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Engineering *Escherichia coli* to bind to cyanobacteria

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We engineered *Escherichia coli* cells to bind to cyanobacteria by heterologously producing and displaying lectins of the target cyanobacteria on their surface. To prove the efficacy of our approach, we tested this design on *Microcystis aeruginosa* with microvirin (Mvn), the lectin endogenously produced by this cyanobacterium. The coding sequence of Mvn was C-terminally fused to the ice nucleation protein NC (INPNC) gene and expressed in *E. coli*. Results showed that *E. coli* cells expressing the INPNC::Mvn fusion protein were able to bind to *M. aeruginosa* and the average number of *E. coli* cells bound to each cyanobacterial cell was enhanced 8-fold. Finally, a computational model was developed to simulate the binding reaction and help reconstruct the binding parameters. To our best knowledge, this is the first report on the binding of two organisms in liquid culture mediated by the surface display of lectins and it may serve as a novel approach to mediate microbial adhesion.

[**Key words:** Surface display; Lectin; Microvirin; Binding; Cyanobacteria; *Escherichia coli*]

Cyanobacteria are autotrophic prokaryotes which have developed a great variety of forms. They are reported to be possibly the most genetically diverse microorganisms and can live in a broad range of environments including marine, fresh water and terrestrial habitats spanning all latitudes (1). Being diverse and ubiquitous, cyanobacteria fulfill vital ecological functions around the world as some of the most important contributors to the world's oxygen and nitrogen budgets (1,2). Apart from maintaining ecological homeostasis, they also represent important natural resources because of their high nutritional value (3,4). However, these vast biological values remain far from being broadly utilized, and large-scale exploitation is impeded by technical limits and high costs (3,5). Aquatic cyanobacteria are also widely known for the visible algal blooms that can form due to their excessive biomass in both freshwater and marine ecosystems, and these bloom events have become increasingly common during the past decades (6,7). In addition to the fact that cyanobacterial blooms can greatly disorder the balance of ecosystems and jeopardize other aquatic organisms, the toxic metabolites found in some species can also pose a direct threat to humans and livestock via food chain links as well as direct water supply (8–10). Thus, finding efficient ways to control cyanobacteria under normal conditions and especially during bloom events represents a crucial and as of yet unresolved problem.

Escherichia coli is the most widely used bacterial host for biotechnological applications due to its amenability to genetic engineering and capability to produce recombinant proteins in high yields. It is therefore expedient to implement applications relating to the control and utilization of cyanobacteria in *E. coli*, which can then be used in aquatic systems. In this report, lectins from the target cyanobacteria were used to bind *E. coli* cells to the cyanobacteria. Lectins are carbohydrate-binding proteins that are highly specific for certain sugar moieties (11). They mediate surface recognition on the molecular level and play various roles in biological processes. Additionally, lectins can also mediate the binding of bacteria and viruses to their respective targets (12). Historically, the uses of lectins were mostly limited to clinical and biochemical applications and they were rarely used in biological engineering (12). However, considering the ubiquitous presence of lectins in nature and their high affinity of binding, we surmised that they have the requisite properties for controlling cyanobacteria.

Herein, we report an attempt to specifically bind *E. coli* cells to cells of the toxin-producing cyanobacterium *Microcystis aeruginosa* as a proof of principle. A lectin produced by *M. aeruginosa*, named microvirin (Mvn), was discovered to bind to the lipopolysaccharide (LPS) fraction of this cyanobacterium by Kehr et al. (13). Although Mvn and other lectins have been reported to be expressed in *E. coli* in previous research (13,14), it has to our knowledge never been displayed outwards and used to mediate interactions between different microorganisms. The ice nucleation protein NC (INPNC) was chosen among the various commonly used cell-surface display systems because it has been reported to be one of the most stable systems for displaying bulky proteins (15,16). In order to maintain the native structure and function of Mvn, a flexible linker was added between Mvn and INPNC (Fig. 1). The expression of the binding module was tested with 2 promoters of different strengths

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(J23116 and J23118). In order to analyze the experimental data using a quantitative approach, we built a computational model to simulate the binding process. By fitting the model to the microscopic data, we were able to obtain the kinetic parameters of the binding reaction, which can be used to facilitate further modelling as well as future practical applications.

