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Optogenetics Manipulation Enables Prevention of Biofilm Formation of Engineered *Pseudomonas aeruginosa* on Surfaces

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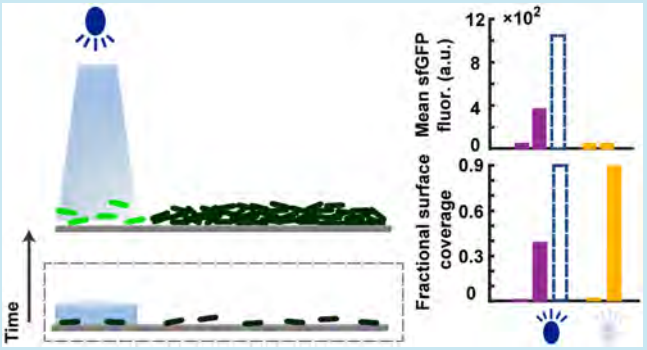
Supporting Information

ABSTRACT: Synthetic biologists have attempted to solve real-world problems, such as those of bacterial biofilms, that are involved in the pathogenesis of many clinical infections and difficult to eliminate. To address this, we employed a blue light responding system and integrated it into the chromosomes of *Pseudomonas aeruginosa*. With making rational adaptions and improvements of the light-activated system, we provided a robust and convenient means to spatiotemporally control gene expression and manipulate biological processes with minimal perturbation in *P. aeruginosa*. It increased the light-induced gene expression up to 20-fold. Moreover, we deliberately introduced a functional protein gene *PA2133* containing an EAL domain to degrade c-di-GMP into the modified system, and showed that the optimally engineered optogenetic tool inhibited the formation of *P. aeruginosa* biofilms through the induction of blue light, resulting in much sparser and thinner biofilms. Our approach establishes a methodology for leveraging the tools of synthetic biology to guide biofilm formation and engineer biofilm patterns with unprecedented spatiotemporal resolution. Furthermore, the findings suggest that the synthetic optogenetic system may provide a promising strategy that could be applied to control and fight biofilms.



KEYWORDS: *optogenetic, biofilms, c-di-GMP, Pseudomonas aeruginosa, light sensing*

Bacterial cells are often present in biofilms,[1] which are associated with surfaces, or exist as floating cell aggregates embedded in a hydrated extracellular polymeric substances. Biofilms establish an essential and protective lifestyle for bacteria in various natural and man-made environments.[2] Bacterial biofilms have been implicated as a cause of most persistent infection and contamination in medical,[3] industrial[4] and food processing settings[5] because of their inherent resistance to antimicrobial agents and host defenses. Thus, the development of useful strategies that inhibit biofilm formation or promote biofilm detachment has long been a concern in biomedical research and environmental science.

Generally, biofilm dispersal appears to be a complex and dynamic process that involves numerous environmental signals, signal transduction pathways, and effectors.[6] Several physiological processes and regulatory mechanisms are involved in biofilm dispersal, including the intracellular c-di-GMP levels. In prokaryotes, c-di-GMP[7] stimulates the biosynthesis of adhesions and exopolysaccharide matrix substances in biofilms and inhibits various forms of motility: it controls switching between the motile planktonic and sedentary biofilm-associated lifestyles of bacteria.[8] The mass sequencing of genomes has revealed the highly abundant proteins having domain of GGDEF or EAL. Studies have showed that the GGEDF and EAL domain[11] are generally involved in the turnover of c-di-GMP levels whereby the GGDEF domain stimulates c-di-GMP production and the EAL domain stimulates c-di-GMP degradation.

Furthermore, c-di-GMP acts as a second intracellular messenger in *Pseudomonas aeruginosa*, an opportunistic pathogen that causes biofilm-associated chronic diseases in humans and is a model organism for studying c-di-GMP signaling and biofilms. In *P. aeruginosa,* decreased c-di-GMP levels not only lead to increased motility through relieving the repression of binding effector components, such as PilZ[9] and FleQ,[10,11] but also enable transcriptional repression at the pel, psl, and cdrA promoters.[11−14] Therefore, when the levels of intracellular c-di-GMP are sufficiently low, the cells may disperse to the planktonic medium with more active motility and lower secretion of polysaccharides, and consequently lead to biofilm inhibition.

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Because biofilm dispersal is an essentially genetic process, it could be manipulated similarly to other genetic systems by using the tools of synthetic biology.[15−18] Synthetic biology[19] has enabled the development of many engineered genetic modules, including toggle switches,[20] cascades,[21] oscillators,[22] and autoregulatory circuits.[23] Among these genetic modules, one notable achievement was an engineered light-sensing circuit in *E. coli*,[24] which allowed bacteria to switch between different states using red light. In addition to controlling diverse adaptive responses, light sensors, as light regulated actuators, provide the foundation for optogenetics. In fact, organisms in nature are able to detect light and regulate their physiology and performance in response because of biological photoreceptors.[25] Novel photoreceptors[26] have been engineered that establish control through light over manifold biological processes previously inaccessible to optogenetic intervention. In contrast to chemical molecules that regulate gene expression systems, such as IPTG or tetracycline, light is an ideal inducer because it is easy to obtain, highly tunable, noninvasive, has low toxicity, and, most critically, has high spatiotemporal resolution.[27−30]

However, to date, spatiotemporal manipulation of biofilms remains far from complete. In this study, we employed an optogenetic tool, the plasmid pDawn,[31] which exploits a blue light photoreceptor to confer light-induced gene expression in *P. aeruginosa*. By using genetic circuit design and optimization, we initially optimized the optogenetic systems in *P. aeruginosa,* and then further manipulated biological processes in the biofilm development using light with unprecedented spatial and temporal resolution. We inspected the performance of the optogenetic system by examining the fluorescence of sfGFP. Subsequently, we introduced a functional protein gene *PA2133*,[32] which contains an EAL domain to degrade c-di-GMP, showing that its expression effectively inhibits biofilms through the induction of blue light.

