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Genetic Engineering of *Caulobacter crescentus* for Removal of Cadmium from Water

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Abstract Hexa-histidine (6His) peptide was inserted to a permissive site of the surface layer (S-layer) protein RsaA of *Caulobacter crescentus.* The recombinant strain JS4022/p723–6H, 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. This work provides a potential cost-effective solution toward bioremediation of heavy metals from aqueous systems.

Keywords *Caulobacter crescentus* · S-layer · Cadmium · Heavy metals · Bioremediation

Introduction

Pollution caused by heavy metals poses risk to humans and the environment, which has led to stringent regulations over the allowable limits of heavy metals in drinking water. Heavy metals can bioaccumulate 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

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cancer. A recent study [1] has suggested links between heavy metals (particularly cadmium and chromium) and multiple sclerosis and other demyelinating syndromes. Due to their extreme toxicity, there is demand for safe and effective ways to remove metal contaminants from the environment.

Traditional technologies available to remove heavy metals from industrial wastewater are as follows: precipitation, adsorption, ion exchange, reverse osmosis, evaporation, electrolysis, and cementation [2]. These methods are either costly or ineffective to treat large solution volumes at low concentration of heavy metal contaminants, for example at sub-parts-per-million (sub-ppm) levels. 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 [3, 4], fungi [5, 6], bacteria [7, 8], and animal [2] biomass to sequester metals has been well documented. In one example, 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 [7].

In order to achieve specific adsorption to heavy metals, researchers have overexpressed affinity peptides, such as hexahistidines, glutathione *S*-transferase, metallothionein, maltose-binding proteins, and synthetic phytochelatins in microbial cells [9, 10]. The drawbacks of this approach are limited uptake of the metals through the cell membrane and the toxicity of these metals once accumulated inside host cells. Addressing these concerns, scientists have incorporated metal-binding peptides to the microbial cell surface by fusing the peptides to native cell surface proteins [11–13]. Such whole cell adsorbents successfully sequestered dissolved heavy metals from solutions. Effective as these approaches are, challenges remain. One of the common issues is safe retrieval of heavy metals once sequestered. Ideally, intact microbial cells, having the capacity to adsorb heavy metals, would be immobilized on a device ready for deployment. After affinity binding of heavy metals, the device could be retrieved from solution—along with the bound metal ions—and be recycled after the metals have been safely discharged.

Caulobacter spp. is a candidate for such a remediatory approach. The distribution of *Caulobacter* is ubiquitous, including freshwater [14], seawater [15], ground water [16], wastewater [17], drinking water [18–20], soil [21], deep-sea sediment [22], a gold mine [23], and even ancient ice dated back thousands of years [24]. Cells of *Caulobacter* are harmless to animals, humans, and the environment and are well known for their ability to survive in nutrient-poor habitats. To accommodate the need to thrive under limiting conditions, they have evolved complex metabolic pathways to utilize aromatic compounds [25] and to cope with heavy metal stress [26], making them natural bioremediation agents. Genes involved in cadmium resistance in *Caulobacter* have been identified [27].

Mature *Caulobacter* cells bear a stalk-like polar appendage and are called “stalked cells.” Via an adhesive holdfast, the stalk keeps the bacterium attached to a surface so that water currents will not dislodge the cell from its substrate. The force of adhesion of a single *Caulobacter* cell to a borosilicate surface has been measured in the micro Newton range representing the strongest ever measured for biological adhesives and even stronger than some commercial superglues [28–30]. Through this distinct adhesion strength, *Caulobacter* cells are able to overlay a solid surface with monolayer biofilms; the high density biofilms resist starvation, recover rapidly from stress, and self-seal from mechanical abrasion [31]. One can take advantage of these features by immobilizing *Caulobacter crescentus* on a bioremediation reactor to clean up contaminants such as heavy metals.