MATERIALS AND METHODS

Bacterial strains and media *E. coli* strains DH5α and BL21 were used for cloning and protein expression, respectively, and were grown in Luria–Bertani (LB) medium. Ampicillin (50 μg/mL) or chloramphenicol (17 μg/mL) was added where appropriate.

***Microcystis* strains and cultivation** The strain of *M. aeruginosa* used was FACHB1343, isolated from Lake Tai in 2010 by Yan Xiao and purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, Hubei, China). The cyanobacteria were cultured in BG11 medium in conical flasks and grown at 25°C on a 12:12 h light/dark cycle controlled by an illumination incubator (illuminance: 15,000 Lx, Jiangnan Instrument, Nanjing, China).

Plasmid construction The plasmids used were P1 expressing Mvn-eYFP-His_6, P2 expressing INPNC-rTEV linker-Mvn-mRFP and their respective controls P2_blank: vehicle, P2_delINP: Mvn expressed in the cytoplasm without fusing with INPNC, and P3 expressing INPNC-Mvn + GFP. In the first step, all the required gene sequences with homologous overlaps were generated using PCR with the primers listed in Table S1. Afterwards, the overlapping DNA fragments were purified and selectively assembled using Gibson assembly master mix to produce the three plasmids (17), detailed components of the Gibson assembly master mix are listed in Table S2. During the construction of P1, *mvn* was synthesized and assembled together with *eyfp* with the vector backbone of pET-21a (+). The *inpnc* gene for P2 was provided by the International Genetically Engineered Machine competition (iGEM) organizers and was assembled together with *mvn* and *mrfp* with the vector backbone of pSB1C3. The *gfp* gene for P3 was also provided by the iGEM organizers and was expressed from the pSB1C3 vector. The DNA fragments encoding *inpnc* and *mvn* were assembled on the vector under the control of two independent promoters.

rTEV digestion assay Cells transformed with the plasmid P2 were cultured at 37°C in 5 mL LB medium until the OD_{600} reached 0.8. For each group, a 1 mL aliquot of the culture was transferred into a fresh 1.5 mL EP tube and harvested by centrifugation at 3800 ×*g* for 4 min. The supernatant was discarded and 1480 μL of rTEV (Solarbio, Shanghai, China) buffer and 20 μL of rTEV protease were added into the EP tubes containing the cell pellets. The cells and the reagents were mixed thoroughly and incubated at 30°C for 2 h. The cells were pelleted again by centrifugation at 1900 ×*g* for 6 min, followed by centrifugation at 3800 ×*g* for 4 min. The supernatant was transferred to a fresh EP tube and fluorescence intensity was measured in a flat-bottom 96-well plate with an excitation wavelength of 584 nm and absorbance wavelength of 610 nm, using a microplate reader (Varioskan Flash, Thermo Scientific, Shanghai, China).

Mvn-eYFP purification The C-terminally His_6-tagged MVN-eYFP was expressed from plasmid P1 in *E. coli* BL21 (DE3) cells induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 3800 ×*g* for 15 min, after which 40 mL of lysis buffer and 40 μL of APL (protease inhibitor cocktail containing aprotinin, pepstatin and leupeptin, Sigma–Aldrich, Shanghai, China) were added and the cells lysed by sonication. The crude extracts were subsequently transferred to a 50 mL corning tube and centrifuged at 13,800 ×*g* for 30 min. The resulting supernatant was purified using a needle filter and applied to a Ni^{2+}-nitrilotriacetate column (HisTrap FF crude 5 mL, GE Healthcare, Beijing, China). The column was washed with 500 mL buffer A (40 mM NaH_2PO_4/Na_2HPO_4 pH 7.3, 100 mM NaCl) and eluted with 200 mL buffer B (40 mM Tris–HCl, 10 mM imidazole, pH 8.0, 100 mM NaCl). The eluent was reduced to 1.5 mL using a 30 kDa cutoff centrifugal concentrator tube (Corning, Shanghai, China) and loaded onto a size-exclusion chromatography apparatus (Superdex 200 10/300 GL, GE Healthcare). Finally, 40 mM Tris–HCl pH 8.0, 100 mM NaCl buffer was applied to collect the eluent for further tests. Purified Mvn-eYFP was obtained from the eluent and applied to SDS-PAGE for examination.

