

Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen

Nazanin Saeidi¹, Choon Kit Wong¹, Tat-Ming Lo, Hung Xuan Nguyen², Hua Ling, Susanna Su Jan Leong, Chueh Loo Poh* and Matthew Wook Chang*

School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore

¹ These authors contributed equally to this work

² Present address: Department of Biomedical Engineering, Duke University, Durham, NC, USA

* Corresponding author. CL Poh, Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459, Singapore. Tel.: +65 6514 1088; Fax: +65 6794 7553; E-mail: clpoh@ntu.edu.sg or MW Chang, Division of Chemical and Biomolecular Engineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459, Singapore. Tel.: +65 6513 8063; Fax: +65 6794 7553; E-mail: matthewchang@ntu.edu.sg

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Synthetic biology aims to systematically design and construct novel biological systems that address energy, environment, and health issues. Herein, we describe the development of a synthetic genetic system, which comprises quorum sensing, killing, and lysing devices, that enables *Escherichia coli* to sense and kill a pathogenic *Pseudomonas aeruginosa* strain through the production and release of pyocin. The sensing, killing, and lysing devices were characterized to elucidate their detection, antimicrobial and pyocin release functionalities, which subsequently aided in the construction of the final system and the verification of its designed behavior. We demonstrated that our engineered *E. coli* sensed and killed planktonic *P. aeruginosa*, evidenced by 99% reduction in the viable cells. Moreover, we showed that our engineered *E. coli* inhibited the formation of *P. aeruginosa* biofilm by close to 90%, leading to much sparser and thinner biofilm matrices. These results suggest that *E. coli* carrying our synthetic genetic system may provide a novel synthetic biology-driven antimicrobial strategy that could potentially be applied to fighting *P. aeruginosa* and other infectious pathogens.

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Introduction

Synthetic biology aims to engineer genetically modified biological systems that perform novel functions that do not exist in nature, with reusable, standard interchangeable biological parts. The use of these standard biological parts enables the exploitation of common engineering principles such as standardization, decoupling, and abstraction for synthetic biology (Endy, 2005). With this engineering framework in place, synthetic biology has the potential to make the construction of novel biological systems a predictable, reliable, systematic process. While the development of most synthetic biological systems remains largely *ad hoc*, recent efforts to implement an engineering framework in synthetic biology have provided long-awaited evidences that engineering principles can facilitate the construction of novel biological systems. Synthetic biology has so far demonstrated that its framework can be applied to a wide range of areas such as energy, environment, and health care. For example, novel biological systems have been constructed to produce drugs (Ro *et al.*, 2006) and biofuels (Steen *et al.*, 2010), to degrade contaminants in water (Sinha *et al.*, 2010), and to kill cancer cells (Anderson *et al.*, 2006).

Despite these encouraging advances, synthetic biology has not yet been exploited to develop new strategies for tackling infectious disease, a leading cause of death worldwide, especially in poor countries. Given the stalled development of new antibiotics and the increasing emergence of multidrug-resistant pathogens, using synthetic biology to design new treatment regimens for infectious disease could address an urgent need. Consequently, in this study, we have made progress toward developing a novel antimicrobial strategy, based on an engineered microbial system, using the synthetic biology framework. Toward this aim, we designed and constructed a genetic system, based on standardization, decoupling, and abstraction, that allows sensing and killing of *Pseudomonas aeruginosa*, a human pathogen, in a non-pathogenic chassis, *Escherichia coli*. The biological parts of our devices were designed and synthesized in compliance with the BioBrick assembly standards (Canton *et al.*, 2008). Each of the biological devices was characterized to understand its behavior, and the correlation between the input and output of one key biological device was studied in detail.

P. aeruginosa colonizes the respiratory and gastrointestinal tract (Fujitani *et al.*, 2011), and causes life-threatening infections to the patients with immunodeficiency such as

cystic fibrosis and cancer (Chang *et al*, 2005a; Small *et al*, 2007). Despite a wide range of antibiotics available in the market, *P. aeruginosa* is still among the leading causes of nosocomial infection primarily because it is intrinsically resistant to many antibiotics and antimicrobials, in part because of its effective efflux systems (Chang *et al*, 2005b). Contemporary treatments against *P. aeruginosa* infection include antibiotic chemotherapy and bacteriophage therapy. In antibiotic chemotherapy, a combinatorial treatment involving multiple antimicrobial agents is usually preferred over monotherapy due to the rapid acquisition of drug tolerance in *P. aeruginosa*. This approach, however, promotes unspecific killing of bacteria and upsets a healthy human microbiome. Phage therapy involves strain-specific bacteriophages that invade and destroy the cellular integrity of pathogens (Wright *et al*, 2009). The therapeutic potential of employing virus in bacterial infection, however, is limited, as a directed treatment cannot be re-employed after the infected host develops specific antibodies against the introduced virus (Häusler, 2006).

Certainly, combating *P. aeruginosa* infections now requires the development of novel, unconventional antimicrobial strategies that do not entirely rely on current antibiotics. To this end, we have exploited *P. aeruginosa*'s own strategy for survival in a competitive environment by means of bacteriocins, ribosomally synthesized antimicrobial peptides. Bacteriocins are specific and effective against closely related species, and thus have garnered attention as a new generation antibacterial agent (Baba and Schneewind, 1998). Pyocins are narrow-spectrum bacteriocins produced by *P. aeruginosa*. Contrary to traditional antibiotics, the acquisition of pyocin resistance by lateral gene transfer between bacteria has not yet been encountered, supporting the use of pyocins in targeting *P. aeruginosa* infection (Scholl and Martin, 2008). In general, pyocins are classified into three types: R, F, and S. An earlier study estimated that R and F type pyocins are synthesized by 90% of all *P. aeruginosa* strains and S type by 70% (Smith *et al*, 1992). S-type pyocins are soluble and comprise two protein molecules associated in complex, with parallel structural and functional features similar to DNase-based colicins. The larger protein has been identified as the functional protein for killing while the smaller protein is regarded as the immunity protein that confers the host cell with defense mechanism against its own pyocin (Seo and Galloway, 1990). Our group has recently reported that pyocin S5 exhibits strong bactericidal activity against *P. aeruginosa* clinical isolates through membrane damage but is ineffective against *E. coli* (Ling *et al*, 2010). Together with its characteristics of being soluble and medium sized at 498 amino acids, pyocin S5 presents itself as an ideal candidate for a killing molecule in an *E. coli* chassis.

Further, to enable our engineered microbes to produce pyocin S5 only in response to the presence of *P. aeruginosa*, we exploited the quorum sensing mechanisms of *P. aeruginosa*. Quorum sensing is an organic chemical signaling cascade that regulates a myriad of physiological activities such as cell motility, virulence, biofilm formation, and growth (Chang *et al*, 2005b). This sensing mechanism is mediated by various diffusible, chemical signals known as autoinducers that are produced by the synthase genes of the bacteria. The extracellular concentration of signaling molecules increases as a function of cell density and is permeable to cell

membrane. Upon attaining a threshold concentration of the chemical signals, the quorum sensing cascade is activated to elicit expressions or repressions of multiple genes, including those that are functional for production of autoinducers such as acyl homoserine lactones (AHLs; Asad and Opal, 2008). Although similar production mechanisms are present in some Gram-negative bacteria, each synthase homolog producing AHLs differs in either length or functional groups (e.g., hydroxyl and carbonyl groups) on the acyl side chain. Thus, with each bacterium possessing disparate synthase sequence, a high level of specificity can be achieved during intercellular quorum communication (Schuster, 2004; Smith *et al*, 2008).