RESULTS

Construction of the Optogenetic System in *P. aeruginosa*. We adopted the chromosome gene integration system mini-Tn7,[33] which is more stable than the plasmid cloning system in bacteria, to facilitate integration of the synthetic blue light-sensing system pDawn into *P. aeruginosa*. Furthermore, to prevent the undesired read-through from chromosomal promoters into cloned sequences, we made modifications to the delivery vector pUC18T-mini-Tn7T-Gm (detailed description in the Methods). As shown in Figure 1, all the functional components of pDawn were assembled into the reconstructed vector pTn7-L(T0T1), resulting in a pDawn-Tn7 system. The light-sensing element was based on the YF1/FixJ system. YF1[34] is a blue light-sensitive histidine kinase that employs a light-oxygen-voltage blue light photosensor domain.[35] In the absence of blue light, YF1 phosphorylates its cognate response regulator, FixJ. FixJ drives the expression of the λ phage repressor cI from the pFixK2 promoter, which, in reverse, restrains expression of the strong λ promoter pR. Upon light absorption, the net kinase activity of YF1 and consequently gene expression (cI) is greatly reduced, which relieves the transcriptional repression at the promotor pR. Target genes can be introduced in a single step through a multiple cloning sites (MCS) downstream of the pR promoter of pDawn-Tn7, and light increases their expression.

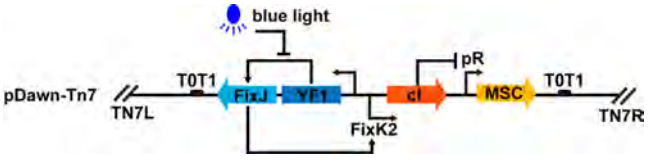

Figure 1. Plasmid pDawn-Tn7 system for being integrated into chromosome of *P. aeruginosa* for light-activated gene expression.

Performance of Light-Activated System in *P. aeruginosa*. We first tested our pDawn-Tn7 system through cloning the green fluorescent protein sfGFP as a reporter into the MCS to yield psfGFP-pDawn-Tn7, in which the expression could be measured through green fluorescence. *P. aeruginosa* carrying psfGFP-pDawn-Tn7 were grown for 12 h at 37 °C in the dark or under constant blue light of 100 nW cm^{-2} to determine the effect of light on gene expression; Wild type PAO1 was cultured under the same conditions for the control group. As shown in Figure 2a, the mean fluorescence intensity of PAO1 psfGFP-pDawn-Tn7 in darkness was as low as that of the wild type, which demonstrates the low background activity of the pDawn-Tn7 system in *P. aeruginosa*. Compared with dark-treated bacteria, an approximately 20-fold increase in sfGFP expression from PAO1 psfGFP-pDawn-Tn7 under blue light illumination suggested that our modified light-sensing system is available in *P. aeruginosa*. Illumination with conventional white light yielded similar effects on gene expression, as the mean sfGFP fluorescence of PAO1 psfGFP-pDawn-Tn7 was approximately 16-fold higher than in the darkness. Therefore, dedicated lamps or lasers could be replaced with a conventional white light source. We routinely confirmed that the growth of the PAO1 psfGFP-pDawn-Tn7 was not affected by the insertion of the pDawn-Tn7 system or light, because the strains under light or in the dark produced a nearly identical pattern of growth compared with the PAO1 (Figure 2b). The insertion of an empty Tn7-L(T0T1) vector also had no effect on growth or biofilm formation (Supporting Information, Figure S1).

We investigated the time course of light-induced and dark-relaxed sfGFP expressions. First, two cultures were grown at 37 °C under constant blue light of approximately 100 nW cm^{-2} or in the dark to stationary phase (OD600 of approximately 2.0) for approximately 24 h. After measuring the initial sfGFP fluorescence intensities, the two cell cultures were diluted to an OD600 of approximately 0.2 and incubated under inverse conditions. Specifically, the dark-cultured cells were exchanged for incubation with blue light and vice versa. Fluorescence intensities were measured every hour. After each measuring, the cell cultures were rediluted to an OD600 of approximately 0.2 to maintain the bacteria at the logarithmic phase. We found that bacteria transferred from an illumination-saturation state to darkness exhibited a slow decay in the amount of sfGFP. The fluorescence intensity decreased to 20% of its initial intensity approximately 3 h after the removal of light, and dropped by 95% after 9 h to reach its minimum (Figure 2c). The time course of light activated fluorescence intensity exhibited an approximately15-fold increase in sfGFP expression after 6 h of blue light illumination compared with original dark-treated bacteria (Figure 2c and Figure 2d). After 9 h, the mean sfGFP fluorescence intensity was increased by 2000% compared with its initial value and had reached a plateau (Figure 2c). In Figure 2b, cells were allowed to grown under light without repeated dilution. the fluorescence had a steep rise during time-period of 8−10 h (Figure 2b, gray bar part) compared with that of the