Mvn – *M. aeruginosa* binding assay The purified MVN protein was thawed on ice, and an appropriate amount of elution buffer (80 mM Tris–HCl, 4 M imidazole, pH 7.9, 2 M NaCl) was added during the procedure to prevent partial precipitation of the protein. The protein solution was later diluted with elution buffer to a concentration of 0.1 mg/mL. A 1 mL aliquot of the cyanobacterial culture with OD_{670} of the cyanobacteria set to 0.45–0.50 after breaking up the colonies, was transferred to an EP tube. The cyanobacteria were washed with phosphate-buffered saline (PBS) and resuspended in 1 mL aliquots. Mild sonication consisting of five pulses at 20 Hz, 250 W and 10% duty ratio, was used to break the colonies. Subsequently, a 20 μL aliquot was spread on a glass slide and fixed by drying at room temperature (RT, 22°C). The cells were covered with 20 μL of the protein solution described above and incubated at RT for 30 min. Excess solution was carefully removed using filter paper. The glass slide was washed with three times 100 μL of PBS and carefully drawn off with filter paper. Finally, the cells were inspected visually using a fluorescence microscope (Ti-E Microscope, Nikon, Beijing, China).

***E. coli* – *M. aeruginosa* binding assay** *E. coli* were grown in liquid LB medium with chloramphenicol for 12 h in order to obtain actively growing cells. The *E. coli* culture was transferred into 1 mL LB medium with chloramphenicol and incubated until the OD_{600} reached 0.6. At the same time, a 1 mL aliquot of cyanobacterial culture was also transferred to a fresh EP tube, with OD_{670} of the cyanobacteria set to 0.45–0.50 after breaking up the colonies. Both the cyanobacteria and *E. coli* were washed with PBS and resuspended in 1 mL buffer aliquots. Mild sonication consisting of five pulses was used to break the cyanobacterial and *E. coli* colonies and 45 μL of cyanobacterial culture, 50 μL of *E. coli* culture and 5 μL of PBS were mixed in a fresh EP tube. The resulting co-culture was incubated at 30°C for 1 h. After incubation, 20 μL of the co-culture was spread on a glass slide and fixed by drying at RT. Finally, 4 μL of PBS was added onto the dried co-culture and the slide was immediately inspected visually using a fluorescence microscope (Ti-E Microscope, Nikon).

Computational simulation We simulated the binding process between the cyanobacterial targets and engineered *E. coli* via particle simulation. The cyanobacteria and *E. coli* were simplified as rigid spheres, with radii of 2.0 and 0.5 μm, respectively. The movement of *E. coli* and cyanobacterial particles was modelled as a random walk. The model was used to assess the likelihood of particle collision, attachment, and separation of attached particles. We assumed that this binding process resembled microscopic chemical reactions in solution, in which particles move randomly, collide and bind each other. The elementary binding reaction between the microbial particles could be expressed as:

$$AE_x + E \rightleftharpoons AE_{x+1} \quad x = 0, 1, 2, \ldots \qquad (1)$$

where *A* denotes cyanobacteria, *E* denotes *E. coli* and *x* stands for the number of *E. coli* cells bound to a cyanobacterium. The simulation was carried out in a cubic area with a side length of 500 μm for every 0.1 s. Each simulation was run for 100 h to reach the equilibrium. Although primary binding may influence the secondary binding reactions, the actual binding sites that each binding bacterium blocks are