To enable an effective release of pyocin S5, we utilized E7 lysis protein to lyse the *E. coli* chassis. The E7 lysis protein is a key component of the SOS response system in colicin-producing cells and functions to export bacteriocins into the extracellular space under stressful environmental conditions (Chak *et al*, 1991). Recent studies have shown that the E7 lysis protein is effective in causing inner membrane damage and maybe associated with the activation of outer membrane phospholipase A for outer membrane modification (Lin *et al*, 2009). In addition to being specific to *E. coli*, the E7 lysis protein is small at 47 amino acids and can be easily utilized as a modular part in the assembly of novel genetic circuits.

Taken together, our final system was designed to (i) detect AHLs produced by *P. aeruginosa*; (ii) produce pyocin S5 upon the detection; and (iii) lyse the *E. coli* cells by E7 lysis protein so that the produced pyocin S5 is released from the cells, leading to the killing of *P. aeruginosa*. In this paper, we demonstrate that our engineered *E. coli* effectively senses and kills *P. aeruginosa*, which provides a novel synthetic biology-based antimicrobial strategy that could be applied to eradicating other infectious pathogens.

Results and discussion

Design of the sensing and killing genetic system

Figure 1 shows a schematic of our sensing and killing genetic system. The sensing device was designed based on the Type I quorum sensing mechanism of *P. aeruginosa*. The *tetR* promoter, which is constitutively on, produces a transcriptional factor, LasR, that binds to AHL 3OC₁₂HSL. The *luxR* promoter, to which LasR-3OC₁₂HSL activator complex reportedly binds, was adopted as the inducible promoter in our sensing device (Gray *et al*, 1994). Next, the formation of the LasR-3OC₁₂HSL complex, which binds to the *luxR* promoter, activates the killing and lysing devices, leading to the production of pyocin S5 and lysis E7 proteins within the *E. coli* chassis. Upon reaching a threshold concentration, the lysis E7 protein perforates membrane of the *E. coli* host and releases the accumulated pyocin S5. Pyocin S5, which is a soluble protein, then diffuses toward the target pathogen and damages its cellular integrity, thereby killing it. Supplementary Figure 1A shows the plasmid map of the final system.

Characterization of the sensing device

To evaluate and characterize the sensing device, the gene encoding the green fluorescent protein (GFP) was fused to the

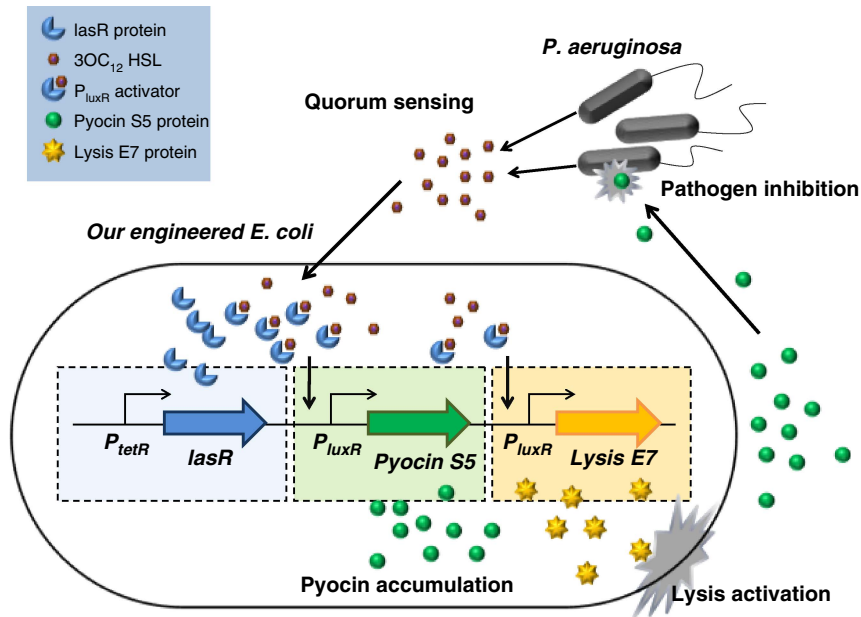


Figure 1 Schematic of 'Pathogen Sensing and Killing' system. *luxR* promoter is activated by LasR-3OC₁₂HSL complex, leading to production of E7 lysis protein and S5 pyocin within *E. coli* chassis. After the E7 protein attains the threshold concentration that causes the chassis to lyse, the accumulated S5 is released into the exogenous environment and kills *P. aeruginosa*.

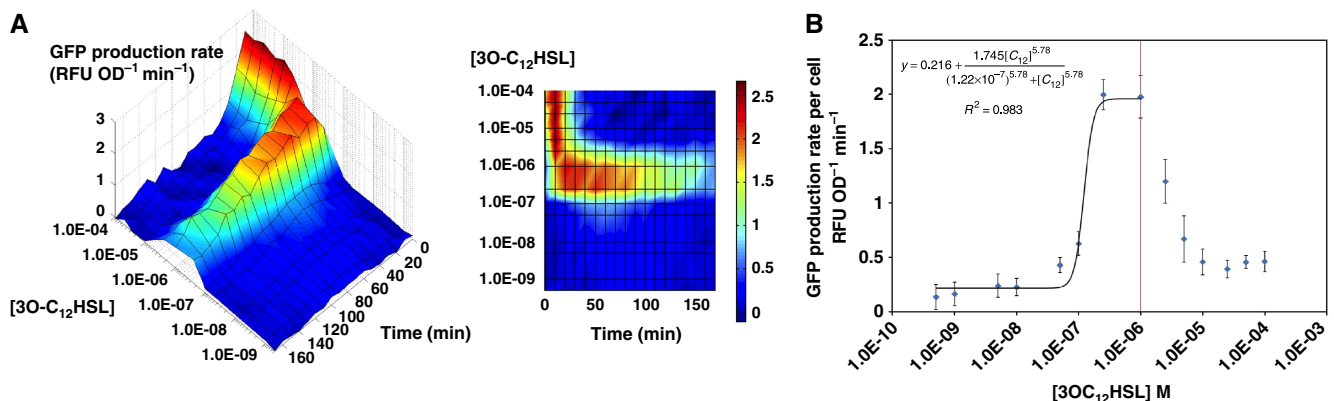


Figure 2 Characterization results of sensing device coupled with GFP reporter. **(A)** GFP production rate per cell over time at different 3OC₁₂HSL inducer concentrations. **(B)** Time-averaged GFP production rate per cell at different input 3OC₁₂HSL concentrations, showing that the optimal operating concentrations for the sensing device range from 1.0E-7 to 1.0E-6 M 3OC₁₂HSL. Error bar represents the standard deviation of statistical means between 20 and 80 m after induction. The experiment was performed with six replicates. Source data is available for this figure at www.nature.com/msb.

sensing device (i.e., pTetR-LasR-pLuxR-GFP; the plasmid map is shown in Supplementary Figure 1B) and the GFP expression was monitored at a range of concentrations of 3OC₁₂HSL. From the measured GFP synthesis rates (Figure 2A), we observed a basal expression level of 0.216 RFU per OD per minute without induction, followed by a sharp increase in GFP production rate as the concentration of 3OC₁₂HSL was increased beyond 1.0E-7 M. This transition peaked at 1.0E-6 M of 3OC₁₂HSL and exhibited a sharp decline afterward. These results suggest that the optimal detection range of the sensing device was between 1.0E-7 and 1.0E-6 M 3OC₁₂HSL. Note that previous studies estimated extracellular concentration of 3OC₁₂HSL to be in the range of 1.0E-6 to 1.0E-4 M within proximity to the site of *P. aeruginosa* infection (Pearson *et al*, 1995; Charlton *et al*, 2000).