In common with many other prokaryotic organisms, *Caulobacter* cells are coated with orderly structured surface layers (S-layers), which are composed of almost 40,000 identical

subunits of protein RsaA. S-layer proteins are arranged in monomolecular crystalline arrays covering the entire cell surface during all stages of cell growth and division. Microbial S-layers likely provide the first line of defense against phages, lytic enzymes, host immune systems, etc. [32]. Due to their location and high copy number, S-layer proteins become ideal carriers to display foreign peptides on the surface of a host cell. Small fragments of antigens of pathogenic bacteria and viruses were fused with RsaA and were able to boost high immunological responses in animal hosts [33, 34]. The secretion system of RsaA has also demonstrated an attractive prospect for the production of particulate biocatalysts, for example, β-1,4-glucanase [35]. The present study aims to employ RsaA as a delivery system to anchor hexa-histidine (6His) peptide to the *Caulobacter* cell surface and construct a recombinant bioremediation agent to sequester heavy metals from aqueous solutions (Fig. 1).

Materials and Methods

Bacterial Strains and their Cultivation

The S-layer negative *C. crescentus* strain JS4022 [36] was used as the host strain to express RsaA derivatives. *Escherichia coli* strain S17–1 [37] was used for DNA manipulations. *E. coli* strains were grown at 37 °C in Luria Broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.5% agar for plates. *C. crescentus* strains were grown at 30 °C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% $CaCl_2 \cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$) with 1.5% agar for plates. Chloramphenicol was supplemented when necessary at 20 and 2 μg mL^{-1} for *E. coli* and *C. crescentus*, respectively. Cell growth was monitored by measuring the optical density of cell cultures at 600 nm (OD_{600nm}). 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 diamine tetra acetic acid (EDTA) as previously described [38].

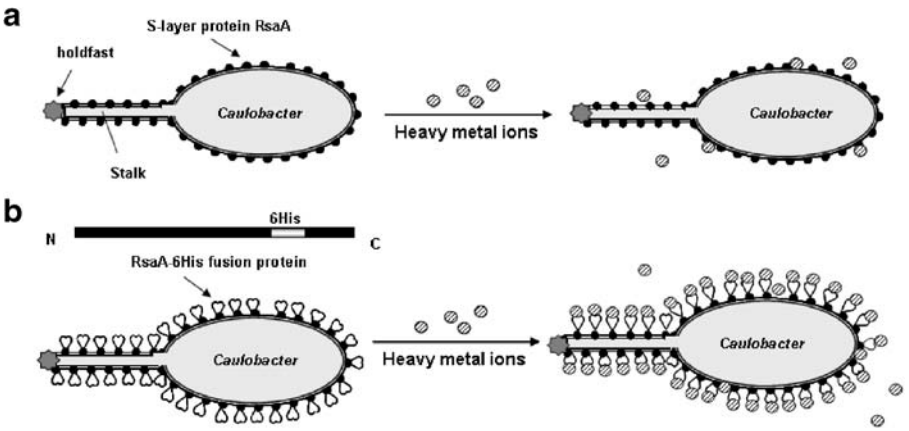

Fig. 1 Scheme showing sequestration of heavy metals using surface expressed foreign peptides (not to scale). **a** Wild-type *Caulobacter* strains adsorb low levels of heavy metal ions through nonspecific ionic attractions. **b** Engineered *Caulobacter* strains retrieve heavy metals by both nonspecific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