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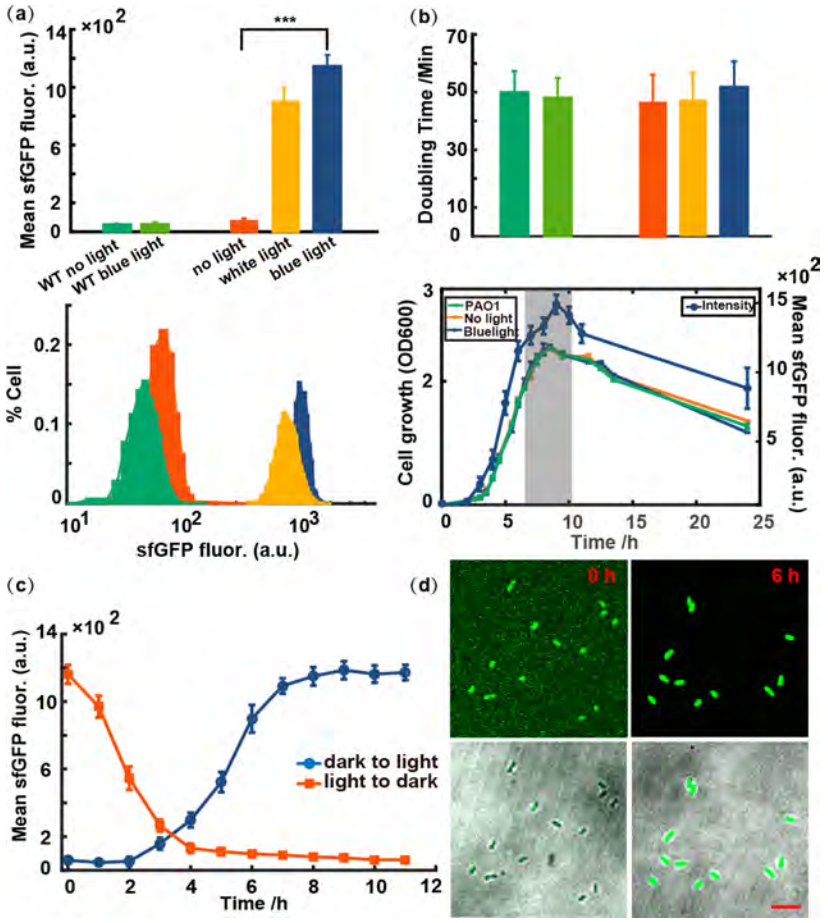

Figure 2. (a) Mean sfGFP fluorescence of PAO1 psfGFP-pDawn-Tn7 grown at 37 °C illuminated with no light, white light and blue light for 12 h (upper) and the proportion (%) of different intensity of sfGFP fluorescence cells (lower). Wlid type PAO1 was as the control group. (b) Doubling time of wild type and PAO1 psfGFP-pDawn-Tn7 illuminated with no light and blue light (upper) and their time course of cell growth (lower). Intensity of sfGFP fluorescence of illumination bacteria was measured along with the growth (lower; blue line). (c) Intensity of sfGFP fluorescence measured at indicated times after incubation with illumination under continuous 100 nW cm^{-2} blue light and then by 12 h darkness, or after incubation in darkness and then illumination under continuous 100 nW cm^{-2} blue light. (d) sfGFP fluorescence microscopy images characterized in c at 0 h (left) and 6 h (right) (upper) and corresponding overlay of bright-field and sfGFP fluorescence microscopy images (lower), Scale bar, 5 μm. In a, b, c, error bars represent means ± s.d (a, b, c n = 3), NS = not significant, ***P < 0.001 (Student's t-test).

cells being cultured with continuous dilution (Figure 2c). It may because that the undiluted cells are suffering at the stationary phase along with the decrease in protein removal caused by cell division decline. Furthermore, when bacteria were regularly in the decline phase, protein degradation accelerated and the fluorescence decreased (Figure 2b).

Characterization of the Light-Induced System at the Single-Cell Level. Given the nature of heterogeneity, the aforementioned measurements were represented using the ensemble average of fluorescence, which is unable to identify whether bacterial populations respond homogeneously to illumination or not. Therefore, we adopted spinning-disk confocal microscopy to characterize the performance of bacteria at the single-cell level. We grew bacteria harboring psfGFP-pDawn-Tn7 on an agarose slab and used a confocal microscope to expose a limited area (see Methods). Every hour for 12 h, we measured the fluorescence of sfGFP in each cell of a growing microcolony within the illumination area and its surroundings (7 × 7 fields). Consistent with the ensemble measurements, the mean fluorescence of psfGFP-pDawn-Tn7 under blue light was increased with time (Figure 3 a, 3b, 3c).Within 3 h, sfGFP expression could be detected within the illuminated area. Conversely, in noninducing dark regions, the fluorescence intensity of bacteria barely increased. We reconstructed the genealogical trees of one microcolony using MATLAB codes developed by our lab[36,37] (Figure 3b). Along each lineage, the fluorescence per pixel in cells increased slowly over 8 h (by approximately 50%). During the subsequent 4 h, fluorescence intensity rapidly increased by 50% in one generation. The mean sfGFP fluorescence per cell fluctuated strongly at the same time point (by approximately ±20%), especially after 3 h. Notably, the increase of fluorescence with lineage is nonlinear (Figure 3b and 3c). In the study, light acted as an inducer to activate the reporter expression from gene *sfGFP* which is under the control of the promoter of pR and the kinetics could be modeled by a single exponential approach to steady state.[38] Specifically, the production of protein sfGFP is not constant over time and the fluorescence varied nonlinearly. Based on these results, we present the first attempt, to the best of our knowledge, to analyze the dynamics of optogenetic system in individual cells using genealogical trees, and demonstrate that heterogeneity is indeed natural, including in optogenetic research.