Transfer function of the sensing device

One important characteristic of the sensing device was the transfer function that describes the static relationship between the input (3OC₁₂HSL) and output (GFP production rate) of the sensing device. The transfer function was determined by fitting an empirical mathematical model (Hill equation) to the experimental data where the input 3OC₁₂HSL concentration is <1.0E-6 M. The resulting best fit model demonstrated that the static performance of the sensing device follows a Hill equation below the input concentration of 1.0E-6 M 3OC₁₂HSL (Figure 2B). The model showed that the sensing device saturated at a maximum output of 1.96 RFU per OD per minute at input concentration >3.3E-7 M but <1.0E-6 M

3OC₁₂HSL, and the switch point for the sensing device was 1.2E-7 M 3OC₁₂HSL, the input concentration at which output is at half-maximal. Since this switch point concentration is smaller than the concentration of 3OC₁₂HSL present (1.0E-6 to 1.0E-4 M) within proximity to the site of *P. aeruginosa* infection as earlier reported in the literature (Pearson *et al*, 1995; Charlton *et al*, 2000), the sensing device would be sensitive enough to detect the amount of 3OC₁₂HSL natively produced by *P. aeruginosa*.

Detection of the native autoinducer produced by *P. aeruginosa*

As stated above, the characterization of the sensing device indicated that it produced an optimal output at 1.0E-7 to 1.0E-6 M 3OC₁₂HSL. To verify that the sensing device would be able to sense the amount of 3OC₁₂HSL natively produced by *P. aeruginosa*, the sensing device coupled with a GFP reporter (i.e., pTetR-LasR-pLuxR-GFP) was induced using the filtered culture of *P. aeruginosa* ln7, a clinical isolate that is sensitive to pyocin S5 (Ling *et al*, 2010). Results show that GFP synthesis rate measured for the isolate ln7 was 1.375 RFU per OD per minute. This value was above the minimum synthesis rate and greater than the half-maximal of the sensing device. This result confirmed that the sensing device was able to detect the natively produced 3OC₁₂HSL. Further, we used the GFP synthesis rate measured and the model (Equation 1, Materials and methods) derived in the earlier experiment to gain an insight into the amount of 3OC₁₂HSL natively produced by the isolate. The average concentration of 3OC₁₂HSL in the liquid culture of the *P. aeruginosa* strain was estimated to be ~1.0E-6 M 3OC₁₂HSL. This result was coherent with previous studies that had estimated that the extracellular concentration of 3OC₁₂HSL is between 1.0E-6 and 1.0E-4 M (Pearson *et al*, 1995; Charlton *et al*, 2000).

Characterization of the lysing device

As stated above, our system was designed to release pyocin S5 through lysis upon detection of *P. aeruginosa*. To determine the lysis activity of the system, we characterized the behavior of the E7 lysis protein under the transcriptional control of the sensing device before integrating both the pyocin S5 and E7 genes into the system. The E7 lysis gene was ligated downstream to the sensing device (i.e., pTetR-LasR-pLuxR-E7; the plasmid map is shown in Supplementary Figure 1C) and its performance was evaluated in the *E. coli* chassis over time by measuring absorbance at OD₆₀₀ at a range of concentrations of 3OC₁₂HSL. Figure 3A shows that at 0 and 1.0E-8 M 3OC₁₂HSL, the growth rates of *E. coli* underwent no noticeable transition into a lysis state. However, at higher concentrations of 3OC₁₂HSL (i.e., 1.0E-6 and 1.0E-4 M), the cells exhibited a significant reduction in optical density, likely due to the lysis activity. Our results imply that 1.0E-6 M or higher concentrations of 3OC₁₂HSL cause observable cell lysis with a delay of ~120 m. To verify the effect of the lysis, cell integrity was examined with and without 1.0E-6 M 3OC₁₂HSL using field-emission scanning electron microscopy (FESEM). Figure 3B shows that *E. coli* containing pTetR-LasR-pLuxR-E7 and

induced with 3OC₁₂HSL appeared shriveled with corrugated surface morphology, in contrast to the distinct 'rod-like' features of the cells that were not induced with 3OC₁₂HSL. To further confirm that the lysis activity can be sustained in the final system including pyocin S5, the morphology of *E. coli* containing the final system (i.e., pTetR-LasR-pLuxR-S5-pLuxR-E7; Figure 1) was monitored using FESEM. Figure 3C shows that *E. coli* cells having the final system and induced with 3OC₁₂HSL also appeared shriveled with corrugated surface morphology, whereas *E. coli* cells having the final system but not induced with 3OC₁₂HSL remained 'rod-like' in shape. These observations were similar to that obtained in our earlier experiment with *E. coli* containing pTetR-LasR-pLuxR-E7. This result suggests that 3OC₁₂HSL induced the lysis of the *E. coli* containing the final system.

In line with the overall objective of the E7 lysis device in mediating the export of pyocin, we studied the efficiency of the lysis device in the final system by measuring the amount of the released protein. After induction with 1.0E-6 M 3OC₁₂HSL, histidine-tagged S5 protein was purified by immobilized metal affinity chromatography from the filtered supernatant and analyzed by SDS-PAGE and Bradford assay. Figure 4A shows that distinct bands that corresponded to pyocin S5 were observed on the SDS-PAGE of the final system (i.e., pTetR-LasR-pLuxR-S5-pLuxR-E7), while no bands were seen in lanes without the lysis device (i.e., pTetR-LasR-pLuxR-S5; the plasmid map is shown in Supplementary Figure 1D). We further validated the results by estimating the protein concentrations in the supernatant with Bradford assay and showed that the amount of pyocin released by our final system was eight times higher than the system without the lysis device (Figure 4B). The dynamic performance of the lysis device in the final system was characterized by an impulse release of protein 2 h after induction, followed by a steady-state response.