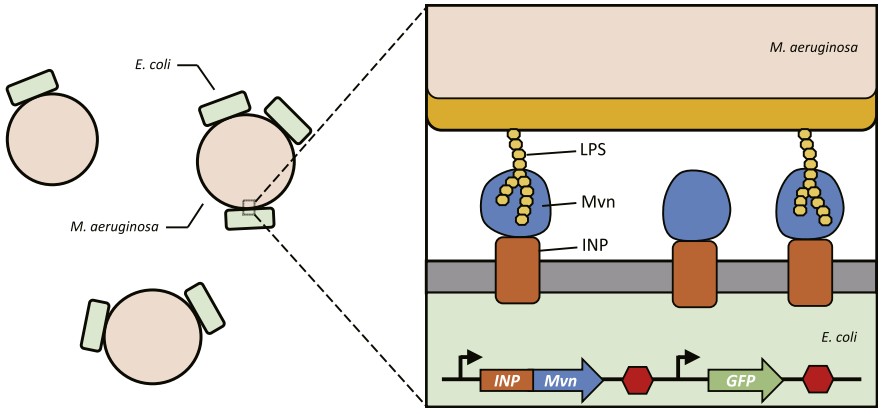

FIG. 1. A schematic representation of the project design. INP::Mvn fusion protein enables *E. coli* to bind to *M. aeruginosa* cells while GFP reports the location of the engineered *E. coli*. Black arrows and red hexagons represent promoters and terminators, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

<1% of the total in our particle simulation model; the proportion of occupied solid angle is $2\pi(1-\cos\theta)/4\pi \approx 0.97\%$, where $\theta = \tan^{-1}(0.5/2+0.5)$. Therefore, we assumed that both the forward and the backward reaction rates, k_+ and k_-, were constant for any value of x ($x < 5$).

According to the law of mass action, the forward reaction rate is proportional to effective collision frequency and to the probability of binding for one collision, i.e., $k_+ \propto P_b$. On the other hand, the backward reaction rate could be expressed as the reciprocal of the average binding time $1/T$, which obeys the law of exponential distribution due to its stochastic nature. Thus, we redefined the binding constant $K = P_b T$, so as to make it sufficient to describe the binding number distribution in steady state. Subsequently, the binding constant was tuned to fit to the experimental data.

Other parameters involved were selected based on judgement of their plausibility and are listed in supplementary Table S3. The concentrations of the modelled cyanobacteria and *E. coli* were chosen based on the experimental data.

RESULTS

Expression and purification of Mvn-eYFP eYFP was expressed as a fusion to the C-terminus of Mvn in order to visualize the binding process. A purified protein fraction was applied to SDS-PAGE for examination and the result is shown in Fig. 2A. A conspicuous band at the position corresponding to a molecular weight of 40 kDa indicated the presence of the Mvn-eYFP fusion protein, based on the molecular weights of Mvn and eYFP being 12 and 28 kDa, respectively.

Mvn-eYFP binding assay To investigate the binding properties of the purified Mvn produced by *E. coli*, we carried out a binding assay with *M. aeruginosa* cells. The Mvn protein was genetically labeled with an eYFP marker to help observe it using fluorescence microscopy. Purified Mvn-eYFP fusion protein was applied to the cyanobacterial cultures after mild sonication and observed immediately afterwards. Fig. 2B shows representative images of cyanobacteria emitting typical red fluorescence of chlorophyll A, Mvn-eYFP emitting yellow fluorescence and the merged picture comprising both channels. It can be seen that all *M. aeruginosa* cells in the field were covered with fluorescent MVN-eYFP fusion protein. Taken together with the fact that *M. aeruginosa* cell cultures alone showed no yellow fluorescence (data not shown), this result demonstrates the high affinity of Mvn towards *M. aeruginosa* cells.

Characterization of the INPNC surface display system In order to characterize the efficiency and robustness of the INPNC protein display system, we transformed *E. coli* cells with plasmid P2 to express the INPNC-Mvn-mRFP fusion protein, which comprises a digestible rTEV linker between INPNC and MVN. The rationale was that rTEV protease added to the solution would digest the linker and fluorescence intensity of the supernatant could therefore be used as a proxy for the amount of Mvn-mRFP that was actually displayed on the cells' surface and could subsequently be liberated into the supernatant by the extracellular rTEV protease.

