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Self-immobilization of Recombinant *Caulobacter crescentus* and Its Application in Removal of Cadmium from Water

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Abstract Microbial biofilms can be valuable in many biotechnological applications, including bioremediation. We have previously constructed a recombinant strain of *Caulobacter crescentus* JS4022/p723-6H by inserting a hexahistidine peptide to a permissive site of the host surface layer (S-layer) protein RsaA. This engineered strain is highly effective in removal of cadmium from water as free cells. In this communication, we examined the biofilms formed by self-immobilized JS4022/p723-6H and evaluated their ability to retrieve cadmium from contaminated water samples. According to light and electron microscopic observations, JS4022/p723-6H cells developed a uniform monolayer biofilm on borosilicate surfaces through their intrinsic appendage, a stalk with an adhesive holdfast. The density of the biofilms reached a maximum after 48 h of incubation and was not affected by exposure to at least 1 ppm cadmium. When 0.4 ppm Cd(II) was added to the growth medium, immobilized JS4022/p723-6H removed 76.9% of the total metal, whereas the control strain only removed 13.5%. When a water sample collected from Lake Erie was spiked with various amounts of $CdCl_2$, immobilized JS4022/p723-6H was able to sequester 44~51% of the total metal, compared to 37~42% accumulated by the control strain. By combining two powerful techniques, cell surface display and self-immobilization, we achieved complete separation of dissolved heavy metals from contaminated water in a single step. This study laid down the foundation to cost-effectively construct large-scale bioreactors with high efficiency and specificity to retrieve environmental contaminants from water.

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Introduction

Each year, several million tons of heavy metal wastes from industrial and agricultural sources are generated in the USA, and a large portion of them are in liquid forms. Heavy metals bioaccumulate in the food chain, and overexposure to them may lead to neurological degeneration, muscular dystrophy, autoimmune diseases [1], cancer, or abnormal child birth [2]. As the awareness of their toxicity rises, the need to retrieve heavy metals from contaminated sites increases. Traditional technologies such as precipitation, adsorption, ion exchange, reverse osmosis, evaporation, electrolysis, and cementation are costly and can be ineffective at removing heavy metals to acceptable levels, thus making biological processes an attractive alternative. The efficacy of using biomass from plants [3, 4], animals [5], and microbes [6–9] to sequester heavy metals is well documented. One popular approach is to incorporate heavy-metal-binding peptides such as polyhistidines [10, 11], metallothionein [12, 13], and synthetic phytochelatins [14] to the cell surface of microbes through genetic manipulations and to use the constructed strain as whole-cell adsorbents for heavy metals. Display of peptides that have high affinity to heavy metals at the bacterial cell surface minimizes uptake of metals across cell membranes, thereby alleviating toxicity to host cells. The high surface area to volume ratio of most bacterial cells, compared to plant and animal tissues, promises enhanced heavy metal adsorption capacity.

Although metal binding capacity of microbial cells may be improved by genetic engineering, retrieval of the metal-laden cells from treated water remains a technical obstacle. Large-scale centrifugation is a possible solution, but the requirement of expensive equipment and the significant operation cost carry economic disadvantages. Immobilization of bacterial biomass to chemical or biological matrices has been attempted by many as an alternative approach. The most popular immobilization means for microbes fall into two categories: artificial entrapment in gel beads [15–17] and natural colonization on solid substances, such as activated carbon [18], ceramic rings [19], glass beads [20], loofah sponge [21], sand columns [22], and even live macroalgae [23]. For the entrapment approaches, additional time of a skilled staff is required to carry out the immobilization procedures. The cost of the trapping materials also needs to be taken into consideration because it is difficult to recover the materials once the system is beyond regeneration. High level of nonspecific binding to the matrices is another problem. Natural colonization of microbial communities often occurs when microbial cells aggregate and secrete adhesive extracellular polymers to hold themselves on specific supporting surfaces, a process often mediated by chemical communications within the community. Physiological heterogeneity caused by differential gene expressions among the community members is characteristic to these biofilms. When they are employed for industrial processes, the heterogeneity affects the efficiency and consistency of the biofilm reactors. For microbial remedial technologies to be economically competitive, the quest for effective immobilization should continue.