Verification of the final system with the sensing, killing, and lysing devices

We have so far demonstrated that our engineered microbes are able to sense natively produced AHL 3OC₁₂HSL, which subsequently triggers cell lysis. To further determine whether the sensing of 3OC₁₂HSL also leads to killing of *P. aeruginosa* as designed, the growth of *P. aeruginosa* was monitored in the presence of the engineered *E. coli* containing the final system. First, to determine the concentration of 3OC₁₂HSL that causes a significant growth inhibition and confirm that the concentration falls within the range of concentrations of 3OC₁₂HSL naturally produced by *P. aeruginosa*, the engineered *E. coli* was exposed to commercial 3OC₁₂HSL at 0, 1.0E-8, 1.0E-6, and 1.0E-4 M, and the filtered supernatants were added onto *P. aeruginosa*-grown agars. We observed that the growth of *P. aeruginosa* was clearly inhibited by the filtered supernatants of the *E. coli* cultures exposed to 1.0E-6 and 1.0E-4 M 3OC₁₂HSL, whereas very faint inhibition zones were observed at 0 and 1.0E-8 M, likely due to the basal expression of pyocin S5 and E7 (Supplementary Figure 2A). Second, to further confirm the inhibition effects, *P. aeruginosa* was examined upon exposure to the supernatant of the *E. coli* cultures with

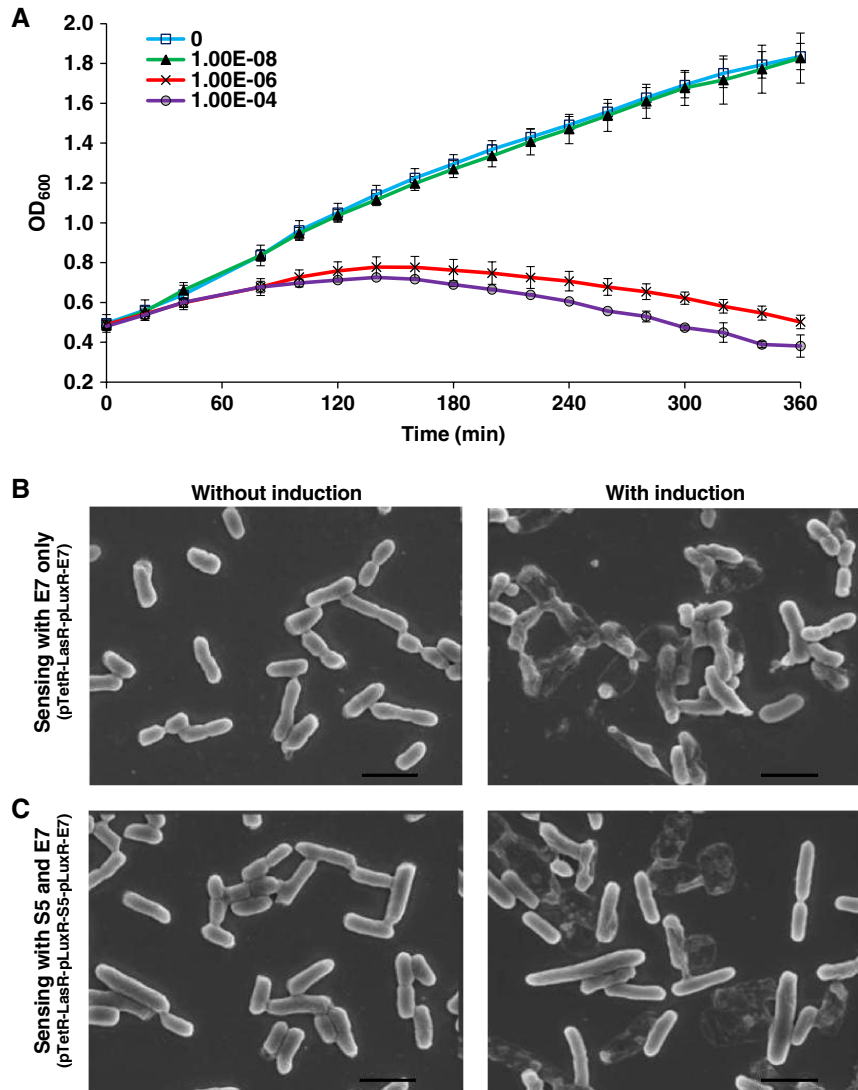


Figure 3 Characterization results of lysis device using 3OC₁₂HSL. **(A)** Growth curve of *E. coli* expressing E7 lysis protein after induction with different concentrations of 3OC₁₂HSL. **(B, C)** Effects of lysis protein on *E. coli* surface morphology as observed using FESEM. The results show that the surface of the *E. coli* was damaged when *E. coli* carrying pTetR-LasR-pLuxR-E7 and *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) were induced with 3OC₁₂HSL. Scale bar: 1 μ m. Error bar represents the standard deviation of four replicates. Source data is available for this figure at www.nature.com/msb.

1.0E-6 M 3OC₁₂HSL using the LIVE/DEAD cell viability assay. As seen under microscope, many *P. aeruginosa* cells exposed to the supernatant of the engineered *E. coli* were stained with the PI dye, which stains a dead cell, whereas those that were incubated with the wild-type *E. coli* were mostly stained with the SYTO 9 dye, which stains a live cell (Supplementary Figure 2B). This result suggests that our engineered *E. coli* carrying the final system can kill *P. aeruginosa* in response to as low as 1.0E-6 M 3OC₁₂HSL. Since our earlier estimation indicated that the concentration of 3OC₁₂HSL natively produced by *P. aeruginosa* was \sim 1.0E-6 M, this outcome may imply that this killing activity would be sustained against *P. aeruginosa* in response to its producing 3OC₁₂HSL.

Therefore, subsequently, to confirm the killing activity by the native 3OC₁₂HSL produced by *P. aeruginosa*, the filtered supernatant of *P. aeruginosa* cultures was mixed with the

E. coli cultures, whose supernatant was then added to *P. aeruginosa*-grown agars. Figure 5A shows that *P. aeruginosa* growth was significantly inhibited by the engineered *E. coli* cultures exposed to the supernatant of *P. aeruginosa* cultures, while neither with the wild-type *E. coli* cells nor without the *P. aeruginosa* supernatant led to growth inhibition. This result indicates that our final system produces pyocin S5 and E7 in response to the 3OC₁₂HSL natively produced by *P. aeruginosa*, which resulted in the killing of *P. aeruginosa*.

To further visualize the inhibition effects on *P. aeruginosa* by our engineered *E. coli*, *P. aeruginosa* cells were stained using the LIVE/DEAD cell viability assay. Figure 5B shows that many *P. aeruginosa* cells exposed to the supernatant of the engineered *E. coli* induced with native 3OC₁₂HSL were stained with the PI dye, whereas the cells incubated with the wild-type *E. coli* were mostly stained with the SYTO 9 dye (green). This

