Engineering *Escherichia coli* for light-activated cytolysis of mammalian cells

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Abstract

By delivering payloads in response to specific exogenous stimuli, smart bacterial therapeutics have the potential to overcome many limitations of conventional therapies, including poor targeting specificity and dosage control in current cancer treatments. Although not yet explored as a trigger for bacterial drug delivery, light is an ideal induction mechanism because it offers fine spatiotemporal control and is easily and safely administered. Using recent advances in optogenetics, we have engineered two strains of *Escherichia coli* to secrete a potent mammalian cytotoxin in response to blue or red light. The tools in this study demonstrate the initial feasibility of light-activated bacterial therapeutics for applications such as tumor cytolysis, and their modular nature should enable simple substitution of other payloads of interest.

Graphical Abstract

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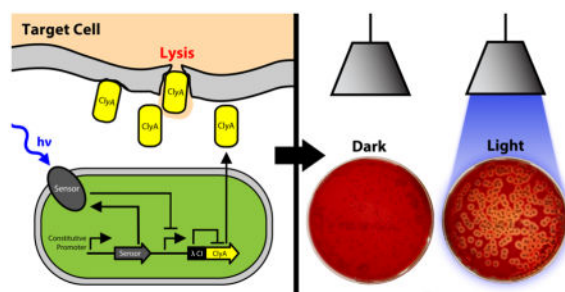
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INTRODUCTION

Synthetic biology has the potential to create novel therapeutic strategies to improve the treatment of diseases such as cancer. Conventional cancer treatment regimens involving chemotherapy, radiotherapy, or surgical excision are often limited by low targeting specificity, inadequate dosage control, or damage to healthy tissue, but these obstacles could be overcome by the development of 'smart' living therapeutics through synthetic biology approaches[1]. In particular, bacteria hold promise as potential therapeutic agents for cancer due to the relative ease of engineering genetic circuitry within them, the innate propensity of certain strains to target tumors, and their ability to actively penetrate solid tumors, as opposed to drugs which rely on passive diffusion[1,2]. Bacteria have been engineered with genetic circuits that allow for tumor cell invasion or therapeutic delivery in response to stimuli such as L-arabinose, hypoxia, cell density, γ-irradiation, and acetylsalicylic acid[2].

In contrast to these stimuli, light can enable safe triggering of gene expression with fine spatiotemporal control without reliance on passive diffusion. Fiber optic systems enabling targeted and accurate light delivery into tumors are already used for photodynamic therapy[3]. Optogenetic control of drug delivery has been demonstrated in mammalian cells with blue light[4], but this approach has not yet been demonstrated in bacteria or extended to light inputs of different wavelengths. In this study we exploited bacterial optogenetic components to enable light-activated delivery of a cytolytic payload using *E. coli*. We achieved blue light-mediated cytolysis of mammalian cells with a common laboratory strain of *E. coli* and replicated these findings with a minimally-immunogenic strain that is being explored for clinical applications. Since the longer wavelength of red light makes it more advantageous for therapeutic applications of optogenetics, we also constructed a plasmid (pMars) which allows for red light-controlled expression of a gene of interest, and successfully utilized it to control cytolysis. Our results provide proof of concept that optogenetic control of cytotoxicity can be achieved in bacteria with multiple wavelengths of light.

RESULTS AND DISCUSSION

We selected the pore-forming toxin cytolysin A (ClyA) as the payload for our system because it is readily secreted *via* outer membrane vesicles[5] and it has been shown to reduce tumor growth when expressed by bacteria *in vivo*[6]. To characterize this toxin, we first codon optimized and synthesized the *clyA* gene for expression in *E. coli* and this gene was cloned into the pET26b(+) expression vector with a C-terminal His tag for protein purification (ClyA: BBa_K811000, ClyA-His: BBa_K811002). To verify that the synthesized product can lyse cancer cells, purified ClyA (0.3 nM) was added to cultured SKBR3 cells and cytotoxicity was determined using the commercial Cytotox-Fluor Cytotoxicity Assay (Promega). ClyA exhibited a significant 6-fold increase in cytotoxicity over the negative control vehicle (Figure 1A, $p < 0.001$) and a 1.2-fold increase in cytotoxicity over the digitonin positive control ($p < 0.001$), verifying that ClyA can potently lyse cancer cells. For more readily visualizing mammalian cell lysis by ClyA-producing bacteria, the remaining experiments were performed using classical blood agar assays.