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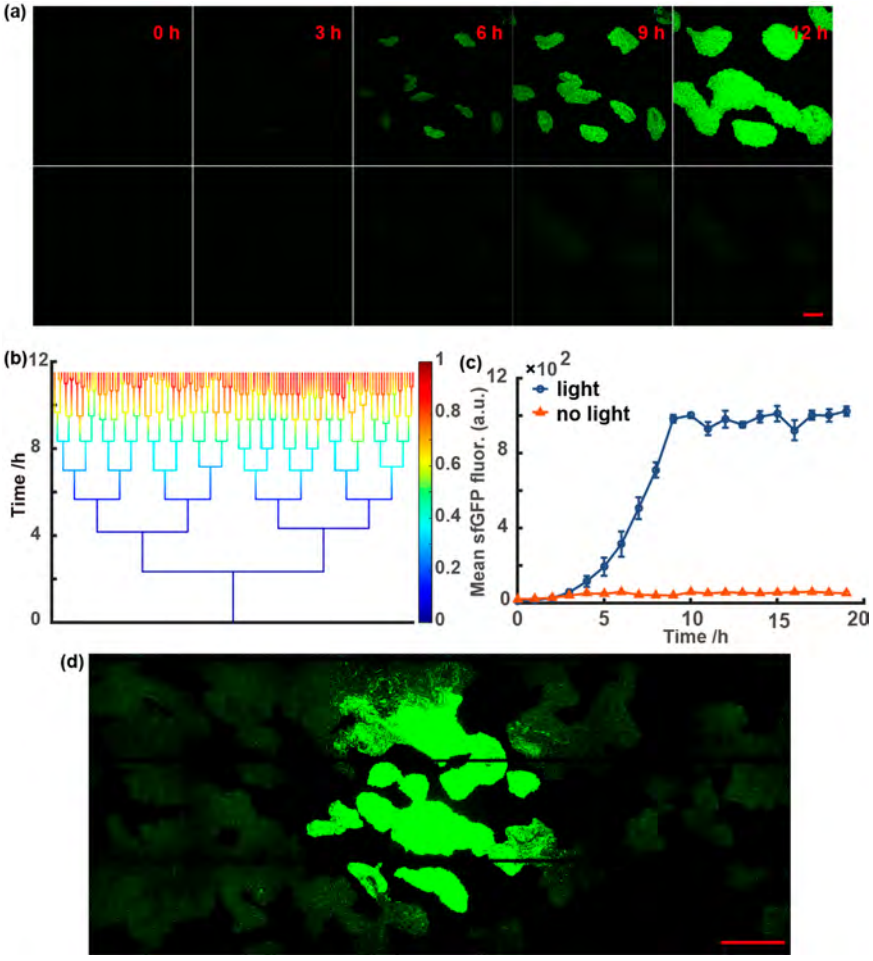

Figure 3. (a) Time-lapse fluorescence image of growing microcolonies with illumination (upper) and without light (lower). Scale bar, 10 μm. (b) Genealogical tree of the colony. The level of sfGFP fluorescence intensity in each cell is color-coded along each lineage (for clarity, only a subset of the tree is displayed). (c) Mean sfGFP fluorescence detected in dark-treated and illuminated bacteria shown in a. Error bars represent means ± s. d with n = 3 biological replicates. (d) 7 × 3 fields sfGFP fluorescence microscopy montage image shows spatial control over blue-light-induced gene expression from PAO1 psfGFP-pDawn-Tn7. The central zone is the illuminated area during a 12 h incubation period at 30 ± 1 °C. Each of the visible microcolonies derives from single cells. Scale bar, 50 μm.

One specific advantage of light-activated systems over other induced systems, such as chemically induced systems, is the superior degree of spatial control.[24,39] We observed that after 10 h, sfGFP expression within the illuminated area was approximately 20-fold higher than that in the surrounding area (Figure 3d). Thus, the system provides a powerful and convenient method to spatially control gene expression in *P. aeruginosa*.

Disassembly of Biofilms through Blue Light Induction. Because the modified optogenetic system worked well after insertion into the chromosomes of *P. aeruginosa*, realizing stringent control by light of the expression of arbitrary target genes through light might be feasible, even for genome-residing genes. Subsequently, we introduced *PA2133* into the system, a gene on the *P. aeruginosa* genome that expresses protein function as phosphodiesterase to degrade the c-di-GMP signal.[13,32]

We accomplished control of the biofilms of PAO1 with blue light through the optimized optogenetic system, which was engineered through exploiting and reassembling different standardized biological parts using the techniques of synthetic biology (see Methods). Finally, the resulting strain, PAO1 p(GTG)PA2133(Mtag)-B0033sfGFP-pDawn-Tn7 (P for short; Figure 4), was constructed. The strain enabled us to simultaneously supervise the degree of response in bacterial cells through the expression of fluorescence.

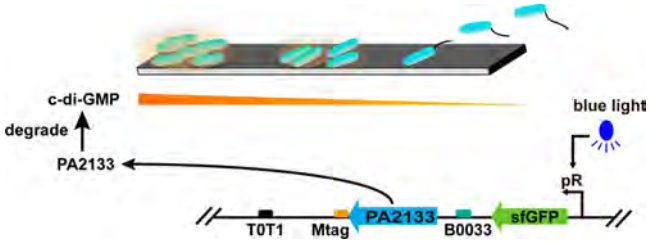

Figure 4. Schematic illustration for blue light-induced expression of *PA2133* to degrade c-di-GMP, consequently causes dispersion of biofilms.