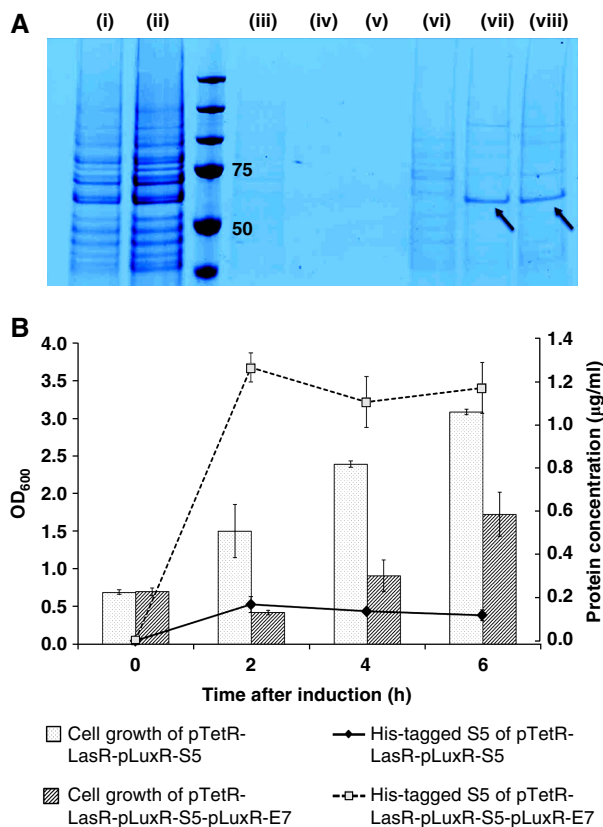


Figure 4 Characterization results of the lysis device in the final system using 3OC₁₂HSL. **(A)** SDS-PAGE of (i, ii) total extracellular proteins and (iii–viii) IMAC purified His-tagged S5 protein sampled from the extracellular supernatant. Total extracellular proteins exported from (i) *E. coli* carrying pTetR-LasR-pLuxR-S5 (without lysis device) was significantly lesser than that exported from (ii) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) as indicated in darker lanes of (ii) relative to (i). (iii–v): *E. coli* carrying pTetR-LasR-pLuxR-S5 (without lysis device) at 0, 2, and 4 h after induction. (vi–viii) *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (the final system) at 0, 2, and 4 h after induction. The results show that pyocin S5 (57 kDa; arrowed) was only detectable in lanes that corresponded to *E. coli* carrying the final system and not in lanes of *E. coli* without the lysis device. Ladder used was Bio-Rad's Precision Plus Protein standards. **(B)** Characterization of lysis device in the final system by optical density (bar graphs) and concentration of pyocin released (lines) after induction. The results show an impulse release of pyocin S5 at 2 h after induction, followed by a sustained steady-state release in the final system (dotted lines). Optical density of the final system was characterized by an initial decrease at 2 h after induction, indicative of the onset of lysis, after which the regrowth of engineered *E. coli* occurs (shaded bar). Correspondingly, the concentration of pyocin released in *E. coli* without the lysis device (solid line) was 1/8 that of the final system with a continually increasing optical density (unshaded bar). Error bar represents the standard deviation of two replicates. Source data is available for this figure at www.nature.com/msb.

result suggests that our engineered *E. coli* carrying the final system can kill *P. aeruginosa* in the presence of native 3OC₁₂HSL produced by *P. aeruginosa*.

To verify that our engineered *E. coli* that contains the final system (i.e., pTetR-LasR-pLuxR-S5-pLuxR-E7) exerts a killing activity against *P. aeruginosa* in a mixed culture, we monitored the growth of *P. aeruginosa* co-cultured with the engineered *E. coli* in the ratio 1:4. To determine the growth inhibition of *P. aeruginosa* in the mixed culture, we used *P. aeruginosa* that constitutively expresses GFP and *E. coli* that is without either the pyocin S5 or E7 lysis devices as negative controls.

Figure 5C shows that the GFP expression level of the *P. aeruginosa* co-cultured with the *E. coli* that carries the final system remained low and almost constant, whereas the GFP level underwent an exponentially increase when *P. aeruginosa* was cultured with the negative control *E. coli* systems. To verify the efficiency in growth inhibition, we also performed CFU count on mixed cultures using *P. aeruginosa* that was transformed with chloramphenicol-resistant plasmid. Figure 5D shows that our engineered *E. coli* inhibited the growth of *P. aeruginosa* by >99% while continuous growths were apparent in *P. aeruginosa* co-cultured with incomplete *E. coli* systems missing either the pyocin S5 or E7 lysis devices. The result also implies that our engineered system was activated only after the pathogen entered the late exponential and stationary phase when the autoinducers were released (Supplementary Figure 2C).

To examine the potential application of our engineered system against a *pseudo* disease state of *Pseudomonas*, a static biofilm inhibition assay was performed by culturing *P. aeruginosa* carrying a chloramphenicol-resistance plasmid with the engineered *E. coli*. Figure 6A shows that our engineered *E. coli* inhibited the formation of *P. aeruginosa* biofilm by close to 90%. This observation is in stark contrast to the pyocin-resistant control strain PAO1 and pyocin-sensitive clinical isolate In7 subjected to treatment with *E. coli* having the systems missing either the pyocin S5 or E7 lysis gene.

To visualize the extent of biofilm inhibition, biofilm cells with green fluorescence were grown in the presence of engineered *E. coli* on glass slide substrate and examined with confocal laser scanning microscopy (CLSM). Figure 6B shows that the morphology of *Pseudomonas* biofilm treated with the engineered *E. coli* appeared sparse while elaborated honey-combed structures were apparent in the control experiments. This observation implies that our engineered *E. coli* has the capability to inhibit biofilm formation during the initial attachment phase and prevent subsequent progression into mature microcolonies. Collectively, our results suggest that our engineered *E. coli* carrying the final system, which contains the sensing, killing, and lysing devices, can effectively inhibit the growth of *P. aeruginosa* in both planktonic and sessile states. Nonetheless, we recognize the need for further extensive experimental studies in direct disease-relevant models. In particular, future studies should examine the *in vivo* efficacy of the engineered *E. coli* in controlling the growth of *P. aeruginosa* in infected murine models.

Conclusion

In summary, we engineered a novel biological system, which comprises sensing, killing, and lysing devices, that enables *E. coli* to sense and eradicate pathogenic *P. aeruginosa* strains by exploiting the synthetic biology framework. We designed and synthesized all the biological parts in compliance with the BioBrick assembly standards. The sensing, killing, and lysing devices were characterized to elucidate their functionalities, which subsequently aided in the construction of the final system and the verification of its designed behavior. Based on the characterization and modeling results, we verified that the sensing device had an optimal activity approximately in the