It is clearly shown that the fluorescence intensity of the experimental group is higher than that of the control groups by a large margin (Fig. 3). This result indicates that the Mvn-mRFP fusion protein is displayed at the surface with high efficiency by the INPNC surface display system, as well as that mRFP can be maintained in a functional form.

***E. coli* binding assay** In a more application-oriented proof-of-principle, we experimented with engineered *E. coli* and *M. aeruginosa* cells to directly test our binding system. *E. coli* cells expressing INPNC-Mvn fusion protein on the cell surface and GFP in the cell matrix were co-cultured with *M. aeruginosa* cells for

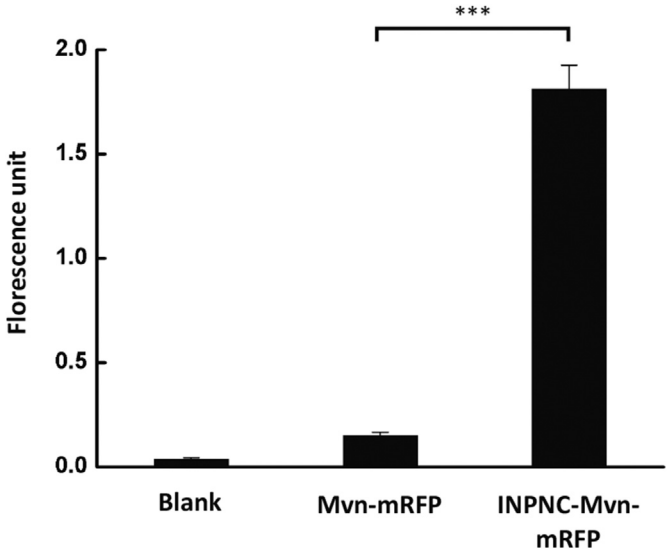

FIG. 3. Fluorescence intensities of released Mvn-mRFP measured by a microplate reader. ***$p < 0.001$.

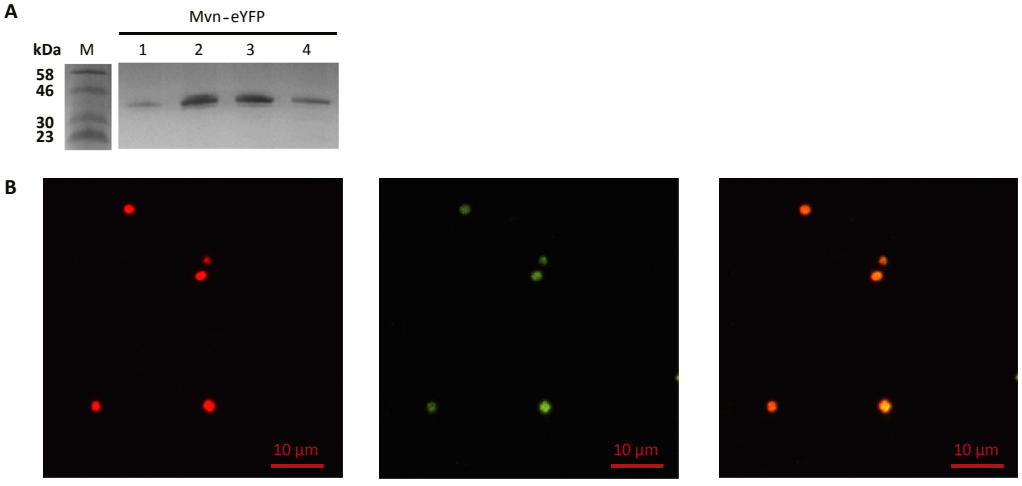

FIG. 2. (A) Purification of MVN-eYFP fusion protein. The band on the SDS-PAGE indicates the purified Mvn-eYFP at the position corresponding to a protein size of 40 kDa. Lane M denotes the marker, lanes 1, 2, 3, 4 stand for 4 consecutively collected eluent fractions from the same experiment. The original gel image is available as Fig. S1. (B) Binding of Mvn-eYFP to *M. aeruginosa* cells. Left: intrinsic red fluorescence of chlorophyll A in *M. aeruginosa* cells; middle: Mvn-eYFP fluorescence; right: merged image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 h and subsequently spread on slides for microscopic observation. Twenty views were taken for each slide of which a representative picture can be seen in Fig. 4A. It is clearly visible that binding was achieved by displaying Mvn on the cell surface.