Construction of *rsaA-6his* Chimeric Gene

The plasmid p4ArsaA(723Δ)GSCCΔ (chl^r) contains an *rsaA* mutant gene with several unique restriction sites inserted at the *Bam*HI linker site corresponding to amino acid 723 of RsaA [36]. The protein product of this gene is denoted as RsaA(723) in this paper. Primers ZPMS-001 (5′-GGAAGATCTTCTAGAccaagcggaCATCACCATCACCATCAC-3′) and ZPMS-002 (5′-ttggCTGCAGaACTAGTccaagcggaGTGATGGTGATGGTGATG-3′) were used to amplify the *6his* fragment, which contains *Bgl*II and *Xba*I sites upstream and *Spe*I and *Pst*I sites downstream. ZPMS-003 (gatcacggactcggaagc) and ZPMS-004 (gcgttggccagggtcac) were used as sequencing primers to verify the correct insertion of *6his* to *rsaA*. Plasmid p4ArsaA(723Δ)GSCCΔ and *6his* polymerase chain reaction fragment were double digested with *Bgl*II and *Pst*I, purified from agarose gels, and ligated to generate plasmid p723–6H. The sequence of the *rsaA-6his* fusion gene was confirmed by DNA sequencing. Plasmids were transformed into *E. coli* strain S17–1 by the calcium chloride method and were introduced into *C. crescentus* by conjugation with *E. coli* S17–1. DNA manipulations were carried out according to standard procedures.

Extraction and Analysis of S-layer Proteins

S-layer proteins were recovered from the cell surface by a low-pH extraction method, as described previously [36]. In brief, cells were collected by centrifugation of a 10 ml overnight culture at 12,000×*g* for 5 min, followed by two washes with 750 μl of 10 mM HEPES (pH 7.2). The volume of the cell pellet was estimated and was suspended in equal volume of 100 mM HEPES (pH 2.0). After incubation of no more than 5 min, 2.8 μl of 10 M NaOH was added for every 240 μl of 100 mM HEPES (pH 2.0). The mixture was centrifuged for 3 min and the supernatant, which contained soluble S-layer proteins, was subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, followed by visualization with the GelCode™ Blue Stain Reagent (Thermo Scientific). HisDetector Western Blot Kit (Kirkegaard & Perry Laboratories, Inc.) was used for colorimetric immunodetection of 6His following the recommendations of the manufacturer.

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 to 1.0 OD_{600nm}. Various amounts of 1,000 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 3,300×*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.

Statistical Analysis

Data were subject to one-way analysis of variance (ANOVA) followed by a Tukey Honestly Significance Difference (HSD) test using VassarStats: Website 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.

Results

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 was detected in both recombinant strains but was missing from the host strain JS4022 (Fig. 2a). 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. 2b) 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).

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. 3a). 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. 3b). 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; $P<0.05$)

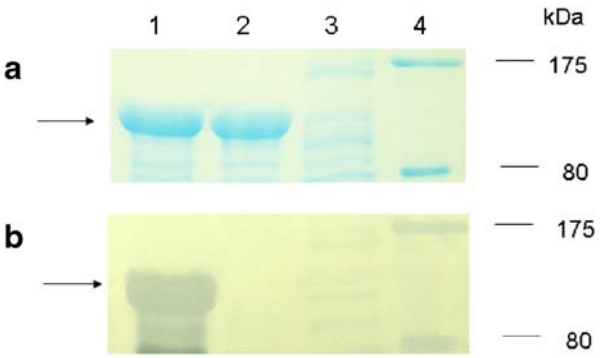

Fig. 2 Expression of RsaA-6His proteins in *Caulobacter* JS4022. S-layer proteins were extracted by a low-pH method and were analyzed with 7.5% SDS-PAGE (**a**) and Western blotting (**b**). *Lane 1*, JS4022/p723–6His; *lane 2*, JS4022/p4ArsaA(723Δ)GSCCΔ; *lane 3*, JS4022; *lane 4*, prestained protein molecular weight markers. *Arrows* indicate the position of RsaA-6His