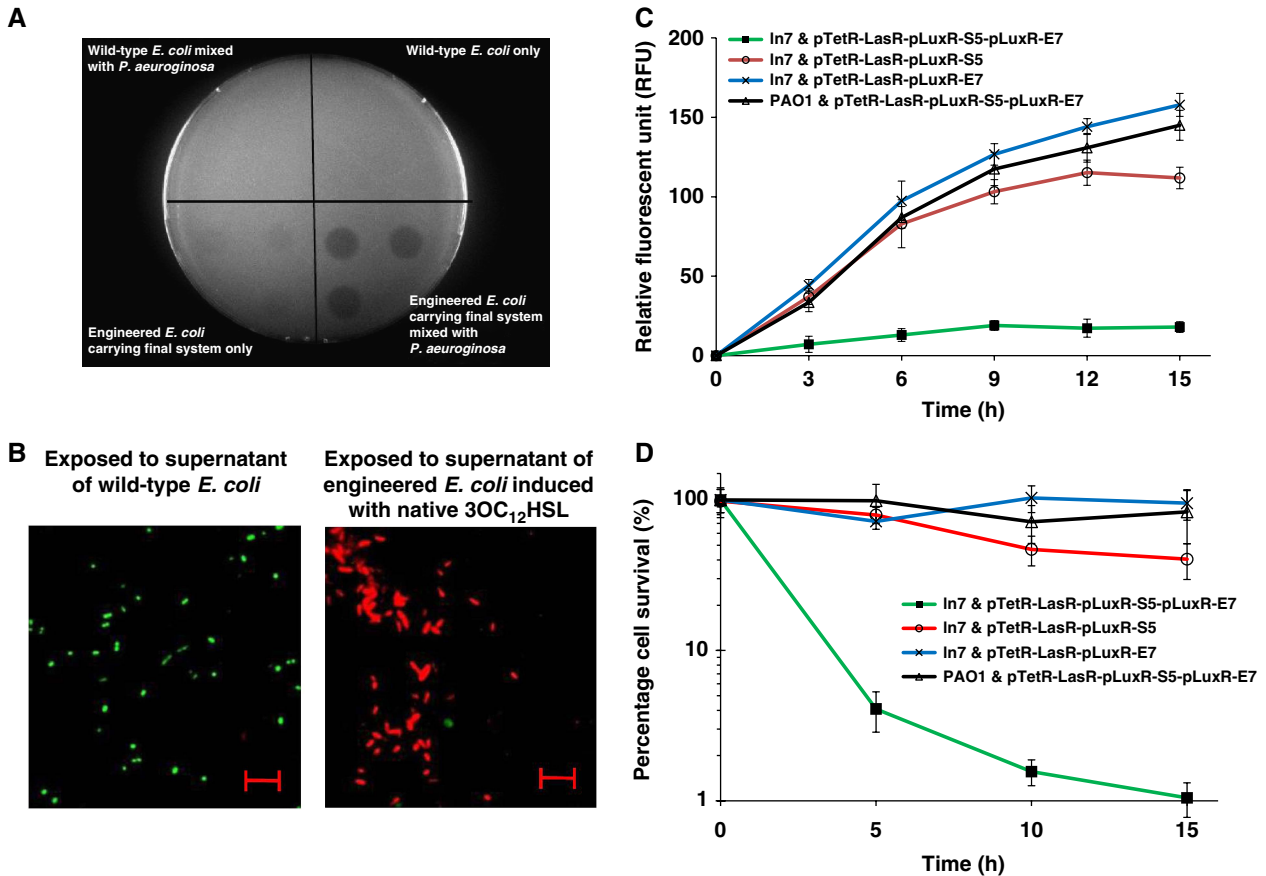


Figure 5 Inhibition of *P. aeruginosa* by the engineered *E. coli* induced with native 3OC₁₂HSL produced by *P. aeruginosa*. **(A)** Agar overlay assay of *P. aeruginosa* after exposure to supernatant of four different cultures. First, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* showed no bactericidal activity. Second, *P. aeruginosa* exposed to supernatant of wild-type *E. coli* mixed with *P. aeruginosa* produced no inhibition zones. Third, exposure to supernatant of *E. coli* carrying pTetR-LasR-pLuxR-S5-pLuxR-E7 (final system) did not produce any inhibition as well. Fourth, only *P. aeruginosa* exposed to supernatant of *E. coli* carrying final system with *P. aeruginosa* displayed clear inhibition zones, which suggested that the system produced sufficient pyocin S5 to exhibit bactericidal activity. **(B)** *P. aeruginosa* cells stained using the LIVE/DEAD cell viability assay. Many *P. aeruginosa* cells were stained with PI dye, which indicate dead cells, when exposed to supernatant of engineered *E. coli* carrying the final system that was induced by native 3OC₁₂HSL produced by *P. aeruginosa*. Scale bar: 5 μ m. **(C)** Fluorescence measurement of *P. aeruginosa* that constitutively expresses GFP in mixed culture with engineered *E. coli* carrying pTetR-LasR-pLuxR-S5 shows an exponential increase in the fluorescence readings, whereas the mixed culture with *E. coli* carrying pTetR-LasR-pLuxR-E7 (the final system) exhibited no increase in the readings. This suggests that the growth of *P. aeruginosa* was significantly inhibited in the mixed culture with engineered *E. coli* carrying the final system. PAO1, which pyocin S5 was derived from, was included as a negative control. Error bar represents the standard deviation of six replicates. **(D)** Percentage survival of *P. aeruginosa* carrying chloramphenicol-resistant plasmid in mixed culture with the engineered *E. coli*. *Pseudomonas* in the mixed culture was quantified by viable cell count using chloramphenicol selection. The result shows that our engineered *E. coli* inhibited the growth of *Pseudomonas* by 99%. In contrast, inhibition was less observed in *Pseudomonas* co-cultured with incomplete *E. coli* systems missing either the pyocin S5 killing device or E7 lysis device. Error bar represents the standard deviation of three replicates. Source data is available for this figure at www.nature.com/msb.

range of 1.0E-7 to 1.0E-6 M 3OC₁₂HSL, which was in line with the concentration of 3OC₁₂HSL secreted by *P. aeruginosa*. We further confirmed the activity of the killing and lysing devices in response to this range of 3OC₁₂HSL concentration. Finally, we demonstrated that our engineered *E. coli* with the complete system effectively kills *P. aeruginosa* in both planktonic and biofilm states when those two microbes were grown together. As a proof of concept, *E. coli*, a natural inhabitant of the gastrointestinal tract, was chosen as the chassis in this study. The synthetic biology framework and genetic devices developed in this work could potentially be transferred into other microbial chassis such as probiotics and residential microbes of the upper respiratory tract (Brook, 2005; Charlson *et al*, 2010). Further, this study presents the possibility of engineer-

ing potentially beneficial microbiota into therapeutic bioagents to arrest *Pseudomonas* infection. Given the stalled development of new antibiotics and the increasing emergence of multidrug-resistant pathogens, this study provides the foundational basis for a novel synthetic biology-driven antimicrobial strategy that could be extended to include other pathogens such as *Vibrio cholera* and *Helicobacter pylori*.

Materials and methods

Strains and media

All cells involved in cloning and characterization experiments are *E. coli* TOP10 (Invitrogen) unless otherwise stated. Commercial