The pDawn plasmid, a well-characterized optogenetic tool[7], was used to achieve blue (480 nm) light-activated gene expression. To obtain light-dependent ClyA expression, the *clyA* gene was ligated into pDawn while adding an N-terminal His tag (His-ClyA: BBa_K811001), creating the plasmid pDawn-ClyA. *E. coli* BL21(DE3) cells were transformed with pDawn-ClyA and induced with blue light (or kept in the dark) at 25°C for 24 hours. ClyA was purified and a 34-kDa band was visible only in the blue light condition, verifying light-dependent production of ClyA (Figure 1B). To visualize light-activated cytolysis, we subsequently plated pDawn-ClyA-bearing bacteria onto blood agar plates. To further demonstrate efficacy in a clinically-relevant strain, we also transformed pDawn-ClyA into *E. coli* Nissle 1917, a nonpathogenic and minimally immunogenic strain which is already used in clinical applications[8] and has tumor-targeting capabilities in mice[9]. By illuminating plates with blue light, we achieved inducible cytotoxicity with both BL21(DE3) and Nissle 1917, as evidenced by the clear zones of lysis surrounding only those colonies which were both transformed with pDawn-ClyA and illuminated with blue-light (Figures 1C,D). When bacteria were transformed instead with the pDawn plasmid containing the non-cytotoxic protein mCherry, no light-dependent cytolysis was observed.

A key advantage of light-activated cytotoxicity is that the amount of protein produced can be finely tuned by controlling the illumination time. In a preliminary demonstration, we switched the output of our pDawn plasmid to mCherry and demonstrated control over total protein production by varying illumination time (Figure 1E). In the future, this tool could be calibrated based on the potency of ClyA to precisely produce the minimum amount of protein needed for cytolysis of a given tissue volume.

For eventual *in vivo* applications, the ideal light-activated therapeutic would be triggered by wavelengths longer than 480 nm because blue light is relatively poor at penetrating tissue. We thus sought to construct a new tool for red light-activated payload delivery. We developed a new plasmid backbone, pMars, which uses the well-characterized cph8[10] red-light sensor to activate gene expression with 650 nm light. A pMars-mCherry plasmid (red-light activated mCherry expression, Figure 2A) or a pMars-ClyA plasmid (red-light activated ClyA expression, Figure 2B) was transformed into *E. coli* JT2. The pPL-PCB(S) plasmid, which encodes the chromophore synthesis genes necessary to functionalize cph8[10], was co-transformed with the above plasmids. Red light- and chromophore-dependent mCherry expression was demonstrated with pMars-mCherry (Figure 2A, $p < 0.001$) and red light-induced expression of ClyA was achieved with pMars-ClyA (Figure 2B). JT2 is a knockout strain that is not optimized for protein expression and purification and we did indeed observe lower purity of His-tagged ClyA recovered from JT2 (Figure 2B) compared to BL21(DE3) (Figure 1B), as evidenced by additional ~25-kDa and ~9-kDa light-dependent bands (hypothesized to be either cleavage-products of the 34-kDa ClyA protein or endogenous JT2 proteins that are co-purified with ClyA) and a ~67-kDa constitutive band (hypothesized to be an endogenous JT2 protein that interacts with the purification column). Nevertheless, the level of ClyA expression achieved with this strain was sufficient for eliciting red light-mediated cytotoxicity, with clear zones of lysis surrounding pMars-ClyA bearing colonies plated onto blood agar (Figure 2C). Given the natively low on:off ratio of the cph8 sensor[10], successful light-induced cytolysis is likely due to the strong potency of the ClyA toxin (Figure 1A). In our experiments, the better performance of pDawn relative to

pMars is due to the properties of the strains and circuits themselves, because no diffusion barriers were placed between the light sources and the bacterial colonies.