Caulobacter spp. are ubiquitously distributed and are harmless to humans and the environment. Each new *Caulobacter* cell possesses a single polar flagellum and is called a "swarmer cell," which is motile and nonproductive (Fig. 1a). In response to a cellular developmental cue, a swarmer cell sheds its flagellum, synthesizes a stalk-like structure at the same location, and becomes a "stalked cell," which is sessile and productive (Fig. 1a). A stalked cell attaches itself to a surface via an adhesive holdfast located at the tip of the

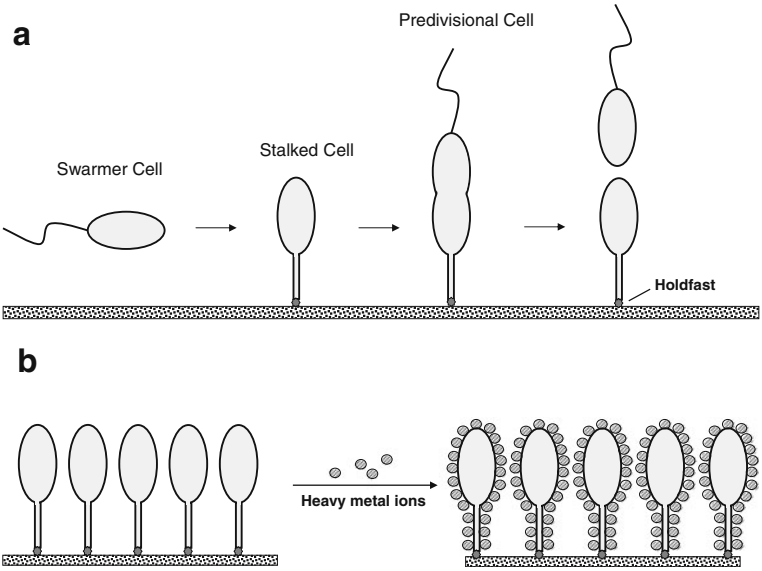

Fig. 1 Schematic drawings of **a** life cycle of a *C. crescentus* cell and **b** bioremediation of heavy metals using *Caulobacter* cells self-immobilized on a solid surface through their holdfasts. Not to scale

stalk. The bond strength has been measured in the micronewton range, "the strongest ever measured for biological adhesives," stronger than some commercial superglue [24]. This unique feature of holdfast makes us envision the potential of constructing a remedial bioreactor with self-immobilized *Caulobacter* cells (Fig. 1b).

Smit et al. visualized the spontaneous biofilms formed by *Caulobacter* spp. for the first time using phase-contrast and epifluorescence microscopy and estimated the density of biofilms by image processing software [25]. It was concluded that cells of *Caulobacter crescentus* CB2A/Rif^r form uniform monolayer biofilms in 24 h and the density of biofilms increases with time and peaks at about 9 days. A more recent study, however, reported biphasic biofilms formed by *C. crescentus* CB15, which contain densely packed, mushroom-shaped structures scattered on the lawn of monolayer biofilms [26]. The mushroom structures started to take shape after 3–4 days of growth under confocal laser scanning microscope, but the monolayer lawn only appeared after 5 days of growth [26]. For practical applications, mushroom-like structures are less desirable than monolayer biofilms because cells enclosed inside of the three-dimensional structures do not have immediate access to the water contaminants and thus the overall adsorption rate and/or capacity will likely be comprised. To address the discrepancy of the previous descriptions about the morphology and development of *Caulobacter* biofilms, additional investigations are necessary.

Besides spontaneous immobilization, *Caulobacter* has another attractive feature that makes it an outstanding agent for remedial applications—the possession of around 40,000 surface layer (S-layer) proteins RsaA. Due to its high copy number, outermost location, and readiness for genetic manipulation, RsaA has become a popular carrier for display of foreign peptides on the surface of *Caulobacter* cells. In a previous study, we inserted hexahistidine (6His) polypeptide to a permissive site of the surface layer (S-layer) protein RsaA of *Caulobacter* [10]. The RsaA–6His fusion protein was successfully expressed to the outer surface of the recombinant strain JS4022/p723-6H and was able to specifically

retrieve Cd(II) from solutions in the presence of an excess of other divalent ions, such as calcium and magnesium. The constructed strain removed 16 mg Cd(II) g^{-1} cell dry weight within 30 min. Under optimal conditions, the sequestration efficiency was as high as 99.9%. To completely separate these metal-laden bacterial cells from water without the need of centrifuges or entrapment matrices, we propose to employ self-immobilized *Caulobacter* cells to construct specialized remedial bioreactors. As a proof for the concept, this study aims (1) to characterize the biofilm formed by self-immobilized JS4022/p723-6H on a borosilicate surface and (2) to evaluate the potential of the biofilms in heavy metal sequestration using cadmium as an example.

