANOVA; $P < 0.01$). An increase of divalent ions in the growth medium provided the cells extended protection against EDTA. When grown in PYE medium containing 0.3% $CaCl_2 \cdot 2H_2O$, only 35% of the JS4022/p723-6H cells experienced lysis after 100 min of exposure to EDTA whereas for JS4022/p4ArsaA(723Δ)GSCCΔ, only about 10% of the cells were subject to lysis (data not shown).

Optimization of contact time for removal of cadmium.

Recombinant strain JS4022/p723-6H was tested for its metal-binding dynamics in solution. Within 15 min, JS4022/p723-6H removed 94.3% of cadmium from the aqueous phase, whereas, the control strain JS4022/p4ArsaA(723Δ)GSCCΔ removed only 13.2% (Fig. 5). 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/p4ArsaA(723Δ)GSCCΔ, respectively. These results suggest that the time required by JS4022/p723-6H to sequester heavy metals can be as short as 15 min. For the convenience of handling multiple samples, we chose to adopt the contact time of 30 min for further studies.

Effect of the amount of biomass on sequestration of cadmium.

When cell density was as low as 0.03 g L^{-1} (CDW), non-specific binding of Cd(II) was dominant in JS4022/p723-6H; it removed just 3% more cadmium than did the control strain (Fig. 6). As the cell density was increased, so to did the specific binding of metal ions delivered by RsaA-6His. As 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 fluctuated 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/p4ArsaA(723Δ)GSCCΔ to 37%.

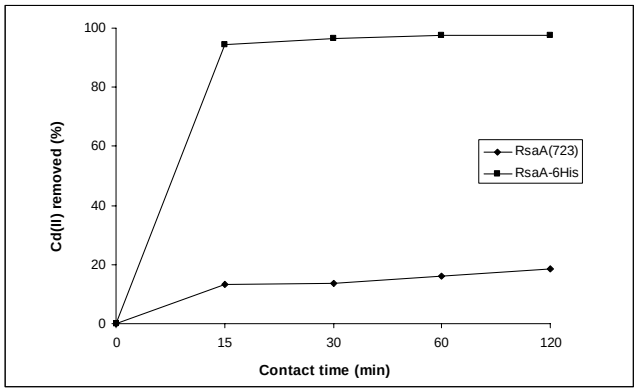

Fig. 5. *The percentages of cadmium removed by JS4022/p723-6H (indicated as RsaA-6His, square) and JS4022/p4ArsaA(723Δ)GSCCΔ (indicated as RsaA(723), diamond) as a function of time. Total input of Cd(II) was 1 ppm.*

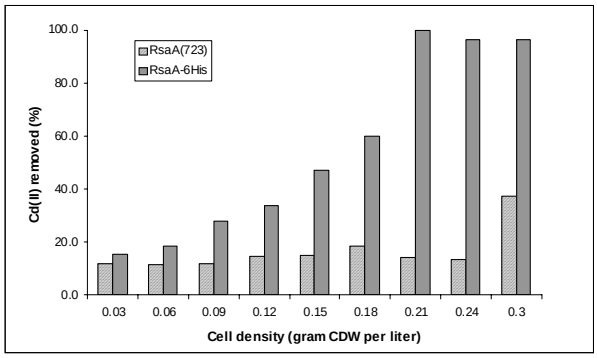

Fig. 6. *The percentages of cadmium removed by recombinant strains at various cell densities. Solid bars, JS4022/p723-6H, shown as RsaA-6His. Crossed bars, JS4022/p4ArsaA(723Δ)GSCCΔ, shown as RsaA(723). Total input of Cd(II) was 1 ppm.*

Removal of cadmium at different initial concentrations.