Engineered bacteria hold promise as potential therapeutic agents, and one of the factors that will determine the success of this approach is the choice of induction mechanism for therapeutic delivery. While other triggering methods have been explored previously[2], here we have introduced light-activated bacterial drug delivery, which is uniquely suited for achieving precise dosage control not only temporally but also spatially. Optogenetic control of mammalian cell cytotoxicity was demonstrated using two engineered strains of *E. coli*, including clinically-relevant Nissle 1917. Blue or red light was used to successfully induce the desired cytolytic response, although further optimization of the red-light-activated system will be necessary to improve its dynamic range and reduce its leakiness, especially for *in vivo* applications. More generally, this approach could be used for light-activated expression of molecular payloads other than ClyA, including cytokines or other cytotoxic agents for specific cancer applications[2].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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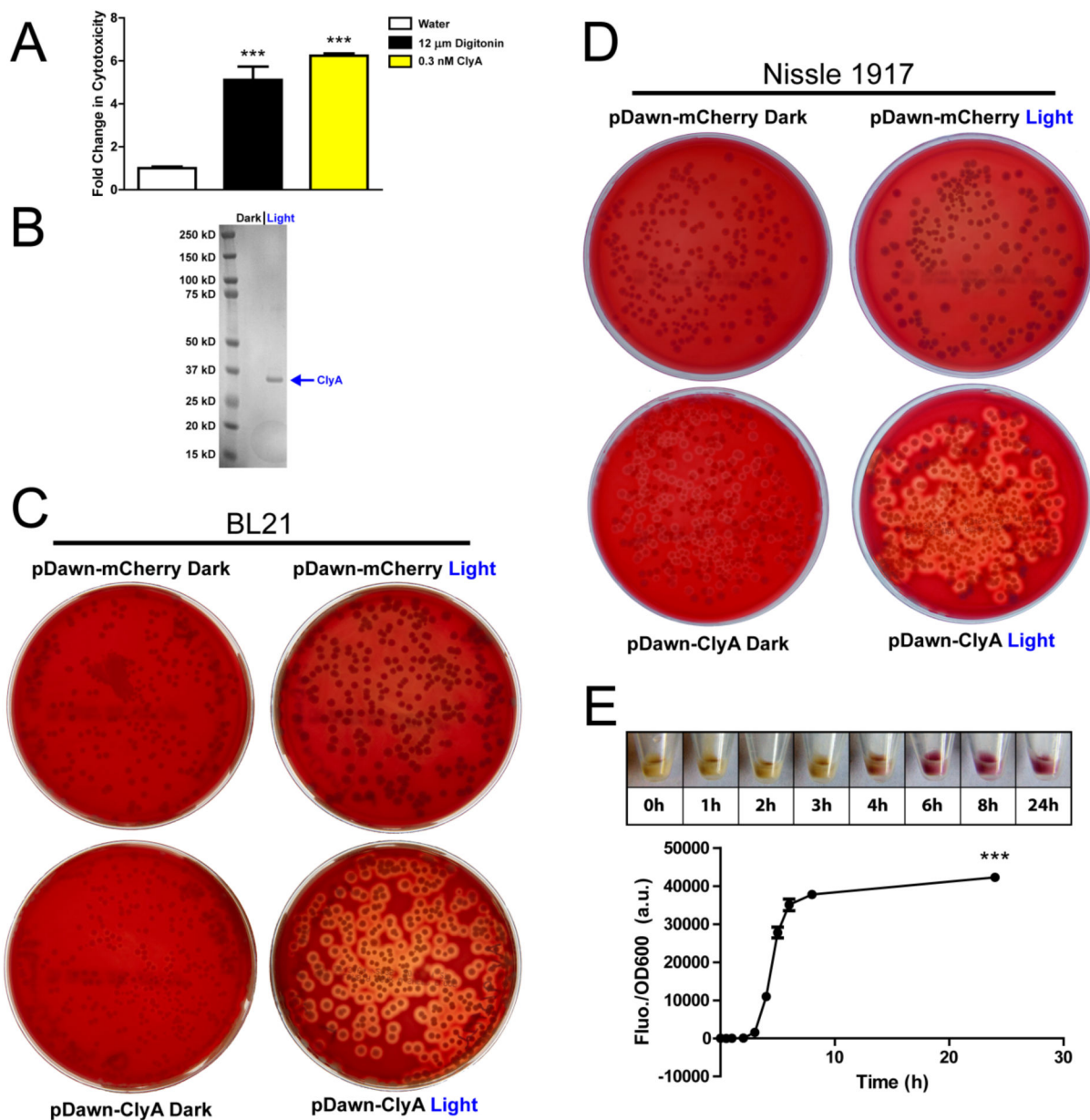
Figure 1.
Blue light-activated cytolysis in *E. coli* BL21 and Nissle 1917 using pDawn. **(A)**
Synthesized and purified ClyA lyses SKBR3 cancer cells *in vitro*. Purified ClyA (0.3 nM)
resulted in a 6-fold increase in cytotoxicity, significant ($p < 0.001$) over both the negative
control vehicle (water) and the positive control (digitonin, 12 μM) (n = 6 samples per group).