To exclude effects on the light-on system after the introduction of PA2133, we tested the light-dependent impact on the expression level of the fluorescence reporter in strain P. The cells were illuminated with blue light at 37 °C for 12 h and then used to measure the sfGFP expression. Strain P showed a marked light-induced reporter gene expression, which was

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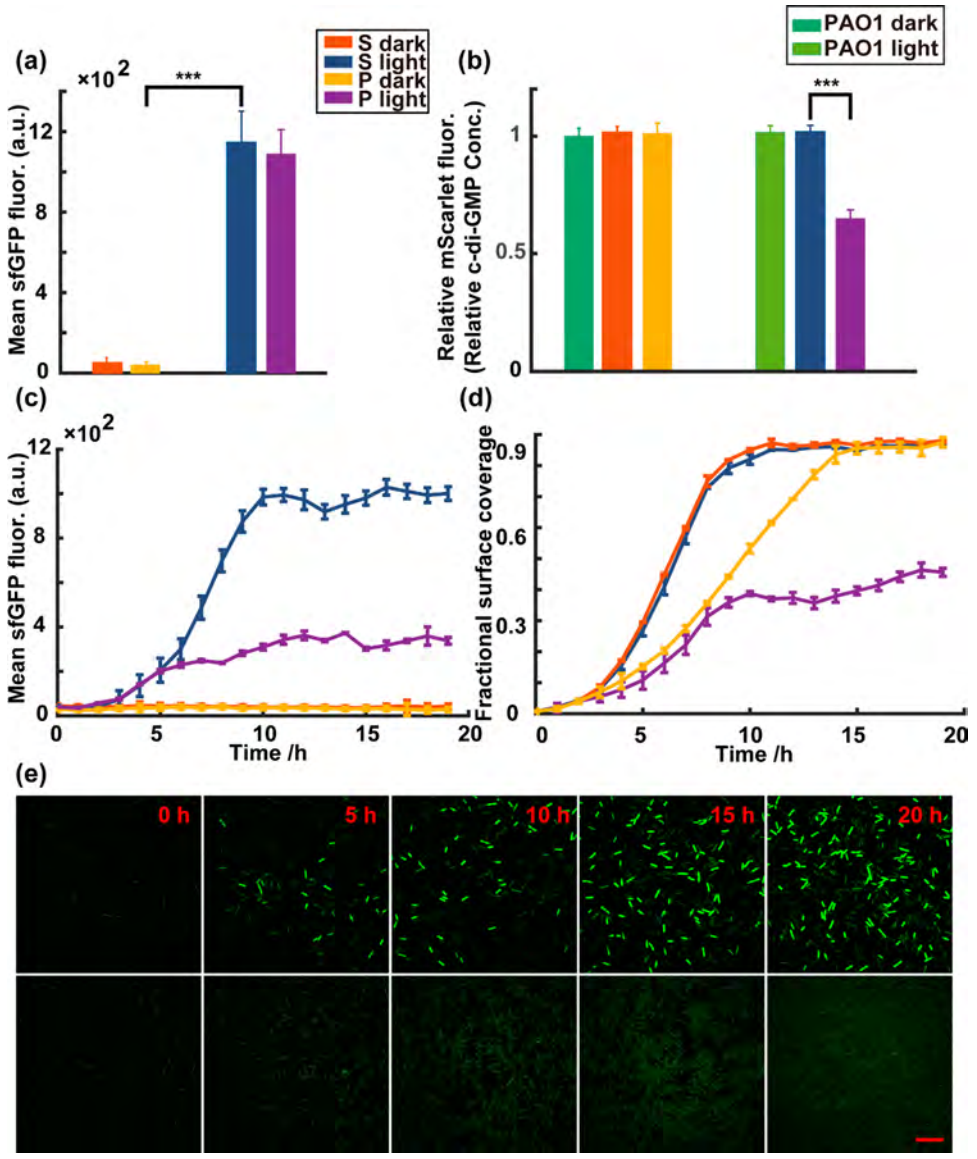

Figure 5. (a) Means sfGFP fluorescence of bacteria grown in shaker for 12 h. Here, S is short for PAO1 psfGFP-pDawn-Tn7, P is short for PAO1 p(GTG)PA2133(Mtag)-B0033-sfGFP-pDawn-Tn7. Orange denotes S incubated in dark, while S grown under illumination is marked as blue. Yellow denotes P incubated in dark, while P grown under illumination is marked as purple. It is described as the same pattern in b, c and d. (b) Expression of relative mScarletI fluorescence of PAO1, strain S, strain P carrying c-di-GMP reporter cultures at 37 °C for 12 h in the dark or under light. (c) Time course of intensity of sfGFP of bacteria grown in flow cell. Mean sfGFP intensity of P in illumination area could not catch the maximum shown in b, but S could. It was because that sfGFP and PA2133 expressed at the same time, once the protein amount reached certain point, the bacteria would detach from surface. (d) Time course of fractional surface coverage of bacteria grown in flow cell. It is quantitative characteristics for PA2133 expression. (e) Time-lapse fluorescence image of growing bacteria in flow cell with illumination (upper) and without light (lower) in the same experiment. Scale bar, 10 μm. In a, b, error bars represent means ± s. d (n = 3), ***P < 0.001 (Student's t-test). In c, d, error bars represent means ± s. d, each data point represents the mean values from n = 5 fields.

comparable to that of the mutant of PAO1 psfGFP-pDawn-Tn7 (S for short; Figure 5a). To verify that PA2133 could indeed degrade c-di-GMP, we constructed a c-di-GMP levels reporter by fusing the c-di-GMP-responsive *cdrA* promoter[40] to genes encoding the red fluorescent protein mScarletI[41] (Supporting Information Figure S3) and introduced it into strain P, strain S and wild type PAO1. After culturing the strains under blue light or in darkness for 20 h, we found that the level of fluorescence of strain P with the reporter in darkness was roughly equal to that for strain S and wild type PAO1 either in darkness or under illumination. However, strain P with the reporter under illumination exhibited a substantially lower level of fluorescence than in darkness (Figure 5b). This indicated that the intracellular c-di-GMP levels of strain P decreased upon blue light illumination.