Materials and Methods

Bacterial Strains and Their Cultivation

Recombinant *C. crescentus* strain JS4022/p723-6H expressing RsaA–6His fusion proteins was constructed as described previously [10]. Strain JS4022/p4ArsaA(723Δ)GSCCΔ expressing RsaA with a cloning linker at its 723-amino-acid residual position [27] 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% $CaCl_2{\cdot}2H_2O$, and 0.02% $MgSO_4{\cdot}7H_2O$) supplemented with 2 μg ml^{-1} of chloramphenicol. For plates, 1.5% (*w*/*v*) agar was added. Bacterial growth in liquid was monitored by measuring the optical density of cell cultures at 600 nm ($OD_{600\ nm}$).

Cultivation of Biofilms

The procedure for cultivation of biofilms was modified from Smit et al. [25]. Briefly, circular glass coverslips with a diameter of 12 mm (catalog #26023, Ted Pella Inc., Redding, CA, USA) were cleaned and sterilized by flaming with ethanol and were 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 characterization analyses or Cd(II) removal assays.

Biofilm Imaging and Characterization

For phase-contrast imaging, one side of the coverslips coated with *C. crescentus* biofilms was cleaned with a cotton ball and ethanol and was 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 an RS Princeton Instruments digital camera (Trenton, NJ, USA) using Metamorph software (Universal Imaging Co., Westchester, PA, USA).

For electron microscopic imaging, 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 followed by an additional dehydration treatment with hexamethyldisilazane for 30 min. After being coated with gold palladium, biofilms were examined under a Hitachi S-2700 scanning electron microscope (SEM). Five areas in size of 100 μm^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 to determine the density of biofilms.

Cd(II) Removal from Bacterial Growth Medium

After the biofilms were cultivated for up to 7 days, liquid culture was withdrawn from each well and discarded. The biofilms were washed in the same wells three times with 1.5 ml Millipore 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.

Cd(II) Removal from Environmental Water Samples

Biofilms aged 2 days were cultivated and rinsed as stated above and were used to sequester cadmium from a water sample collected from Lake Erie Station 84 (41° 56.07 N, 81° 39.11 W, depth 22.3 m). The lake water was filtered through a 0.45-μm membrane right after sampling and was filtered one more time with a 0.22-μm membrane prior to use. The water was acidified to pH 5.4 with concentrated HCl and was spiked with $CdCl_2$ to reach final concentrations of Cd(II) ranging from 0.4 to 1.0 ppm. One and a half milliliters of such treated water was added to each well containing one biofilm-coated coverslip and incubated at 30 °C for 30 min at 100 rpm. Cadmium measurement was done as described above.

Statistical Analysis

Data were subjected to one-tailed *t* test for independent samples or one-way analysis of variance followed by a Tukey honestly significant difference test using VassarStats: Web Site for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html).

Results

Self-immobilization of Recombinant Strain JS4022/p723-6H

Sterile coverslips were cultivated with cell culture of JS4022/p723-6H or JS4022/p4ArsaA (723Δ)GSCCΔ for up to 7 days in PYE medium and were examined for formation of biofilms by phase-contrast (Fig. 2a) or scanning electron microscopy (Fig. 2b). No mushroom-shaped structures were observed with either strain. *Caulobacter* cells appear to form monolayer biofilms coating the surface of the coverslips with no distinguishable differences observed between the two strains (data not shown), which is not surprising since the two strains share the same type of holdfast and stalk. Monolayer biofilms had already formed after 24 h of cultivation (data not shown), and this basic morphology remained unchanged for at least 7 days. The SEM photos reveal that cells constituting the biofilms are either stalked cells or predivisional cells (Figs. 3 and 4); no swarmer cells were identified. The attachment of the cells to the glass coverslips is presumably through their holdfast because, when the biofilm was scratched by the tips of forceps, cell bodies were dislodged, but their holdfasts remained; cross-bands that are characteristic to *Caulobacter* stalks were clearly visible (Fig. 3b). The monolayer biofilms formed by *Caulobacter* differ