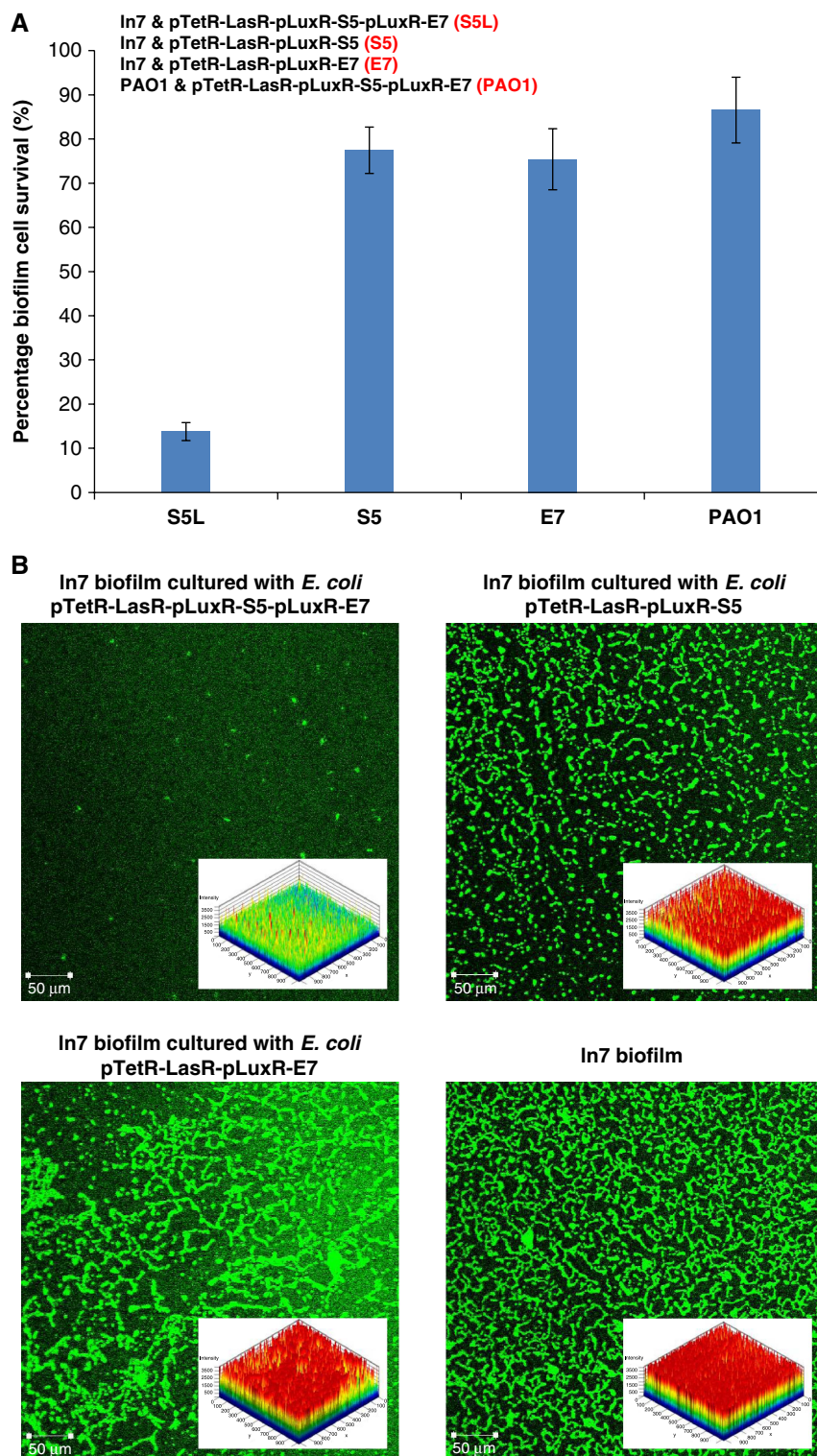


Figure 6 Biofilm inhibition assay with engineered *E. coli*. **(A)** Percentage survival of *P. aeruginosa* biofilm carrying chloramphenicol-resistant plasmid. *Pseudomonas* biofilm was grown in a polystyrene 24-well plate in the presence of the engineered *E. coli* for 18 h and quantified by viable cell count using chloramphenicol selection. The results imply that the formation of *Pseudomonas* biofilm was inhibited by close to 90% with the engineered *E. coli* carrying the final system (pTetR-LasR-pLuxR-S5-pLuxR-E7) as compared with biofilm grown with wild-type *E. coli* or incomplete *E. coli* system missing either pyocin S5 or E7 lysis genes. *P. aeruginosa* PAO1, which pyocin S5 was derived from, was included as a negative control. Error bar represents the standard deviation of six replicates. **(B)** Biofilm inhibition observed under CLSM microscopy. *Pseudomonas* biofilm with green fluorescence was grown on glass slide in the presence of the engineered *E. coli* and visualized under CLSM microscope after 18 h. Images reconstructed from biofilm Z-stacks using Zeiss 2.5D software implied that the initialization and progression of biofilm cells into multilayers were strongly inhibited for *Pseudomonas* grown with *E. coli* carrying the final system as opposed to lush and elaborated biofilm formation observed in *Pseudomonas* grown alone or with incomplete *E. coli* system missing either pyocin S5 or E7 lysis genes. Scale bar: 50 μ m. Z-stack: 40 μ m. Source data is available for this figure at www.nature.com/msb.

Luria-Bertani (LB) and Muller Hinton (MHB) were used as the medium for cloning and inhibition studies unless otherwise stated. Supplemented M9 (M9 salts, 1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 0.1 M MgSO₄, 0.5 M CaCl₂) was used as the medium for the characterization. Ampicillin (100 µg/ml) was added to the culture media for antibiotic selection where appropriate. Homoserine lactone (3OC₁₂HSL; Sigma-Aldrich) was used for characterization experiments. All restriction and ligation enzymes were purchased from New England Biolabs (NEB). Supplementary Table 1 summarizes all plasmids, Biobrick parts, and devices used in this study. Genetic mapping of representative engineered constructs is illustrated in Supplementary Figure 1.

System assembly

The genetic constructs developed in this study were assembled using standard synthetic biology protocols (Canton *et al*, 2008). Briefly, for front insertion of Biobrick parts, purified insert and vector plasmids were digested with *EcoRI/SpeI* and *EcoRI/XbaI* respectively. For back insertion to upstream vector, the insert and vector plasmids were digested with *XbaI/PstI* and *SpeI/PstI* in that order. Digested fragments were separated by DNA gel electrophoresis and ligated with NEB Quick Ligase in accordance with the manufacturer's instructions. Plasmids from chemically transformed cells were purified by affinity columns and verified by DNA sequencing.

Characterization of pTetR-LasR-pLuxR-GFP with 3OC₁₂HSL

Single colonies of pTetR-LasR-pLuxR-GFP (Top10) were each inoculated into 5 ml of prewarmed supplemented M9 ampicillin for overnight culture in a shaking incubator at 37°C. After overnight growth, the cultures were diluted to OD₆₀₀ of 0.002 and allowed to incubate further to OD₆₀₀ of 0.5 or 5.0E7 cfu/ml under the same condition. Cultures were then transferred into a transparent, flat-bottom 96-well plate in triplicate aliquots of 200 µl for induction with 3OC₁₂HSL at varying molar concentrations (0, 5.0E-10, 1.0E-9, 5.0E-9, 1.0E-8, 5.0E-8, 1.0E-7, 2.5E-7, 5.0E-7, 1.0E-6, 5.0E-6, 1.0E-6, 5.0E-5, and 1.0E-4 M). The plate was incubated at 37°C with rapid shaking in a microplate reader (Biotek) and assayed for green fluorescence. Time-series fluorescence and OD₆₀₀ data were obtained at intervals of 10 m for a total run time of 3 h. The result was zeroed with supplemented M9 to remove background fluorescence and OD₆₀₀. A relative GFP production rate was derived as a ratio of background subtracted green fluorescence to OD₆₀₀ value. A time-averaged GFP synthesis rate was obtained by averaging the relative GFP production rates between 20 and 80 m after induction with 3OC₁₂HSL. The experimental results were fitted using an empirical mathematical model (Hill equation),

$$y = A + \frac{B[C_{12}]^n}{C^n + [C_{12}]^n} \quad (1)$$

Equation 1 models GFP synthesis rate (y) as a function of input concentration of 3OC₁₂HSL ($[C_{12}]$). The four parameters (A , B , C , n) were estimated to obtain the best fit curve by performing a non-linear curve fitting using the experimental results. This curve fitting was performed using MATLAB Curve Fitting Toolbox (The Mathworks, Natick, MA, USA).

