Development of a light-regulated cell-recovery system for non-photosynthetic bacteria

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Abstract

Background: Recent advances in the understanding of photosensing in biological systems have enabled the use of photoreceptors as novel genetic tools. Exploiting various photoreceptors that cyanobacteria possess, a green light-inducible gene expression system was previously developed for the regulation of gene expression in cyanobacteria. However, the applications of cyanobacterial photoreceptors are not limited to these bacteria but are also available for non-photosynthetic microorganisms by the coexpression of a cyanobacterial chromophore with a cyanobacteria-derived photosensing system. An *Escherichia coli*-derived self-aggregation system based on Antigen 43 (Ag43) has been shown to induce cell self-aggregation of various bacteria by exogenous introduction of the Ag43 gene.

Results: An *E. coli* transformant harboring a plasmid encoding the Ag43 structural gene under a green light-regulated gene expression system derived from the cyanobacterium *Synechocystis* sp. PCC6803 was constructed. Ag43 was inserted downstream of the *cpcG2* promoter P_{cpcG2} and its expression was regulated by green light induction, which was achieved by the functional expression of cyanobacterial CcaS/CcaR by coexpressing its chromophore synthesis gene cassette in *E. coli*. *E. coli* transformants harboring this designed system self-aggregated under green light exposure and precipitated, whereas transformants lacking the green light induction system did not. The green light induction system effectively functioned before the cell culture entered the stationary growth phase, and approximately 80% of the cell culture was recovered by simple decantation.

Conclusion: This study demonstrated the construction of a cell recovery system for non-photosynthetic microorganisms induced by exposure of cells to green light. The system was regulated by a two-component regulatory system from cyanobacteria, and cell precipitation was mediated by an autotransporter protein, Ag43. Although further strict control and an increase of cell recovery efficiency are necessary, the system represents a novel tool for future bioprocessing with reduced energy and labor required for cell recovery.

Keywords: Antigen 43, Green light induction, Cell recovery, Green light sensor, Optogenetics, *Escherichia coli*
gene, cpcG2, is chromatographically regulated by a sensor histidine kinase, CcaS, and a cognate response regulator, CcaR [18]. Using the endogenous CcaS/CcaR system, the green light regulation of an exogenously induced gene was achieved using a modified promoter of cpcG2, P
\textsubscript{cpcG2}, inserted upstream of the target gene on a vector plasmid [19]. In addition, CcaS, CcaR, and P
\textsubscript{cpcG2} from Synechocystis sp. PCC6803 has been transformed into the marine cyanobacterium Synechococcus sp. NKBG 15041c as an exogenous green light-regulated gene expression system [20]. This system has been applied to the construction of a green light-regulated autolysis system for cyanobacteria that employs a T4 phage-derived lysis system under the control of green light-regulated gene expression [21].

However, the applications of cyanobacterial photoreceptors are not limited to cyanobacteria but are also available for non-photosynthetic microorganisms. A pioneering study by Tabor et al. achieved the functional expression and utilization of a cyanobacterium-derived green light-sensing system in E. coli [22]. Because phycocyanobilin (PCB), a chromophore of CcaS, is not endogenously synthesized in E. coli, the coexpression of a PCB synthesis gene cassette together with CcaS/CcaR resulted in green light-regulated gene expression in E. coli [23]. Tabor and his coworkers also reported a multichromatophore gene expression system employing an engineered CcaS.

In this study, we aimed to construct a novel technology for non-photosynthetic microorganism-based bioprocesses, a light-regulated cell-recovery system. As a light-regulated expression system, the cyanobacterium-derived green light-regulated gene expression system was controlled by the two-component regulatory system CcaS/CcaR. For the cell recovery technology, the E. coli-derived self-aggregation system was selected. Antigen 43 (Ag43), an autotransporter protein from E. coli, is an essential protein for aggregation and biofilm formation during infection. Ag43 is composed of three domains: a signal peptide for secretion into the periplasmic space, a β domain that forms a selective channel in the outer membrane to transfer the α domain for extracellular display, and an α domain, which is a linker for self-aggregation. High affinity among the α domains triggers self-aggregation, which leads to cell precipitation [24–28]. Recently, the structure of the α-domain complex of Ag43 has been reported [29]. In the present study, the Ag43 structural gene was inserted downstream of the cpcG2 promoter, P
\textsubscript{cpcG2}, and its expression was regulated by green light induction, achieved by the functional expression of cyanobacterial CcaS/CcaR by coexpression of its chromophore synthesis gene cassette in E. coli. E. coli transformants carrying this system self-aggregated under green light exposure and precipitated, whereas transformants lacking the green light-induction system did not. The green light-induction system effectively functioned before the cell culture entered the stationary growth phase, and approximately 80% of the cell culture was recovered by simple decantation.