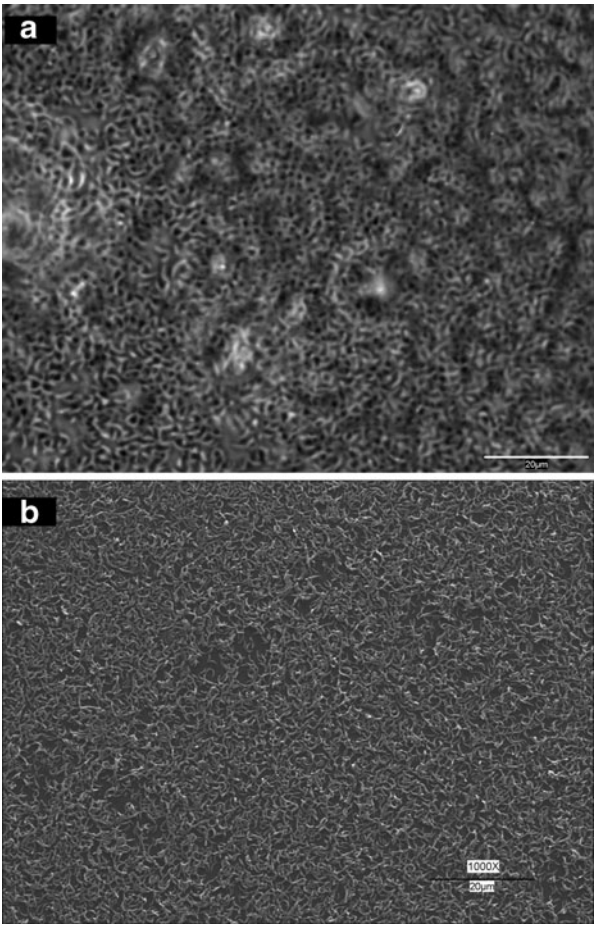

Fig. 2 Phase-contrast light micrograph (**a**) and scanning electronic micrograph (**b**) of the biofilm formed by *C. crescentus* JS4022/p723-6H at day 5. *Bars*, 20 μm

from those community-based biofilms fundamentally. Each *Caulobacter* cell immobilizes itself to the coverslip surface and enjoys equal access to the environment as a free cell does, which ensures the highest reaction rate and capacity in a foreseeable bioreactor.

Cell density changed in response to biofilm age with highest densities associated with 2-day biofilms (61 cells 100 μm^{-2}) and lower densities associated with 5- and 7-day biofilms (45 and 40 cells 100 μm^{-2}, respectively; Fig. 5; $P<0.001$, one-way analysis of variance followed by Tukey honestly significant difference test). It was also noticed that at day 1, the cell density fluctuated markedly among different samples. On the other hand, the number of abnormal filamentous cells started to go up by day 5 (data not shown). For these reasons, further investigations were conducted with biofilms cultivated for 2 days. Based on the size of the coverslips and density of biofilms formed by JS4022/p723-6H, we calculated that each coverslip can host up to 1.38×10^8 cells with both sides.

As a toxic metal, cadmium is harmful to *Caulobacter* cells. The potential intoxication may induce a cascade of physiological changes that could result in detachment of biofilms. Our data revealed that 2-day-old biofilms challenged with 1 ppm Cd for 30 min showed no

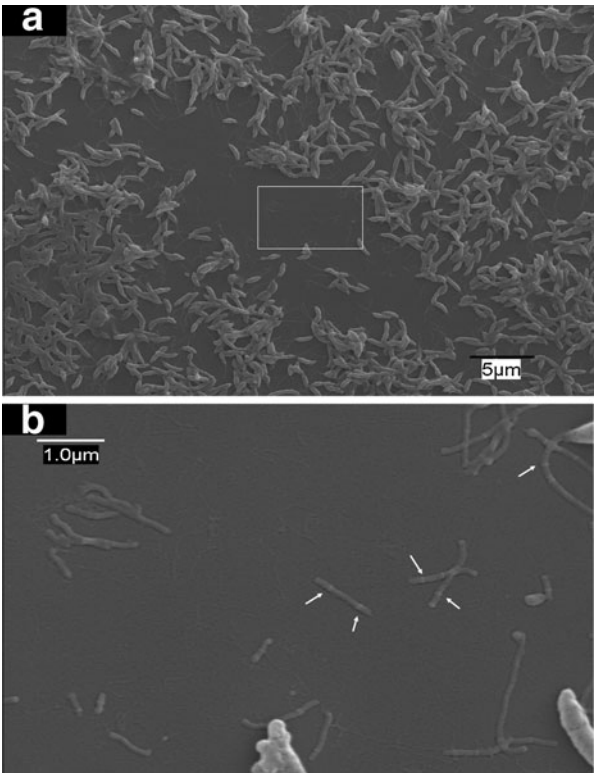

Fig. 3 SEM micrographs of a biofilm formed by JS4022/p723-6H at day 2 with magnification of ×2,500 (**a**) and ×130,000 (**b**). Notice the mechanical abrasion to the biofilm caused by forceps in **a** and the scattered holdfasts in **b**. The *boxed area* in **a** represents the field of view shown in **b**. *Arrows* in **b** point to cross-bands that are characteristic to *Caulobacter* stalks

apparent morphological damage (Fig. 4a) and individual cells expressed no sign of stress from appearance (Fig. 4b). Cell enumeration confirmed that the cell density within the biofilm remained unchanged following exposure to cadmium. An average of 60 cells were identified per 100 μm^2 with metal-treated samples (Fig. 5; two-tailed *t* test, $P>0.05$, $df=5$).

