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Engineering *Escherichia coli* to bind to cyanobacteria

Zijian Zhang,[1,‡] Liuyi Meng,[1,‡] Congjian Ni,[1] Lanqiu Yao,[1] Fengyu Zhang,[1] Yuji Jin,[1] Xuelang Mu,[1] Shiyu Zhu,[1] Xiaoyu Lu,[1] Shiyu Liu,[1] Congyu Yu,[1] Chenggong Wang,[1] Pu Zheng,[1] Jie Wu,[1] Li Kang,[1,3] Haoqian M. Zhang,[2,3,*] and Qi Ouyang[2,3]

Peking University Team for the International Genetically Engineered Machine Competition (iGEM), Peking University, Beijing 100871, China,[1] Center for Quantitative Biology, Peking University, Beijing 100871, China,[2] and Peking-Tsinghua Joint Center for Life Sciences, Peking University, Beijing 100871, China[3]

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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 fulfil 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 homoeostasis, 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

* Corresponding author at: Peking-Tsinghua Joint Center for Life Sciences, Peking University, Beijing 100871, China. Tel./fax: +86 010 62756943.
E-mail address: myelinzhang@pku.edu.cn (H.M. Zhang).
‡ The first two authors contributed equally to this work.

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(J23116 and J23118). In order to analyse 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

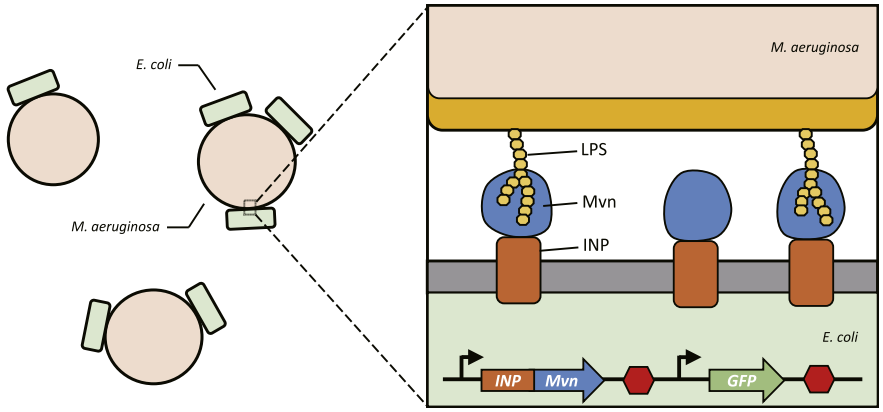

FIG. 1. A schematic representation of the project design. INP::Mvn fusion protein enables *E. coli* to bind to *M. aeruginosa* cells while GFP reports the location of the engineered *E. coli*. Black arrows and red hexagons represent promoters and terminators, respectively. (For interpretation of the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

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