Methods

Construction of a plasmid encoding a green light-inducible aggregation system

Gene encoding the aggregation protein, ag43 derived from E. coli amplified from BioBrick Bba_K317008 (Registry of Standard Biological Parts [30]) was inserted downstream of a P
\textsubscript{cpcG2} promoter corresponding to the Ndel and XbaI sites of pKTGSS, which contains the green light-sensing two-component regulatory system CcaS/CcaR [20]. The resulting plasmid was designated pKTGLAg. The gene cassette containing the green light-inducible aggregation system was amplified by PCR using primers 5′-AGCGGCCGCGAATTCTTGAAGAC-3′ and 5′-TTTTTTTCGCTGCA-GATGGAAGCCGGGC-3′. These amplified products were fused with an InFusion cloning kit (Takara, Otsu, Japan). The constructed vectors comprising the green light-inducible aggregation system (pBRGLAg) are shown in Fig. 1. Control vectors with each component deleted were also constructed by inverse PCR using pBRGLAg with primers designed to eliminate ccaS or ccaR (pBRGLAgAS, pBRGLAgAR, and pBRGLAgASR). These constructed vectors are shown in Fig. 1, and the components are described in Table 1.

Construction of a plasmid encoding PCB synthesis genes

A PCB synthesis gene cassette was constructed by assembling P
\textsubscript{LtetO₁} (Bba_B0040; Registry of Standard Biological Parts [30]), a ribosomal binding site (RBS) (Bba_B0034; Registry of Standard Biological Parts [30]), the heme oxygenase gene ho1 from Synechocystis sp. PCC6803 (Bba_115008; Registry of Standard Biological Parts [30]), the PCB-thioredoxin oxidoreductase gene pcyA from Synechocystis sp. PCC6803 (Bba_115009; Registry of Standard Biological Parts [30]), and a double terminator (Bba_B0015; Registry of Standard Biological Parts [30]) using three antibiotic assembly and inserted at the EcoRI and PstI sites of the plasmid derived from pSTV28, whose construction has been previously described [32, 33, 34]. This plasmid was named pSTVPCB. In this plasmid, ho1 and pcyA were constitutively transcribed independently following RBS in E. coli DH5α by P
\textsubscript{LtetO₁} polycistronically (Fig. 1). The components of this plasmid are shown in Table 1.
Autoaggregation-regulation assay

*E. coli* cells carrying pBRGLAg, pBRGLAgΔS, pBRGLAgΔR, or pBRGLAgΔSR together with pSTVPCB were cultured in LB broth containing 25 µg/ml chloramphenicol and 100 µg/ml ampicillin in a test tube at 37 °C with shaking at 140 rpm overnight. The prepared precultures were inoculated into fresh 40 ml LB broth containing 0.1 M HEPES (pH 6.6), 0.05 mM aminolevulinic acid, 0.05 mM FeCl₃, 100 µg/ml ampicillin, and 25 µg/ml chloramphenicol in 100-ml Erlenmeyer flasks. Cell density was monitored 6 h after the start of culture. Cells were cultured with shaking at 100 rpm and exposed to red light (660 nm, 40 µmol s⁻¹ m⁻²) at 30 °C until the cell density reached OD₅₉₅ or OD₆₀₀ = 0.4–0.6. After this period, each transformant was cultured under either of the following two conditions: one culture in triplicate was exposed to...
green light (520 nm, 40 µmol s⁻¹ m²) instead of red light for 6 h, and the other culture in triplicate was continuously exposed to red light with shaking at 100 rpm and 30 °C. A 10-ml culture was transferred to a 15-ml tube to measure the aggregation-regulation ability of the cells.