Removal of Cadmium from Growth Medium

Biofilms formed by JS4022/p723-6H or JS4022/p4ArsaA(723Δ)GSCCΔ were challenged with different concentrations of Cd(II) to evaluate their metal removal capabilities in the growth medium. At each concentration tested, strain JS4022/p723-6H removed cadmium more effectively than did the control strain (Fig. 6; one-tailed *t* test, $P<0.001$). With addition 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. 6a). As total input of cadmium increased, the removal efficiency of JS4022/p723-6H declined to the treatment of 0.8 ppm Cd(II) ($P<0.001$, one-way analysis of variance followed by Tukey honestly significant difference test), whereas that of the control strain remained constant (Fig. 6a). Considering the absolute amount of metal removed by the *Caulobacter* biofilms, it was evident that the removal

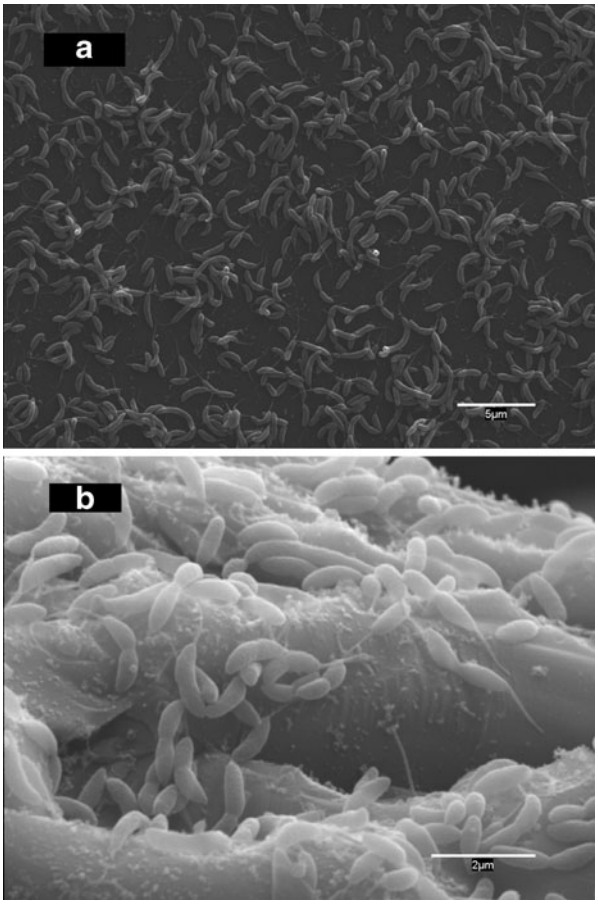

Fig. 4 SEM micrographs of a 2-day biofilm formed by JS4022/p723-6H after exposure to 1 ppm $CdCl_2$ for 30 min. **a** The integrity of biofilm is preserved after treatment with cadmium; **b** metal-laden *Caulobacter* cells remain normal and healthy

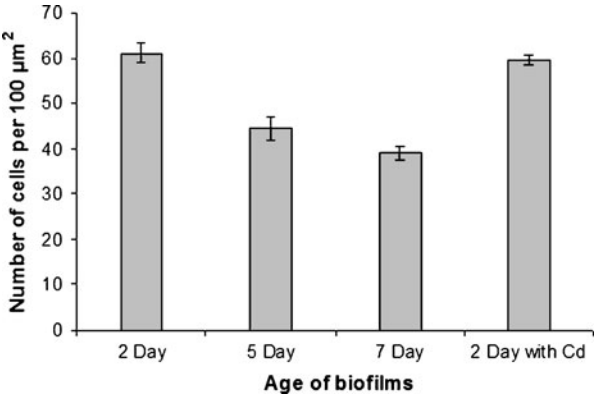

Fig. 5 Cell density of the biofilm formed by JS4022/p723-6H cells at different age. Data of two to three independent experiments

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. 6b). In contrast, the nonspecific binding by the control strain remained around 0.1 μg when up to 0.8 ppm cadmium was applied and reached ~0.2 μg after exposure to 1 ppm Cd(II). This indicates when balanced ratio amount of biomass to cadmium is employed, at least 80% of sequestered cadmium can be contributed by the specific recognition between polyhistidine peptides and cadmium (0.5 vs 0.1 μg). 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, although reaching higher percentage of removal was expected.