(B) BL21(pDawn-ClyA) shows specific, blue light-dependent expression of the ~34 kDa
ClyA protein. **(C)** In contrast to BL21(pDawn-mCherry), BL21(pDawn-ClyA) exhibits blue
light-dependent cytolysis on blood agar. **(D)** Blue light-dependent cytolysis on blood agar is
seen from Nissle 1917(pDawn-ClyA) but not from Nissle 1917(pDawn-mCherry). **(E)** Time
course for mCherry protein expression from BL21(pDawn-mCherry) using 480 nm light.
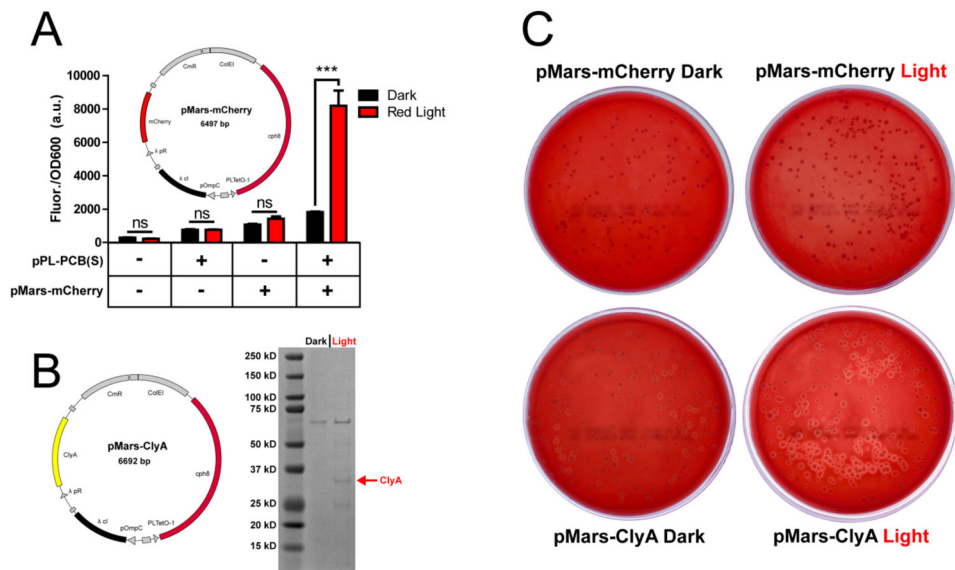Light-dependent mCherry expression in BL21 cultures (n = 3 per time point) is significantly

higher ($p < 0.001$) at all illumination times beyond 3 h (asterisks omitted at 4, 5, 6, and 8 hour time points for clarity). All data are presented as mean ± SD.

Figure 2.
Red light-activated cytolysis in *E. coli* JT2 using pMars. **(A)** The red light-activated system was first tested with pMars-mCherry to ensure functioning of components. Specific, red light-induced expression of mCherry was measured by fluorescence ($p < 0.001$ between dark and light) and this effect was dependent on the presence of both the chromophore synthesis plasmid pPL-PCB(S) and pMars-mCherry ($p > 0.05$ between dark and light in first three conditions). **(B)** Red light-activated system with pMars-ClyA demonstrates weak light-dependent expression of the ClyA protein (~34 kDa). **(C)** JT2(pMars-ClyA) demonstrate red light-activated cytolysis on blood agar plates, while this effect is not observed for JT2(pMars-mCherry). All data are presented as mean ± SD.