Consequently, we used microscopic counting to quantify the results and we observed the effect of promoter strength on binding when two different promoters were used (Fig. 4B). The average numbers of *E. coli* cells bound per cyanobacterium were 1.37 (n = 204), 1.17 (n = 192) and 0.17 (n = 187) for Mvn expressed under the weak promoter J23116, strong promoter J23118 and negative control, respectively. Thus, Mvn-mediated binding was clearly demonstrated and 8.1 fold higher average binding numbers could be achieved. Since the binding process can be viewed as a Poisson process, we used the Chi-square test to examine whether the data indeed obeyed a Poisson distribution. However, the control group and the weak promoter group obeyed ($X^2 = 1.98$ and 7.22, $p < 0.05$) while the strong promoter group did not ($X^2 = 31.91$, $p > 0.05$).

Computational model In order to interpret the experimental data in a more quantitative manner, we developed a computational model to simulate the binding reaction. As displaying Mvn outwards may influence both the possibility of binding (P_b) and the average binding time (T), we assumed that as long as the constant $K = P_bT$ remains the same, changes in specific values of P_b and T would not affect the relative binding distribution of the microbes. To prove this assumption, we set $K = 15.8$ as a constant and simulated the binding reaction for different cases where $P_b = 0.25$, 0.50, 0.75 and 1.00, respectively. We calculated the coefficient of determination R^2 for each case and the results showed that each separate case shared the same form of distribution as when the 4 cases were calculated as a whole (R^2 = 0.9998, 0.9993, 0.991, 0.9990). Therefore, it could be demonstrated as reasonable to set only one variable K during the simulation of the binding reactions.

Afterwards, we successfully fit the computed distribution to the experimental results for both the experimental groups and the control group (Fig. 5). The binding equilibrium constants K for the weak promoter, strong promoter and control are 18.0, 12.8 and 1.8, respectively. The coefficients of determination R^2 for the corresponding fittings are 0.9055, 0.8137 and 0.9996, indicating the high reliability of the parameters as determined. Taken together, we developed a model to faithfully simulate the experimental data and used the model to determine the kinetic constants for different binding reactions.

DISCUSSION

The primary goal of our research is to develop an approach that is both practical and efficient, to specifically bind *E. coli* cells to cyanobacteria. To achieve this, we selected lectins produced by the cyanobacteria themselves as the binding module for the *E. coli* cells. *M. aeruginosa* was chosen to prove the efficacy in our preliminary tests because it is one of the most common species of cyanobacteria that can form toxic cyanobacterial blooms in eutrophic water areas (7). We specifically chose *M. aeruginosa* FACHB1343 due to its prevalence in water blooms.

Microvirin is a lectin discovered in *M. aeruginosa* PCC7806 that is involved in cell–cell attachment (13). It is a lectin consisting of 108 amino acid residues; it has the highest affinity to α(1 → 2) linked mannose residues, as reported in an earlier carbohydrate microarray study (13). Several studies have characterized the properties of Mvn, demonstrating that it is involved in cell–cell recognition and cell–cell attachment of *Microcystis* (13) and the binding target of Mvn was identified in the LPS fraction of the cyanobacterial cells (13,18). We expressed Mvn heterologously in *E. coli* cells and characterized the specificity of purified Mvn binding to *Microcystis*. Our results suggested that Mvn had a high affinity towards Microcystis cells and that it is not restrained to one single strain. To confer this binding ability onto *E. coli* cells, Mvn had to be displayed on the cell surface. Several studies reported the utility of INPNC in bacterial surface display systems, however, previous applications such as for example biocatalysis and epitope mapping, were mostly restricted to binding of smaller molecules (15). In our case, the display system had to be stable enough to mediate the binding of two live organisms successfully and without tensile