Removal of Cadmium from Lake Erie Water Samples

To further evaluate the usefulness of self-immobilized *Caulobacter* cells in bioremediation processes, biofilms formed by JS4022/p723-6H or JS4022/p4ArsaA(723Δ)GSCCΔ were further tested with environmental water samples. A water sample collected from the central basin of Lake Erie was randomly chosen and was spiked with various concentrations of Cd (II). Our preliminary tests with free cells of the recombinant strains indicated that the removal of cadmium from the lake water is more efficient at pH 5.4 than at its natural pH

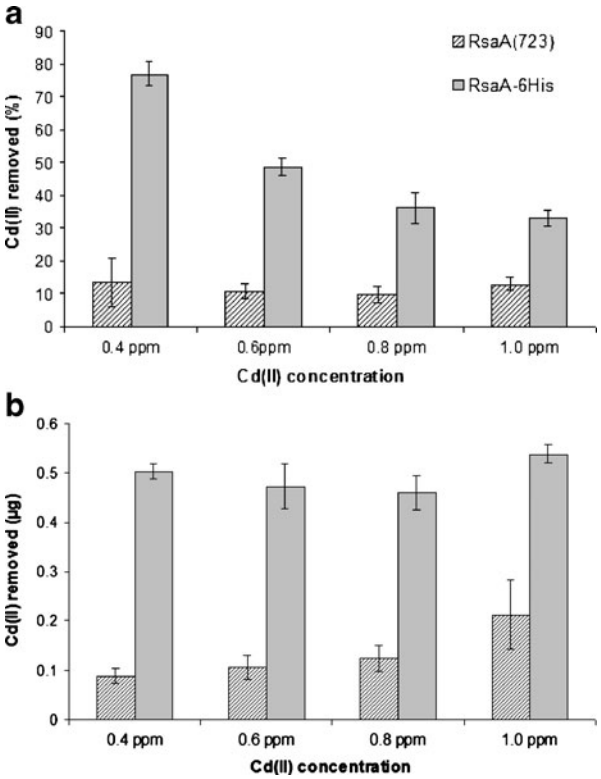

Fig. 6 The removal of cadmium from growth medium PYE by 2-day-old biofilms of JS4022/p723-6H and JS4022/p4ArsaA(723Δ) GSCCΔ depicted as percentages (**a**) and absolute mass amount (**b**). RsaA-6His represents strain JS4022/p723-6H, and RsaA(723) refers to the control strain JS4022/p4ArsaA(723Δ) GSCCΔ. Results of three independent tests; duplicate samples for each test

(pH 8; data not shown). For this reason, all cadmium assays with immobilized cells were done at pH 5.4. At each concentration of $CdCl_2$ tested, JS4022/p723-6H was able to adsorb more dissolved cadmium than did the control strain, even though the nonspecific binding of the control strain was significantly enhanced in the lake water (Fig. 7). Raising the initial input cadmium increased the total amount of sequestered metal (Fig 7b), although the percentage of removal declined slightly (Fig 7a). When the input cadmium increased from 0.4 to 1 ppm, the metal sequestered by JS4022/p723-6H went up from 0.31 μg (51% of the total) to 0.65 μg (45% of the total), and the control strain also showed an increase from 0.25 μg (42% of the total) to 0.54 μg (37% of the total).

Discussion

In this study, we examined the morphology of the biofilms formed by *Caulobacter* cells on glass coverslips with microscopic techniques and tested the heavy metal removal capability