To observe the formation or degradation of biofilms, we incubated bacteria within a flow cell and exposed a limited area under microscopy. Strain S incubated under the same condition as the control group. We investigated the time courses of light-induced expression of sfGFP. Similarly, within 3 h, sfGFP expression of strain P and strain S were detected readily within the illuminated area. However, at 20 h after light absorption, sfGFP expression of remaining bacteria of strain P (Figure 5c) was only approximately 25% of its attainable value (Figure 5a and 5c), but for strain S within the illuminated area, the fluorescence intensity reached the maximum. These results

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suggested that bacteria of strain P with higher sfGFP expression might detach from the surface because of the simultaneous increase in PA2133 expression. As desired, the cells dispersed under blue light. We examined the biofilm variation on the surface within illumination area and the surroundings by fractional surface coverage (FSC) of bacterial cells. As shown in Figure 5d, the light used had no toxicity on biofilm development indicated by the nearly identical kinetic of FSC under either illuminated or unilluminated conditions for strain S; The FSC of strain P increased slightly more slowly than did that of strain S in darkness because of somewhat background expression of PA2133; however, the cells of strain P formed comparable biofilms after 15 h. By contrast, the final FSC of strain P under illumination was less than half that of cells without illumination for 20 h.

We further monitored the illuminated and nonilluminated area using a scanning montage, and demonstrated that after stimulation with blue light, strain P dispersed from the surface with the accumulation of intracellular PA2133, showing much sparser, thinner and poorer biofilms, and that the bacteria in darkness maintained intact biofilms without maturation of fluorescence (Figure 5e).

DISCUSSION

In this study, we validated that the spatiotemporal expression of the target gene could be regulated in the presence of light signals, and our work has made progress toward developing a novel strategy to fight biofilm through degrading c-di-GMP. This strategy could be based on optogenetic methods to manipulate c-di-GMP levels using synthetic biology frameworks.

With the help of genetic circuit design and optimization, we first made the optogenetic systems function well in *P. aeruginosa* and could be used to further manipulate biological processes. We successfully engineered *P. aeruginosa* cells expressing appropriate amount of c-di-GMP degrading protein PA2133 by using optimized optogenetic modules, and this method effectively inhibited biofilms through the induction of blue light. Therefore, for the first time, an elaborate optogenetic circuit has been devised in *P. aeruginosa* that enables biofilm formation to be controlled to the extent that cells can be dispersed using light with ultrafine spatiotemporal resolution. We also showed further evidence of the tight relationship between c-di-GMP and biofilm dispersal.

Although it may be unnecessary in practice to trigger *P. aeruginosa* biofilm dispersal through introducing optogenetic circuits or by employing a strain harboring that, we chose to show this may be accomplished in order to show in principle that biofilms may be controlled. That is, that cells may be controlled and that biofilms may be dispersed with using synthetic optogenetic system. Furthermore, our design provides a prototype of dispersing biofilms by utilizing engineered optogenetic systems and supports the foundational basis for developing novel antimicrobial strategies by using synthetic optogenetic modules that could be extended to include other pathogens such as *Vibrio cholera* and *Helicobacter pylori*. The system may also be adopted as an alternative strategy to overcome the current limitations of biofilm control.

The developed approach has broad potential applications in fundamental bacteriology, particularly in biofilm dispersal. To date, research into biofilm control has nearly been based on temporal resolution.[16−18,42] The spatiotemporal manipulating biofilms has remained unexplored. The optogenetic circuits that we developed could control biofilm dispersal with ultrafine spatiotemporal resolution, creating a synthetic biological platform for the sophisticated patterning of biofilms. The method could be used to pattern *P. aeruginosa* biofilms, and this may indicate the mechanisms involved in coordinating individual activities during complex multicellular behaviors, such as collective movement in *P. aeruginosa* biofilms. Moreover, by utilizing the technique to guide the formation and spatial organization of bacteria in biofilms, we could study individual cell behaviors such as twitching motility and metabolism during detaching or reforming of microcolonies. The study of cell basic biological processes in the development of microbial biofilm formation, particularly at the later stage, represents an important area for future fundamental research, and our method may accelerate this process.

In summary, our study presents a promising platform for biofilm control based on the blue light responding system. In *Pseudomonas aeruginosa*, we first provided an optogenetic approach that enabled us to manipulate the biological processes and further tune bacterial behaviors with unprecedented spatial and temporal resolution. Our approach establishes a methodology for leveraging the tools of synthetic biology to study single-cell metabolism and guide the spatial organization of bacterial cells during biofilm development.