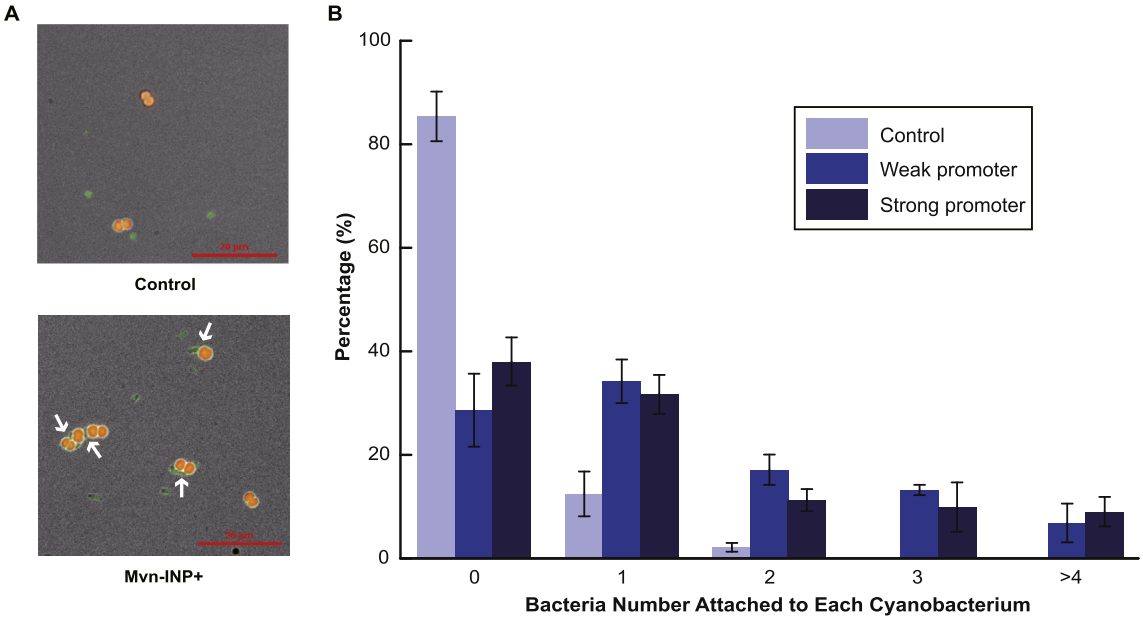

FIG. 4. Direct binding of engineered *E. coli* to cyanobacteria. (A) Representative images of *E. coli* and *M. aeruginosa* co-cultures viewed under a fluorescence microscope. The red ovals are *M. aeruginosa* cells while green rods are *E. coli*. White arrows indicate binding of engineered *E. coli* to the cyanobacterial cells. (B) Quantified data of binding of engineered *E. coli* to *M. aeruginosa*. INPNC-Mvn was absent (control, sky blue bars), expressed under a weak promoter (blue bars), and under a strong promoter (indigo bars), respectively. There is a significant difference between the experiment groups and the control group ($p < 0.05$). Error bars represent S.D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