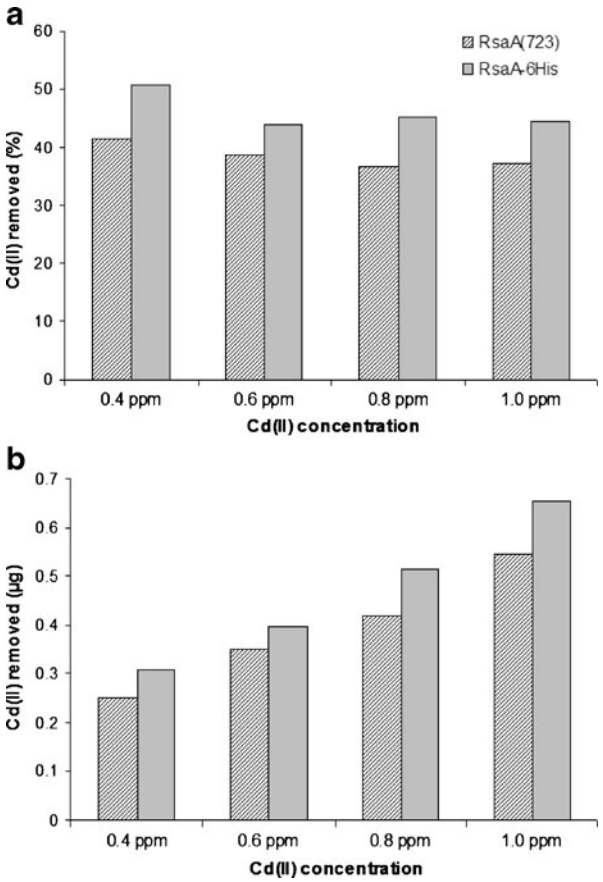

Fig. 7 The removal of cadmium from Lake Erie water samples by 2-day-old biofilms of JS4022/p723-6H and JS4022/p4ArsaA(723Δ) GSCCΔ depicted as percentages (**a**) and absolute mass amount (**b**). RsaA-6His represents strain JS4022/p723-6H, and RsaA(723) refers to the control strain JS4022/p4ArsaA(723Δ) GSCCΔ

of the biofilms. Our investigation confirmed that JS4022 recombinant strains form monolayer biofilms on borosilicate surfaces, an observation consistent with an earlier study using *Caulobacter* CB2A/Rif^r [25]. Both JS4022 and CB2A/Rif^r were derived from strain CB2A. The uniform monolayer biofilms formed by CB2A strains differ significantly from the biphasic biofilms observed with strain CB15 [26]. Besides the different genotypes of CB15 and CB2A, different biofilm cultivation conditions may also have contributed to the divergent morphologies exhibited by the two strains. Although glass coverslips were used as supporting matrix in both cases, the CB2A strains were kept in the wells of tissue culture plates with complex medium PYE, whereas the CB15 strain was incubated in a flow chamber with a defined medium containing 2 mM xylose [26].

Our data also shed new light on the development of the monolayer biofilms. A previous study by Smit et al. [25] suggested that, during the initial 9-day of development, the longer the incubation, the denser are the biofilms. Our results demonstrated, however, that the biofilms mature within 2 days; prolonged incubation only causes deterioration to biofilms (Fig. 5). Since the previous study depended on a phase-contrast microscope to visualize biofilms, the resolution of the images are rather limited, compared to what was captured with electron microscopes. The previous study also relied on photo processing software for enumeration of immobilized cells (and determination of the biofilm densities). The accuracy of this quantification approach can be greatly affected by the resolution of images. We instead employed both phase-contrast microscopy and scanning electron microscopy to characterize the biofilms. With phase-contrast microscopy, we obtained similar images (Fig 2a) to the published ones [25]. With scanning electron microscopy, further details of the biofilms revealed themselves (please compare Fig. 2a, b, both at the same level of magnification). In the SEM images, individual cells can be clearly identified, which allows us to precisely determine the density of biofilms through manual enumeration. The finding that *Caulobacter* biofilms mature within 2 days renders further values for their application in industrial settings. Many microbes require extended period of time to grow or to form biofilms. For example, it often takes cyanobacteria 2–3 weeks of illuminated cultivation to reach a desirable amount of biomass prior to the entrapment procedures [28]. Other studies found that *Streptomyces thermocarboxidus* needed 10 days to establish biofilms on glass beads [20], and *Citrobacter* sp. demanded 6 days to form biofilms on ceramic rings [19].

Recent studies have demonstrated that optimal attachment of *Caulobacter* cells to a solid surface coincides with the synthesis of the holdfast, as swarmer cells differentiate to stalked cells [29, 30]. Once attachment is established, it becomes nearly irreversible due to the unusually strong adhesive force of the holdfast. Results presented in this report support these previous observations. For instance, physical abrasion was harsh enough to separate the bacterial body from the stalk but not the stalk itself from the borosilicate surface (Fig. 3). The attachment was not affected by Cd(II) at 1 ppm (Fig. 4) as well as sodium azide at 0.05% (wt/vol) or formaldehyde at 1.7% (wt/vol) [29]. 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 suited for heavy metal remediation tasks. Release of swarmer cells from immobilized stalked cells should not be a concern for the intended applications as discussed previously [10].