METHODS

Stains and Growth Conditions. The strains and plasmids used in this study are list in Supporting Information (Supplementary Table S1). Standard molecular cloning techniques were used in construction of related plasmids in *E. coli* strain Top10. Unless stated otherwise, both *E. coli* and *P. aeruginosa* stains were grown in LB medium or on LB agar plates at 37 °C. When required, antibiotics were added to medium at the following concentrations (μg mL^{-1}): kanamycin, 30, gentamicin, 15, ampicillin, 100, tetracycline, 20 (*E. coli*); ampicillin, 300, gentamicin, 30, tetracycline, 100 (*P. aeruginosa*). Strains that used for microscopy experiments were freshly streaked on LB agar plates from frozen stocks and incubated overnight at 37 °C. Monoclonal colonies were inoculated into 5 mL polystyrene tubes containing 1 mL of minimal medium FAB[43] without $FeSO_4$ supplemented with 1 mM $FeCl_3$ and 30 mM glutamate. Tubes were wrapped with aluminum foil to achieve a dark condition and incubated in shaker (New Brunswick Innova 40, Eppendorf) with 250 rpm at 37 °C until the culture reached an OD600 of approximately 2.0. For single-cell characterization experiment, the FAB medium-grown cells were diluted to OD600 approximately 0.01 and then put on an agarose slab 2% (w/v) made of agarose in FAB containing 1 mM $FeCl_3$ and 30 mM glutamate. For flow cell experiment, the pregrown cells were diluted 10-fold and then injected into the flow cell system. Blue light and white light were obtained *via* LED for consistent illumination. The light intensity on the surface of tubes was set at 100 nW cm^{-2}, measured with a power meter (model 842-PE, Newport, Darmstadt, Germany) using a silicon photodetector (model 918D-UV-OD3, Newport). Preferable light intensity was chosen for the study according to the expression kinetics of sfGFP reporter of cells under different light intensity, and the light intensities we used are extremely low and proved to be nontoxic to the bacteria (Supporting Information Figure S2).

Plasmids and Strains Construction. The mini-Tn7 vector[44] was adopted for *P. aeruginosa*. To prevent undesired read-through from chromosomal promoters into cloned

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sequences, we first constructed a modified delivery vector pTn7-L(T0T1), adding transcriptional terminators to the left end of original mini-Tn7 plasmid as follow: a 381-bp fragment containing two strong transcriptional terminators T0 and T1[45] was amplified by PCR from pUC18T-mini-Tn7T-Gm with primer T0-*Sma*I-Fand primer *Kpn*I-*Bam*HI-*Stu*I-T1-R. The PCR product digested with *Sma*I and *Kpn*I was inserted into pUC18T-mini-Tn7T-Gm cut with the same enzymes. The resulting vector, pTn7-L(T0T1), carries artificial restriction sites for *Kpn*I, *Bam*HI, *Stu*I and *Sma*I to ensure the multiformity after the reconstruction.

For construction of pDawn-Tn7, a 3601-bp fragment containing the YF1-FixJ-pFixK2-cI-pR, was amplified by PCR from plasmid pDawn with primer *Kpn*I-pDawn-F, carrying an artificial restriction site for *Kpn*I, and primer *Stu*I-pDawn-R, carrying an artificial restriction site for *Stu*I. The PCR product was digested with *Kpn*I and *Stu*I and inserted into pTn7-L(T0T1) cut with the same enzymes. The resulting plasmid carries the main elements of pDawn and the whole mini-Tn7 framework, named pDawn-Tn7 for short. As being the optogenetic tool, it was used for all subsequent gene circuits design and could be integrated into the chromosomes of *P. aeruginosa* for single-copy state. To test the performance of the optogenetic system, we exploited the green fluorescent protein sfGFP as reporter: a 739-bp fragment containing the *sf GFP* gene was amplified by PCR with primer *Hin*dIII-sfGFP-F and *Spe*I-sfGFP-R, digested with *Hin*dIII and *Spe*I, and inserted into the MCS of pTn7-pDawn cut with the same enzymes, resulting in psfGFP-pDawn-Tn7.

To express PA2133 properly, the optimal delivery vector, p(GTG)PA2133(Mtag)-B0033sfGFP-pDawn-Tn7, was finally engineered (see Most Appropriate Gene Expression Level of PA2133). Simply, a 750-bp RBS B0033sfGFP fragment was amplified by PCR with primer *Hin*dIII-sfGFP-F and GTG-B0033-sfGFP-R. A 940-bp Moderate tag[46,47] PA2133-B0033 fragment was amplified by PCR with primer GTGB0033-PA2133-F and*Spe*I-PA2133(Moderate tag)-R. Then two fragments were spliced by overlap PCR with primer *Hin*dIII-sfGFP-F and *Spe*I-PA2133(Moderate tag)-R. The overlap PCR product was digested with *Hin*dIII and *Spe*I, and inserted into pDawn-Tn7 cut with the same enzymes, resulting in p(GTG)-PA2133(Mtag)-B0033-sfGFP-pDawn-Tn7. All plasmids mentioned above were confirmed by sequencing.

In *P. aeruginosa*, Tn7 transposition can be acquired rapidly and efficiently by using a rapid electroporation method[48] and removal of the selection resistance gene marker Gm^r of the inserted strain by Flp-mediated excision[49] is optional. The correct integrated strains were verified by sequencing after PCR from genome.

Construction of c-di-GMP Reporter Plasmids. Gibson assembly[34] was used to join promoter p*cdrA*,[40] terminators (T0T1)[45] and output gene *mScarletI*[41] modules together to implement integrated c-di-GMP reporter plasmids in single-step reactions. Briefly, four DNA fragments, including pcdrA, mScarletI, T0T1 and linearized vector pUCP20, that overlapped in sequence by ∼25 bases were constructed by PCR through the design of PCR primers (Supporting Information Table S1) that contain "overhangs", which provide sequence overlap with adjacent fragments. Next, 100 ng of the linearized vector backbone pUCP20 and equimolar amounts of the other assembly pieces were added to the 10 μL NEBuilder HiFi DNA Assembly Master Mix in a 20 μL total volume assembly reaction mixture. The assembly reaction was incubated at 50 °C for 60 min, and then 5 μL of the assembly reaction was transformed into 100 μL of competent *E. coli*. Finally, the c-di-GMP reporter plasmid pcdrA-mScarletI-T-pUCP20 was confirmed by sequencing.