We next tested the metal removing capacity of JS4022/p723-6H 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 with the rationale 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/p4ArsaA(723Δ)GSCCΔ (one-tailed *t*-test) (Fig. 7). The largest difference in Cd removal efficiency was evident at 1 ppm Cd where JS4022/p723-6H removed 2.7 mg of Cd(II) per gram CDW, which was 154% higher than the control strain JS4022/p4ArsaA(723Δ)GSCCΔ achieved (1.1 mg g^{-1}) (one-tailed *t*-test; $P < 0.0001$). As total Cd

concentration increased, the 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; $P < 0.05$). Whereas adsorption with more concentrated Cd(II) was not tested, the total accumulated metal by each gram of biomass was expected to be higher if more cadmium was added. Similarly, improvement of removal efficiency could be anticipated with an increase of biomass.

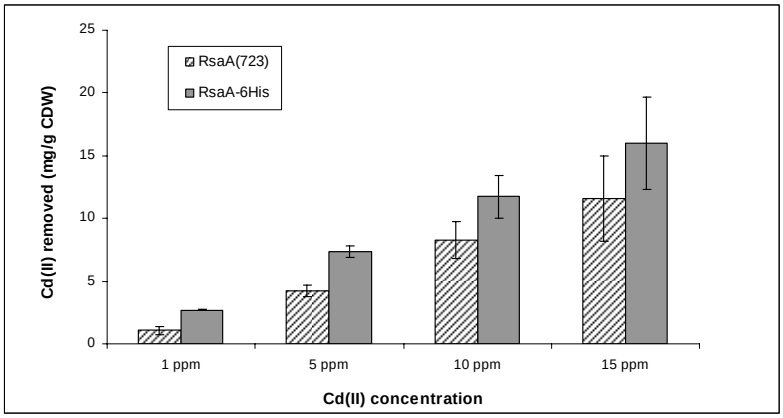

Fig. 7. *Total amount of cadmium removed by recombinant strains at various Cd(II) concentrations. Solid bars, JS4022/p723-6H, shown as RsaA-6His. Crossed bars, JS4022/p4ArsaA(723Δ)GSCCΔ, shown as RsaA(723). Cell density of 0.30 g L^{-1} (dry weight) was used for both strains. Results of five independent tests.*

4. Significance

In this study, we successfully inserted hexa-histidine peptides in a permissive site of RsaA, the S-layer protein of *Caulobacter crescentus* (Fig. 2 and 3). The expression of RsaA-6His fusion proteins sustains normal growth of host cells and offers similar level of protection to the host from the deleterious effects of detergents and chelators as RsaA does (Fig. 4). The constructed recombinant strain JS4022/p723-6H was useful as a whole cell adsorbent to sequester Cd(II) from aqueous solutions. When challenged with 1 ppm Cd(II), strain JS4022/p723-6H demonstrated remarkable specific 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/p4ArsaA(723Δ)GSCCΔ, which expresses RsaA(723), sequestered only 11.4% ~ 37.0% of the added Cd(II) (Fig. 5 and 6). The required contact time can be as short as 15 min. The highest binding capacity achieved by JS4022/p723-6H in this study was found to be 16 mg g^{-1} CDW (Fig. 7), which is also comparable to the findings from other studies.

In conclusion, the construct strain JS4022/p723-6H offers a robust bioremediation agent that is most 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 we only tested Cd(II) in this study, the constructed strain is expected to be effective in removing other divalent heavy metal ions as well, such as nickel, lead, copper, and zinc. To improve the binding capacity or narrow the specificity of the *Caulobacter* recombinant strains, one can incorporate to RsaA other types of heavy metal-

binding peptides, for example, metallothionines, phytochelatins, or desired sequences screened from peptide libraries. This work demonstrates the effectiveness of employing a recombinant *Caulobacter* strain as free cells in the remediation of the heavy metal cadmium and lays the foundation for the construction of bioremediation reactors of high efficiency and low cost.

5. Publications

Patel J, Zhang Q, McKay RM, Vincent R, **Xu Z**. Genetic Engineering of *Caulobacter crescentus* for Removal of Cadmium from Water. Appl Biochem Biotechnol. 2009 Feb 12. [Epub ahead of print]. DOI 10.1007/s12010-009-8540-0.