The transferred culture in each 15-ml tube was exposed to red light for 2 h. During the incubation, a 100-µl culture was periodically transferred from the tube to a 96-well plate every 10 min, and 200 µl of fresh culture was added to the wells to dilute the culture. Cell density was measured using a plate reader (Thermo Fisher Scientific Inc., MA, USA). Cell density measurements were performed in triplicate. In all aggregation experiments, E. coli DH5α was used.

Transcriptional analysis of ag43 by quantitative reverse transcription PCR

E. coli cells harboring pSTVPCB and pBRGLAg were cultured as described above in the aggregation-regulation assay. During culture, a 1-ml culture was periodically removed.

Total RNA was extracted from the cell pellets from 1-ml cultures taken after centrifugation at 12,000 g for 5 min at 4 °C, using a NucleoSpin® RNA Clean-up kit (Takara Bio Inc., Shiga, Japan). The extracted RNA was treated with DNase to eliminate genomic DNA, and reverse transcription from RNA to cDNA was performed using PrimeScript® RT reagent kit with gDNA Eraser (Takara Bio Inc.). Quantitative PCR was performed to measure the transcriptional level of ag43 and 16S ribosomal RNA (rRNA) (housekeeping genes) with SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Takara Bio Inc.). The transcription level was measured using the ΔΔCt method and normalized using the calculated transcription values of 16S rRNA.

Evaluation of cell recovery

Cells harboring the green light-inducible aggregation system were cultured as described above with modification in the timing of the start of exposure to green light.

To determine the timing of gene induction, cultures were induced by green light at different stages of growth. Four separate cultures in triplicate were prepared. For each culture, green light was irradiated at OD₅₉₅ = 0.7, 1.1, or 1.2 or until 10 h had passed after the cell density reached OD₅₉₅ = 1.7. Cultures were then exposed to green light (520 nm, 40 µmol s⁻¹ m²) for 2 h.

Cultures diluted to cell density OD₅₉₅ = 1.0 by the addition of fresh LB broth containing 0.1 M HEPES (pH 6.6), 0.05 mM aminolevulinic acid, 0.05 mM FeCl₃, 100 µg/ml ampicillin, and 25 µg/ml chloramphenicol were transferred to a 15-ml tube and exposed to red light for 180 min for cell precipitation. Then, 7.6 ml of the supernatant was sampled and 400 µl of the culture containing precipitated cells was left behind (decantation procedure). The remained cells in 400 µl of the culture was defined as the recovered cells. In order to quantify the amount of recovered cells and unrecovered cells, thus prepared 400 µl of the culture containing precipitated cells and 7.6 ml supernatant were centrifuged. The cell recovery was calculated as the ratio (%) of the wet weight cells of recovered cells and total (recovered and unrecovered) cells.

Table 1 Vector used in this study

| Plasmid name   | Origin             | Resistance      | Feature                                      | Source                  |
|---------------|-------------------|-----------------|----------------------------------------------|-------------------------|
| pKTG5S        | V ori, p15A ori   | Streptomycin    | cca cluster with gfpuv instead of cpcG2      | Ref. [20]               |
| pBRGLAg       | pMb1 ori          | Ampicillin      | cca cluster with ag43 instead of cpcG2       | This study              |
| pBRGLAgS      | pMb1 ori          | Ampicillin      | cca cluster with ag43 instead of cpcG2 without ccaS | This study              |
| pBRGLAgSR     | pMb1 ori          | Ampicillin      | cca cluster with ag43 instead of cpcG2 without ccaR | This study              |
| pBR322        | pMb1 ori          | Ampicillin/tetracycline | original vector used for construction of pBRGLAg | Ref. [31]               |
| pSTVPCB       | p15A ori          | Chloramphenicol | hox and pcy4 genes for PCB synthesis         | This study              |
| pSTV2B        | p15A ori          | Chloramphenicol | original vector used for construction of pSTV28 | Ref. [33]               |
40 µmol m⁻² s⁻¹) started to decrease in OD₆₀₀ during incubation, indicating that cell precipitation had started. After 60 min of incubation, the decrease in OD₆₀₀ ceased at approximately OD₆₀₀ = 0.5, indicating that cell precipitation had stopped. At this point, >50% of the cells had precipitated. However, no precipitation was observed for transformants harboring pSTVPCB and pBRGLAg, which were exposed only to red light prior to the precipitation assay. In contrast, transformants harboring pSTVPCB and pBRGLAgΔS, pBRGLAgΔR, or pBRGLAgΔSR, cultured under red or green light, showed no decrease in OD₅₉₅ or OD₆₀₀, suggesting that these transformants had no precipitation ability (Fig. 2b, c, d). These results indicated that cell precipitation ability was induced when the transformants harbored both genes, those for PCB synthesis and for the complete two-component regulatory system CcaS/CcaR, and only when cultured under green light.