Most Appropriate Gene Expression Level of *PA2133*. In our study, the expression balance amount of PA2133 between under dark and light inducing condition is the key direction to modify and improve the optogenetic circuit. That is, that under dark, noninducing conditions, the strain harboring the optogenetic circuits could be able to form biofilms rather than being affected by the background activity of PA2133 and that while incubation in blue light, the level of PA2133 expression after induction would be efficient to make the bacteria detaching from the adhered surface.

We optimized the optogenetic circuit using screening for a series of strains carrying modified circuits, which are engineered by exploiting and reassembling different standardized biological parts. As the transcriptional efficiency of gene *PA2133* is under control of λ promoter pR, we considered two aspects to adapt the amount of PA2133: translational frequency and protein degradation rate. We employed two different translational initiation codon GUG or AUG, in which GUG has a slightly lower coding efficiency in *P. aeruginosa*,[50] and different ribosome binding sites (iGEM biobricks: BBa_B0031, BBa_B0033 and BBa_B0034), which have widely varied ribosome binding efficiency ranging from 100% to 0.35%, to adjust the translational level of PA2133. To minimize background activity and avoid excessive accumulation of PA2133, we fused different sequences to the C-terminal of protein PA2133, which code for amino acid sequences that will make the protein susceptible to degradation through SspB-mediated binding to the ClpX protease as well as ClpA protease with different degradation rates (iGEM biobricks: BBa_M0050, very fast; BBa_ M0051, fast; and BBa_ M0052, moderately fast.).[47,51−53] The standardized biological parts used are listed in Table 1. By reassembling the above biological parts including two different start codons, three different ribosome binding sites and four different hydrolysis tags (including the original PA2133 with no tag), we finally found the optimal PA2133 expression strain, named p(GTG)PA2133(Mtag)-B0033sfGFP-pDawn-Tn7. The strains for screening were constructed and their performance are listed in Supporting Information Table S2. We tested their behaviors in flow cell experiment, more detailed experimental information was given in the next section.

Table 1. Summary of Sequences of the Start Codes and RBS for PA2133, and the Amino Acid Sequence Fused to the C-Terminal of PA2133

start codes	ribosome binding sites (RBS)	hydrolysis tags
5′-**ATG**-3′	BBa_B0031, 5′-TCACACAGGAAACC-3′	BBa_M0050, AANDENYALAA
5′-**GTG**-3′	BBa_B0033, 5′-TCACACAGGAC-3′	BBa_M0051, AANDENYNYADAS
	BBa_B0034, 5′-AAAGAGGAGAAA-3′	BBa_M0052, AANDENYADAS

Flow Cell Experiment. Biofilms were grown in the middle channel of three-channel flow cells[54] with individual channel dimensions of 1 × 4 × 40 mm. The substratum consisted of a microscope glass coverslip (Fisherfinest 24 × 50 mm; Fisher Scientific). Bacteria were cultured at 37 °C under darkness overnight, then diluted to an OD600 of proper value. After the

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injection, the flow cell was placed for 3 min to allow attaching of cells to coverslip. Then washed out the unattached cells, and the attached were cultured at 30 ± 1 °C supplied with a flow of 3 mL h^{-1} of FAB medium supplemented with 1 mM $FeCl_3$ and 0.6 mM glutamate. Flow cell was put on XY-stage of microscope, exposed a limited area (3 × 3 microscope fields. one field, 512 × 512 pixel) to blue light. Recorded a 9 × 9 fields sfGFP fluorescence microscopy images every 1 h through appropriate filter (excitation, 488 nm; emission, 524/40 nm).

Image Acquisition. Imaging was performed using spinning disk confocal (CSU-X1, Yokogawa) inverted microscope (IX81, Olympus) equipped with a laser combiner system (Andor Technology), a 100×, 1.4 NA oil immersion objective (Olympus) and an EMCCD camera (iXon 897). The microscope, camera, and stage were actuated with a live cell imaging software Andor iQ. Green channel filter (488 nm exciter and 524/40 nm emitter) and Red channel filter (561 nm exciter and 605/40 nm emitter) set with a dichroic beam splitter (Semrock) was used for detection of sfGFP and mScarletI fluorescence. The light off-on photo conversion of pDawn-Tn7 system was done by illumination with the same filters.

For agarose plate and flow cell experiment, bacteria in a limited area were exposed to 488 nm with a pulse of 20 ms every 10 s. Light was set at 5% and the intensity was estimated about 3 μW cm^{-2} by photodetector. To quantify sfGFP fluorescence, bacteria in the limited area including surrounding areas were imaged every 1 h for 20 h. Intensity of activation laser here was set at 50% with exposure time of 100 ms and camera EM Gain 100.

Statistical Analysis. Images acquired were analyzed using ImageJ v.1.51n software or computed using MATLAB (MathWorks) codes as reported.[36,37] To compute mean fluorescence intensity, we subtracted the average fluorescence per pixel of the background from the average intensity per pixel in the given cell and more than 10^4 cells were analyzed for getting mean fluorescence intensity of bacteria populations. It is notably that the statistical data were analyzed at single cell solution. Mean values and standard deviations were obtained from at least two independent experiments (biological replicates). Statistical analysis was performed with Students unpaired two-sided *t*-test using GraphPad Prism version 7.03. The *P* value was set to <0.001 for significant difference between two groups.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00273.

Figures S1−S3, Tables S1−S2 (PDF)

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Notes

The authors declare no competing financial interest.

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