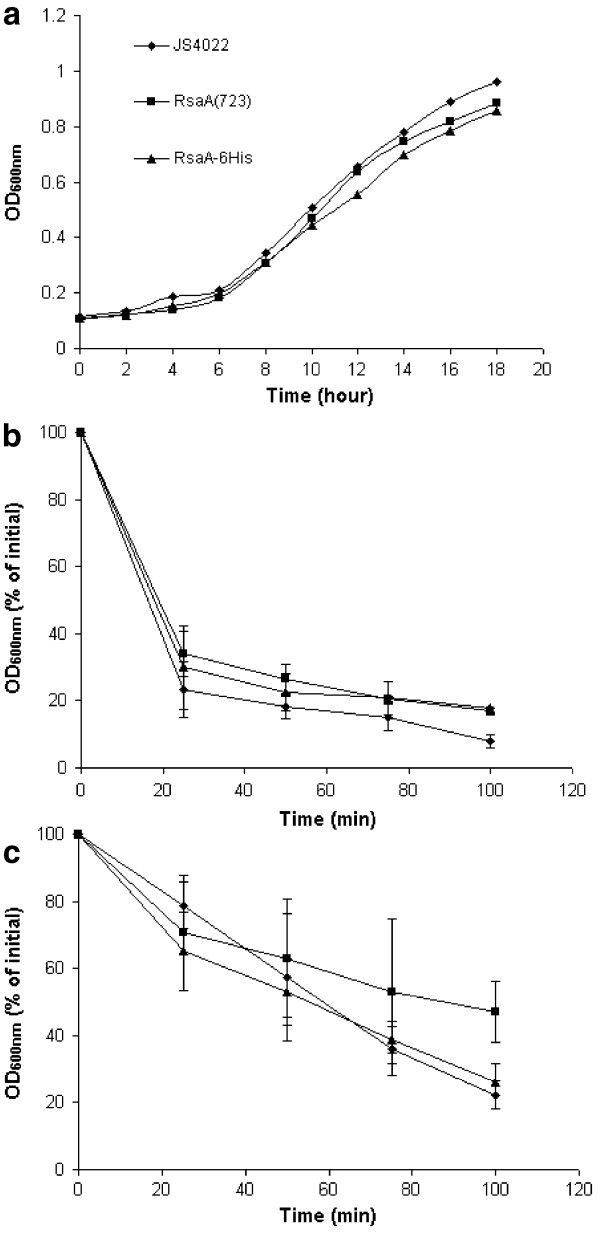

Fig. 3 Physiological characteristics of JS4022/p723–6H. The growth rates of recombinant and control strains are compared in (**a**), as indicated by the optical density of cell cultures at 600 nm. The sensitivities of the strains to 0.1% (*w*/*v*) SDS and 2 mM EDTA are summarized in (**b**) and (**c**), 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

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/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. 4). Extending the incubation time to 120 min increase, 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), nonspecific binding of Cd(II) was dominant in JS4022/p723–6H; it removed just 3% more cadmium than did the control strain (Fig. 5). As the cell density was increased, so did the specific binding of metal ions delivered by RsaA-6His. As cell density was increased from 0.03 to 0.21 g L^{-1}, the Cd(II) removed by JS4022/p723–6H improved stepwise from 15.2% to 99.9%. In contrast, the nonspecific 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 nonspecific binding of JS4022/p4ArsaA(723Δ)GSCCΔ to 37%.

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

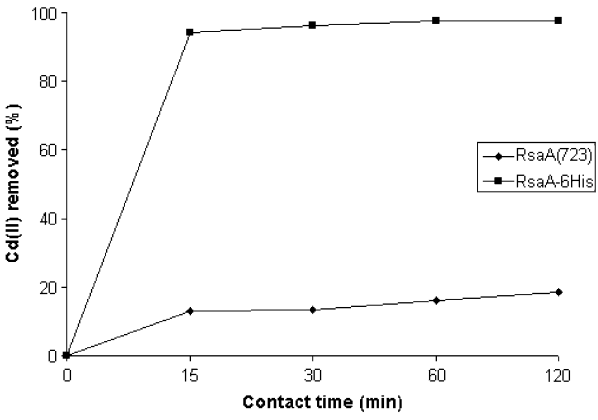

Fig. 4 The percentages of cadmium removed by JS4022/p723–6H (indicated as RsaA-6His, *square*) and JS4022/p4ArsaA(723Δ)GSCCΔ (indicated as RsaA(723), *diamond*) as a function of time. Total input of Cd(II) was 1 ppm