Transcriptional analysis of ag43 in transformants harboring pSTVPCB and pBRGLAg was performed. The transformants were cultured under red light for 12 h and then cultured under green light for the next 2 h, whereas the other transformants were cultured under red light for 14 h. Transcriptional analysis was performed for cells cultured after the first 12 h of culture (Fig. 3). The transcriptional level of ag43 gradually increased after exposure to green light for 20 min. The transcriptional level reached the highest level by 80 min incubation and remained stable for at least the next 20 min at least. Although an increase tendency of ag43 transcription was observed when the culture was exposed to red light, the transcriptional level was <30% of that observed 80–110 min after exposure to green light.

The results of the aggregation assays and transcriptional analysis indicated that the green light regulated aggregation of E. coli cells was achieved by introducing a green light-sensing two-component regulatory system derived from cyanobacteria and Ag43 gene.

Cell recovery
The engineered E. coli cells harboring pSTVPCB and pBRGLAg were then subjected to investigation of cell recovery. Cells were cultured under green or red light (Fig. 4). After an approximately 12-h lag phase,
cell growth entered the logarithmic growth phase and reached the stationary phase after 30 h. Cells at each growth phase, the early logarithmic growth phase (12–18 h; OD595 = approximately 0.7), mid-logarithmic growth phase (18–28 h; OD595 = approximately 1.0), late logarithmic growth phase (28–32 h; OD595 = approximately 1.8), and stationary phase (10 h after the late log phase), were exposed to green light to induce precipitation. Cultures containing cells exposed to green light at each growth phase were diluted to OD595 = 1.0 by addition of LB broth, and an 8 ml culture of each was transferred to a 15-ml tube. Tubes were exposed to red light.

Cells at all growth phases showed precipitation ability, with 2-h exposure to green light resulting in Ag43 expression. Amounts of cells recovered by decantation of the precipitated cells are shown in Fig. 4. From the cells exposed to green light at each growth phase were diluted to OD595 = 1.0 by addition of LB broth, and an 8 ml culture of each was transferred to a 15-ml tube. Tubes were exposed to red light.

Discussion

In this study, we aimed to construct a green light-regulated cell recovery system for non-photosynthetic microorganisms using a green light-regulated gene expression system controlled by a two-component regulatory system from cyanobacteria and using Ag43, an autotransporter protein from E. coli.

Recently, the crystal structure of α-domain of Ag43 has been reported [29]. The crystal structure of this domain shows that the formation of cell aggregates proceeds via a molecular Velcro-like handshake mechanism. Under this mechanism, if Ag43 is expressed on the surface of the outer membrane, cell self-aggregation will occur. The self-aggregation of bacteria using recombinant Ag43 has been previously reported [28]. Exogenously introduced Ag43 led to the self-aggregation of E. coli, Pseudomonas fluorescens, and Klebsiella pneumoniae. Thus, our green light-induced cell recovery system will also be useful in a variety of non-photosynthetic microorganisms if the functional expression of the green light-sensing system is
possible with the introduction of the PCB synthesis gene cassette.