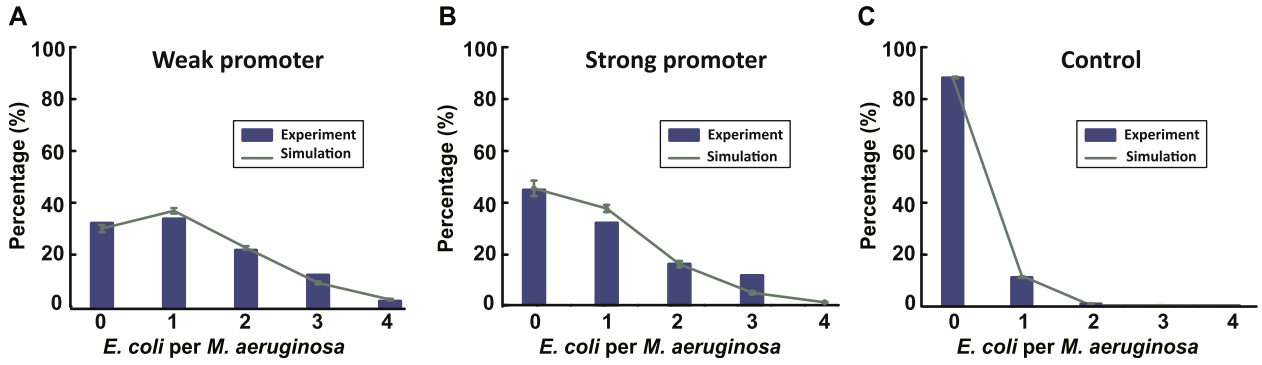

FIG. 5. Computed distributions (green lines) and experimental data (blue columns) of average numbers of *E. coli* cells bound per cyanobacterium for the weak promoter (A), strong promoter (B) and control groups (C). Error bars of fitting distributions are derived from multiple simulations due to the stochastic nature of the binding reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

failure. In addition, the system had to be produced in great amounts to create the necessary binding force. Our rTEV digestion assay demonstrated that INPNC could be produced on the surface in large quantity and that it could maintain the original function of the displayed protein.

The result of the *in vivo* binding experiment was convincing and the average binding number was 8.1-fold higher in the engineered cells than in the control. Additionally, the binding effect should be better than what the average binding number indicates, as the binding of 3 or more cyanobacteria was only observed in the experimental groups. This phenomenon may lead to the use of *E. coli* to crosslink a number of cyanobacteria for future applications. Interestingly, the binding effect was better when Mvn was expressed under modest promoter strength. This result may indicate emerging interactions between *E. coli* cells instead of interactions with cyanobacteria when Mvn is expressed in an exceedingly large amount, considering the inclination for Mvn to dimerize (13). Therefore, an optimal strength of surface display to mediate binding of microbes may exist for different lectin-target pairs.

Engineering *E. coli* to bind to cyanobacteria can have various potential applications in real life. In a previous report by Dziga et al. (19), the successful heterologous expression of microcystinase in *E. coli* shows the potential of engineered *E. coli* to eliminate toxic microcystins. We therefore conjecture that binding toxin-eliminating *E. coli* cells to the cyanobacteria would shorten the reaction range and hence enhance the efficiency of toxin elimination. The detoxification process itself may have greater importance in dealing with algal blooms in water bodies, as it has been reported that microcystins are involved in cyanobacterial colony formation (20). Thus, cutting down the amount of microcystins may help with breaking apart the colonies formed, which may be the crucial point for eliminating algal blooms. However, this application may be relatively limited in scope since Mvn only specifically binds to a few strains of *Microcystis*, and a cocktail treatment may be needed to widen the application scope.

Applications are not limited to detoxification, since cyanobacteria also represent vital natural and ecological resources. Mvn-displaying *E. coli* may help serve as the scaffold to crosslink different cyanobacteria, and make their biomass more accessible to utilization. With further development of microbe-containing devices, the enrichment of cyanobacteria and inducing them to form colonies may be of importance for the recovery of biofuels. Moreover, from a broader point of view, this is to our best knowledge the first reported case of displaying a lectin to achieve binding of two live organisms rather than using antibodies. Since lectins are ubiquitously expressed and easily obtained from plants, this may open up new pathways to designing microbes for various applications, including anti-tumour, anti-HIV, anti-insect and anti-fungal activities (21). Although it is potentially very promising to harness engineered *E. coli* for these diverse applications, there are challenges surrounding the introduction of genetically engineered microbes into the environment. These include off-target effects and the uncontrolled growth of the genetically modified microbes in the environment, which might be addressed by refined microbe-delivering device and programmed cell-elimination circuits (22). Future developments will need to carefully balance these considerations with the advantages that can be obtained, and we hope that lectins will significantly strengthen the toolbox for targeting of cyanobacteria, as well as other target organisms from now on.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbiosc.2016.09.010.

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