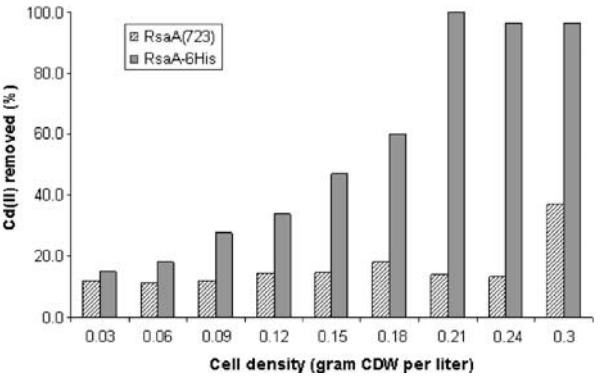

Fig. 5 The percentages of cadmium removed by recombinant strains at various cell densities. *Solid bars*, JS4022/p723–6H, shown as RsaA-6His. *Crossed bars*, JS4022/p4ArsaA(723Δ)GSCCΔ, shown as RsaA(723). Total input of Cd(II) was 1 ppm

compared to control strain JS4022/p4ArsaA(723Δ)GSCCΔ (one-tailed *t* test; Fig. 6). 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.

Discussion

In this study, we successfully inserted hexa-histidine peptides in a permissive site of RsaA the S-layer protein of *C. crescentus* (Figs. 1 and 2). The expression of RsaA-6His fusion proteins sustains normal growth of host cells and offers some protection to the host from the deleterious effects of detergents and chelators (Fig. 3). Detergents disrupt the lipidic structure of the cell outer membrane and chelators weaken the interactions between lipopolysaccharide (LPS) molecules found in outer membranes, which depend on divalent cations, particularly calcium ions to crosslink with each other for a stable leaflet of outer

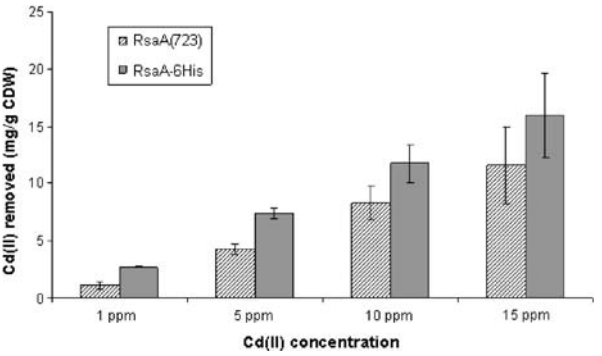

Fig. 6 Total amount of cadmium removed by recombinant strains at various Cd(II) concentrations. *Solid bars*, JS4022/p723–6H, shown as RsaA-6His. *Crossed bars*, JS4022/p4ArsaA(723Δ)GSCCΔ, shown as RsaA(723). Cell density of 0.30 g L^{-1} (dry weight) was used for both strains. Results of five independent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

membrane [39]. Studies also suggest that RsaA attaches to a smooth LPS possibly also via calcium bridging [40, 41]. Insertion of hexa-histidine peptide to the permissive site of RsaA should not alter any structures of the lipidic membrane, including the LPS molecules. Therefore, there was no surprise that JS4022/p723–6H strain was as resistant as JS4022/p4ArsaA(723Δ)GSCCΔ to SDS. The slightly increased sensitivity to EDTA observed with JS4022/p723–6H could be the result of competition for calcium ions between LPS, RsaA, and 6His. This is supported by the fact that increased calcium in the growth medium apparently rendered cells enhanced resistance against the chelator, likely by alleviating this competition. This competition should not interfere the binding of Cd(II) at later remediation stage since 6His has a higher affinity to heavy metal ions (please see below for more discussion).