Cell precipitation was observed in green light-exposed transfectants harboring both pSTVPCB for PCB synthesis and pBRGLAg encoding CcaS/CcaR and Ag43 under \( \text{P}_{\text{ag43}} \) but not in transfectants harboring pSTVPCB and with an imperfect green-light-regulation system (pBRGLAg\( \Delta S \), pBRGLAgAR, or pBRGLAg\( \Delta SR \)) (Fig. 2a–d). However, even in the transfectants with pSTVPCB and pBRGLAg, slight precipitation was observed under red light exposure at 70 min of incubation (Fig. 2a). Although the slight precipitation of the cells exposed to red light was observed at 70 min incubation, the expression of Ag43 under red light was not observed by transcriptional analysis (Figs. 2, 3). However, because the increase tendency was observed in the ag43 transcription under red light, the result suggests undetectable level ag43 transcription under red light led to Ag43 expression and precipitation of cells under red light. The difference in the Ag43 expression levels of transfectants with pSTVPCB and pBRGLAg under red light was obvious in transfectants harboring an imperfect green light-sensing system with ag43. Thus, the expression of Ag43 under red light was not due to the endogenously present \( \text{P}_{\text{ag43}} \) activating factors in E. coli but due to background-level expression under red light in the presence of CcaS/CcaR. It has been reported that CcaS autophosphorylation was repressed under red light. To prevent expression leakage of Ag43 under non-inducing conditions, we cultured E. coli transfectants under red light. However, further repression of kinase activity of CcaS is required to achieve tight regulation using this system.

Cell recovery by exposure to green light was achieved when the cells were induced before entry into the stationary phase. However, when cells were exposed to green light even at the early-logarithmic growth phase, 80 % could be recovered by decantation with 20 % remaining in the culture supernatant. Aggregation is strongly dependent on the cell concentration [27]. With decreasing free cell concentration in the supernatant resulting from the precipitation of flocculate from Ag43-mediated aggregation, aggregation may decrease. To overcome this inherent problem of aggregation-mediated cell recovery, an increase in the expression level of Ag43 per cell would enhance cell precipitation.

Conclusions

In conclusion, this study demonstrated the construction of a cell recovery system for non-photosynthetic microorganisms that is induced by the exposure of cells to green light. The system is regulated by a two-component regulatory system from cyanobacteria, and the cell precipitation is mediated by an autotransporter protein, Ag43. Although further strict control and increase of cell recovery efficiency are necessary, the proposed system provides a novel tool for future bioprocessing with reduced energy and labor for cell recovery.

Authors’ contributions

MN; Mitsuharu Nakajima, KA; Koichi Abe, SF; Stefano Ferri, KS; Koji Sode MN conducted the preparation of recombinant cells and experiments throughout this research. KA designed the genes and vectors used in this research. SF and KS designed the plan of this research and performed the data analysis. KS has supervised this study. All authors participated in design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Leviiskaya A, Weiner OD, Lim WA, Voigt CA. Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature. 2009;461:997–1001.
2. Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, et al. A genetically encoded photoactivatable Rac controls the motility of living cells. Nature. 2009;461:1104–10.
3. Masuda S, Nakatani Y, Ren S, Tanaka M. Blue light-mediated manipulation of transcription factor activity in vivo. ACS Chem Biol. 2013;8:2649–53.
4. Schierling B, Pingoud A. Controlling the DNA cleavage activity of light-inducible chimeric endonucleases by bidirectional photoactivation. Bioconjuate Chem. 2012;23:1105–9.
5. Ohlendorf R, Vidavski RR, Eldar A, Moffat K, Möglich A. From dusk till dawn: one-plasmid systems for light-regulated gene expression. J Mol Biol. 2012;416:534–42.
6. Wang X, Chen X, Yang Y. Spatiotemporal control of gene expression by a light-switchable transgene system. Nat Methods. 2012;9:266–9.
7. van Bergeijk P, Adrian M, Hoogenraad CC, Kapitein LC. Optogenetic control of organelle transport and positioning. Nature. 2015;518:111–4.
8. Liu X, Ramirez S, Pang PT, Puryear CB, Govindarajan A, Deisseroth K, et al. Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature. 2012;484:381–5.
9. Terauchi K, Montgomery BL, Grossman AR, Lagarias JC, Kehoe DM. RacE is a complementary chromatic adaptation photoreceptor required for green and red light responsiveness. Mol Microbiol. 2004;51:567–77.
10. Hirose Y, Narikawa R, Katayama M, Ikeuchi M. Cyanobacteriochrome CcaS regulates phycoerythrin accumulation in nostoc punctiforme, a group II chromatic adapter. Proc Natl Acad Sci USA. 2010;107:8854–9.
11. Bonetti C, Stierl M, Mathes T, van Stokkum IH, Mullen KM, Cohen-Stuart TA, et al. The role of key amino acids in the photoactivation pathway of the Synechocystis Slr1694 BLUF domain. Biochemistry. 2009;48:11458–69.
12. Gao Z, Livoti E, Losi A, Gartner W. A blue light-inducible photophosphodiesterase activity in the cyanobacterium Synechococcus elongatus. Photochem Photobiol. 2010;86:606–11.
13. Enomoto G, Nomura R, Shimada T, Win NN, Narikawa R, Ikeuchi M. Cyanobacteriochrome SesA is a diguanylate cyclase that induces cell aggregation in *Thermosynechococcus*. J Biol Chem. 2014;289:24801–9.