The miniature bioreactors we described in this report are composed of a glass panel of 12 mm in diameter housed in a plastic well of 16 mm in diameter and 6 mm in height—the height of the wet line from a liquid of 1.5 ml shaken at 100 rpm. The highest cadmium binding efficiency (76.9% of removal) and absorption capacity (0.5 μg per miniature reactor) were observed with JS4022/p723-6H exposed to 0.4 ppm Cd(II) (Fig. 6). Exposure

to higher concentrations of cadmium only enhanced the nonspecific binding of the control strain. Similar trends have been noticed in our previous study working with free cell suspensions [10]. To improve the percentage of removal as well as the total amount of captured metals, the ratio of biomass to metal must be increased. Because of the low amount of cells involved in each miniature bioreactor, it is difficult to accurately convert the number of cells to cell weight. Since *Caulobacter* cells are individually immobilized through their holdfast, they are expected to function as free cells. Our previous study indicates that JS4022/p723-6H can remove 16 mg g^{-1} of cadmium (cell dry weight) when challenged with 15 ppm of cadmium. This level of capacity is comparable to similar studies. Live cyanobacterium *Oscillatoria* sp. H1 entrapped in calcium alginate beads was shown to retrieve 32.2 mg g^{-1} of cadmium with an input concentration of 200 ppm [28]. Cyanobacterium, *Synechococcus* sp., isolated from wastewater, was immobilized on loofah sponge disks, and the maximum biosorption was found to be close to 10 mg g^{-1} biomass when 10 ppm cadmium was applied [21]. Granular activated carbon loaded with the bacterium *Arthrobacter viscosus* achieved uptakes of 4.2 mg g^{-1} when 11 ppm of cadmium was used [18].

When tested with environmental water samples collected from Lake Erie, the immobilized recombinant strains were also effective in retrieving dissolved cadmium. The amount of cadmium accumulated increased for both JS4022/p723-6H and the control strain if more cadmium was introduced to the system (Fig 7b). This trend is quite different from what we see with the growth medium, where JS4022/p723-6H reached the maximum capacity (0.5 μg) with the lowest concentration used (0.4 ppm), and the adsorption by the control strain remained around 0.1 μg until 1 ppm Cd(II) was introduced (Fig 6b). The reasons contributing to these different adsorption patterns are unclear, since the lake water is a natural complex containing large amount of colloidal substances and many unknown organic and inorganic compounds. Nevertheless, the data prove that it is indeed feasible to employ *Caulobacter* biofilms for real remedial applications.

It is known that *Caulobacter* cells can also bind to plastic surfaces [29]. In this study, cells attached to the wells of a tissue culture plate also contribute to the removal of Cd(II). We have noticed that if biofilm-coated coverslips were transferred to a fresh well for the metal binding assay, the total removal of Cd(II) by JS4022/p723-6H from the growth medium dropped 40~70% depending on the input Cd(II) amount, whereas the nonspecific binding with the control strain remained at the same levels (data not shown). This again emphasizes the importance of increasing immobilized biomass. Optimization of biofilm cultivation conditions and construction of bioreactors with rich internal surface areas should increase metal removal efficiency and are future objectives of this research.

Desorption of heavy metals sequestered by microbes has been achieved with diluted acids [16, 28], acidified buffers [31], or chelators [15, 19]. After each desorption treatment, the volume of metal-containing waste will be significantly reduced, allowing convenient handling thereafter. These acid or chelator treatments are expected to be similarly effective in releasing the cadmium associated to RsaA–6H fusion proteins for following biosorption–desorption cycles. When desirable, removal of *Caulobacter* biofilm may be achieved by application of detergent solutions enriched with a digestive enzyme such as lysozyme, which has been shown to efficiently weaken the adhesion [32]. The complete removal of microbes within the bioreactors maybe required only occasionally, in addition to the routine acid or chelator washes. Investigations are underway to test these methods.

In summary, through integration of two useful techniques, cell surface display and self-immobilization, we demonstrated a novel method to completely separate heavy metal ions from the aqueous phase in a single step. Intact bacterial cells are directly immobilized via

their intrinsic adhesive appendages, an approach offering economic advantages over alternative immobilization technologies. Biofilms can be cultivated directly inside the reactor and can be ready for deployment immediately; no downstream processing is necessary. After each adsorption operation, the bound metal ions may be washed off by small volumes of acids or chelators and be recycled or disposed properly. The biofilms may be regenerated by self-healing and subject to the next round of application. To the best of our knowledge, this is the first example of using self-immobilized *Caulobacter* cells to achieve bioremediation objectives. The bioreactor prototype developed here are also useful for biosensing, bioconversion, biocatalysis, and related applications.

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