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) (Figs. 4 and 5). The required contact time can be as short as 15 min. When up to 15 ppm Cd(II) was added, the total Cd(II) bound to JS4022/p723–6H was still consistently higher compared to JS4022/p4ArsaA(723Δ)GSCCΔ (Fig. 6). It is worth to point it out that all binding assays were performed in PYE solutions, which 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. Even in the presence of excessive amount of Ca(II) and Mg(II) ions, JS4022/p723–6H still demonstrated remarkable specificity toward Cd(II).

The rapid uptake of Cd(II) indicates that binding occurred at the cell surface as intended rather than internally. The high efficiency of the binding was in good agreement with similar studies. 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 challenged with 11 ppm Cd(II) [42]. In that study, the bioadsorption rate increased rapidly during the first 10 min and remained nearly constant after 40 min [42]. 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) [43].

The highest binding capacity achieved by JS4022/p723–6H in this study was found to be 16 mg g^{-1} CDW, which is also comparable to the findings from other studies. Up to 6.7 mg g^{-1} (equals to 60 μmole g^{-1}) removal capacity for cadmium has been reported with engineered *E. coli* strains when tested with 2–25 ppm Cd(II) [10, 11, 13, 44]. An immobilized live cyanobacterium (*Oscillatoria* sp. H1) generated a net adsorption of 11 mg g^{-1} with initial input of 25–250 ppm of cadmium [45]. In another study, the maximum binding of *Bifidobacterium* spp. and *Lactobacillus* spp. strains was predicted to range from 12.1 to 54.7 mg g^{-1} if over 50 ppm cadmium was added [43]. It was also noted in the latter two studies that bioadsorption of Cd(II) increased as initial concentrations of the metal increased between 1 and 100 ppm.

Certain levels of nonspecific binding are common with bacterial biomass due to the fact that bacterial cell surface is usually negatively charged. This is why the above-mentioned wild-type cyanobacterial and lactic acid bacterial strains were used in bioadsorption. Increase of biomass influences the equilibrium of metal uptake and results in high efficiency of bioaccumulation. In this study, significant nonspecific binding of Cd was observed when the biomass/metal ratio became low. The reason probably lies in the two-dimensional lattice assembly of S-layers. Structural analysis suggested that the S-layer of *C.*

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 [40]. 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 to facilitate exchange of materials in and out of the cells. Cd(II) is one of the small molecules that receives no resistance when crossing the S-layer. When the biomass/metal ratio remains high, 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/metal ratio drops, RsaA-6His molecules become limited and can be quickly saturated with Cd(II). 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 nonspecific binding.

The major advantage of using *Caulobacter* in bioremediation is the prospect of constructing bioreactors with self-immobilized bacterial cells. Cell division events are undesirable in the foreseeable process of remediation. Newborn cells are morphologically different by possessing a single flagellum instead of a stalk and are called "swarmer cells." Through cell-cycle development, these progeny cells will eventually shed their flagellum and synthesize a stalk at the same location to become stalked cells. Swarmer cells cannot be immobilized and can potentially escape the bioreactor along with the heavy metal attached. In reality, however, this risk is low due to the fact that *Caulobacter* grows slowly. The doubling time of *Caulobacter* is about 2 h (versus to 20 min with *E. coli*) under optimal laboratory conditions and becomes much longer if it lives in the environment. When incubated in water samples from the Lake Erie, no evidence of dividing was detected within 12 h even at their optimal growth temperature 30 °C (data not shown; longer incubation was not attempted). In bioreactors, the doubling time of the cells is expected to be at similar low levels. On the other hand, the sequestration of heavy metals with *Caulobacter* takes less than 15 min (Fig. 4). In addition, one may take advantage of the bacterial mutants that have division defects. Under suboptimal conditions, the mutant cells remain vital but cease to divide.

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.

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