14. Enomoto G, Win NN, Narikawa R, Ikeuchi M. Three cyanobacteriochromes work together to form a light color-sensitive input system for c-di-GMP signaling of cell aggregation. *PNAS*. 2015;112:8082–7.

15. Yoshihara S, Shimada T, Matsuoka D, Zikihara K, Kohchi T, Tokutomi S. Reconstitution of blue-green reversible photoconversion of a cyanobacterial photoceptor, PixJ1, in phycocyanobilin-producing *Escherichia coli*. Biochemistry. 2006;45:3775–84.

16. Narikawa R, Suzuki F, Yoshihara S, Higashi S, Watanabe M, Ikeuchi M. Novel photosensory two-component system (PixX-NixB-NixC) involved in the regulation of positive and negative phototaxis of cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol*. 2011;52:2214–24.

17. Songa JY, Chob HS, Choc JI, Jeonc JS, Lagariasd JC, Parka YI. Near-UV cyanobacteriochrome signaling system elicits negative phototaxis in the cyanobacterium *Synechocystis* sp. PCC 6803. *PNAS*. 2011;108:10780–5.

18. Hirose Y, Shimada T, Narikawa R, Catayama M, Ikeuchi M. Cyanobacteriochrome CcaS is the green light receptor that induces the expression of phycobilisome linker protein. *PNAS*. 2008;105:9528–33.

19. Abe K, Miyake K, Nakamura M, Kojima K, Ferri S, Ikebukuro K, et al. Engineering of a green-light inducible gene expression system in *Synechocystis* sp. PCC6803. *Biotechnol Biofuels*. 2014;7:56.

20. Badary A, Abe K, Ferri S, Kojima K, Sode K. The development and characterization of an exogenous green-light-regulated gene expression system in marine cyanobacteria. *Biotechnol Biofuels*. 2015;7:245–51.

21. Miyake K, Abe K, Ferri S, Nakajima M, Nakamura M, Yoshida W. A green-light inducible lytic system for cyanobacterial cells. *Biotechnol Biofuels*. 2014;7:56.

22. Tabor JJ, Levskaya A, Voigt CA. Multichromatic control of gene expression in *Escherichia coli*. *J Mol Biol*. 2011;405:315–24.

23. Gambetta GA, Lagarias J. Genetic engineering of phytochrome biosynthesis in bacteria. *PNAS*. 2001;98:10566–71.

24. Diderichsen B. Flu, a metastable gene controlling surface properties of *Escherichia coli*. *J Bacteriol*. 1980;141:858–67.

25. Owen P, Meehan M, de Loughrey-Doherty H, Henderson I. Phase-variable outer membrane proteins in *Escherichia coli*. *FEBS Immunol Med Microbiol*. 1996;1663–76.