Detection of the native autoinducer produced by *P. aeruginosa*

GFP production rates induced by 3OC₁₂HSL natively produced from *P. aeruginosa* were measured with pTetR-LasR-pLuxR quorum sensor as described above. Briefly, overnight cultures of pTetR-LasR-pLuxR-GFP (Top10) were diluted in Supplemented M9. Diluted *Pseudomonas* cultures were grown to a late logarithmic phase and filtered with a filter membrane (0.22 µm). Sterile filtrates containing 3OC₁₂HSL were mixed with pTetR-LasR-pLuxR-GFP culture to activate GFP production. The resultant mixtures were transferred into a transparent, flat-bottom 96-well plate in triplicate aliquots of 200 µl to be assayed for GFP

production rates in a microplate reader (Biotek) at 37°C with rapid shaking. The rates obtained were then compared with the Hill function mathematical model derived earlier using 3OC₁₂HSL to estimate the native 3OC₁₂HSL concentration from *P. aeruginosa* In7.

Characterization of lysis device with 3OC₁₂HSL

Overnight cultures of pTetR-LasR-pLuxR-E7 (Top10) were diluted in supplemented M9 and harvested at an OD₆₀₀ of 0.5. The resultant cultures were transferred into a transparent, flat-bottom 96-well plate in triplicate aliquots of 200 µl for induction with 3OC₁₂HSL at varying concentrations (i.e., 0, 1.0E-8, 1.0E-6, and 1.0E-4 M). The plate was incubated at 37°C with rapid shaking in a microplate reader (Biotek) and assayed for cell turbidity. Time-series absorbance at OD₆₀₀ was obtained at intervals of 10 m for a total run time of 6 h. The result was zeroed with supplemented M9 to remove background absorbance.

FESEM assay

To examine the effect of E7 lysis protein on cell morphology, re-inoculated cultures of pTetR-LasR-pLuxR-E7 (Top10) and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were induced with 1.0E-6 M 3OC₁₂HSL at OD₆₀₀ of 0.5 and cultured for 2 h. Cell pellets collected after centrifugation at 4000 r.p.m. for 15 m were washed with 0.1 M sodium cacodylate (pH 7.4) three times before fixation with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 2 h of incubation at 4°C. Cell pellets were further washed three times with sodium cacodylate after fixation and resuspended in 0.1 M sodium cacodylate (volume depends on cell amount). In all, 2 µl of sample was loaded onto PEI-coated silicon slide followed by incubation at 25°C for 30 m. The loaded silicon slide was fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate at 25°C for 90 m. Silicon slide was then dehydrated in serial concentrations of absolute ethanol (37, 67, 95% and three times of 100%) for 15 m each before drying in a vacuum evaporator overnight. Coating of silicon slide was performed with 20 nm of gold-palladium alloy (60:40) and examined using a field-emission scanning electron microscope (JSM-6700F FESEM) at 10 kV

Characterization of lysis device by protein release in engineered *E. coli*

To characterize the efficiency of the lysis device in mediating pyocin release, pTetR-LasR-pLuxR-S5-pLuxR-E7 and pTetR-LasR-pLuxR-S5 plasmids were first labeled with hexa-histidine tags on the 3' terminus of S5 gene with *pfu* polymerase (Promega) and transformed into *E. coli* Top10. Overnight cultures of the His-tag version of pTetR-LasR-pLuxR-S5-pLuxR-E7 and pTetR-LasR-pLuxR-S5 were then diluted in LB and harvested at an OD₆₀₀ of 0.7. The collected cultures were induced with 1.0E-6 M 3OC₁₂HSL and incubated for 6 h in a shaking flask culture set at 37°C and 170 rpm. At regular intervals of 2 h, cell cultures were drawn and filter sterilized (0.22 µm). The filtered cultures were mixed with 1/10 volume of 100% (w/v) trichloroacetic acid (Sigma-Aldrich) and incubated on ice for an hour to allow protein precipitation, before being washed with an equal volume of acetone. Precipitated proteins were reconstituted in 1 ml of reconstitution solvent (1 × PBS, 30 mM imidazole and 4 M urea; pH 6.0) and purified by immobilized metal affinity chromatography using Vivapure miniprep MC (Sartorius Stedim Biotech GmbH) in accordance to the manufacturer's instruction. Finally, purified pyocin proteins were analyzed by SDS-PAGE (Chen and Leong, 2009; Huang *et al*, 2009) and Bradford assay (Bradford, 1976).

Overlay inhibition assay with 3OC₁₂HSL and the final system

Overnight cultures of pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10), *P. aeruginosa* In7 and PAO1 were diluted in LB and harvested at OD₆₀₀ of 0.7 and 0.2 separately. Collected cultures of pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were induced with varying molar concentrations of 3OC₁₂HSL (0, 1.0E-8, 1.0E-6, and 1.0E-4 M) and

incubated for 2 h before being filtered with a filter membrane (0.22 μm). In all, 30 μl of sterile filtrate from each induced sample containing soluble S5 was spotted onto trypticase soy agar (TSA) plate in triplicates. Upon drying of spots, 0.1 ml of In7 at OD₆₀₀ of 0.2 in soft agar (1% peptone, 0.5% agar) prewarmed at 55°C was thinly filmed over the spotted TSA and allowed to dry completely. Resultant TSA plate was then incubated for 6 h at 37°C before image analysis with Bio-Rad ChemiDoc XRS. To evaluate the effectiveness of the engineered system coupled with the sensing function, overnight culture of *P. aeruginosa* In7 was also harvested at OD₆₀₀ of 1.0 after redilution. The culture was filtered with a filter membrane (0.22 μm) and the sterile filtrate obtained, containing planktonic 3OC₁₂HSL was used to induce pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10). These procedures were repeated to capture inhibitory images for the engineered system that was activated by 3OC₁₂HSL natively produced from *P. aeruginosa*.

Co-culturing of the engineered *E. coli* and *P. aeruginosa*

GFP reporter plasmid pMRP9-1 and chloramphenicol-resistant plasmid pAWG1.1 were transformed into *P. aeruginosa* In7 and PAO1 using a method described before (Choi *et al*, 2006). Overnight cultures of *P. aeruginosa* (In7/PAO1 with pMRP9-1), pTetR-LasR-pLuxR-S5 (Top10), pTetR-LasR-pLuxR-E7 (Top10), and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were diluted and harvested at an OD₆₀₀ of 1.0. pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) was added to In7 or PAO1 in the ratio 4:1 to obtain a mixed culture with an overall cell density of 1.0E8 cfu/ml in 25 ml of MHB. The resultant mixture was grown for 15 h in a shaking flask culture set at 37°C and 170 rpm. For fluorescence assays, the mixed culture was transferred into a transparent, flat-bottom 96-well plates in aliquots of 200 μl and assayed for background subtracted green fluorescence in a microplate reader (Biotek) at regular intervals of 3 h. The same procedures were repeated for pTetR-LasR-pLuxR-S5 (Top 10) and pTetR-LasR-pLuxR-E7 (Top 10) as negative controls. For cell viability assays, aliquots of *P. aeruginosa* in the mixed culture were quantified by CFU count on chloramphenicol selective agar plates at regular intervals of 5 h. The same procedures were repeated for pTetR-LasR-pLuxR-S5 (Top 10) and pTetR-LasR-pLuxR-E7 (Top 10) as negative controls. Percentage survival of planktonic *P. aeruginosa* was determined as follows:

Percentage cell survival

$$= \frac{\text{CFU of } P. \text{ aeruginosa in treated sample at time } t \times 100}{\text{CFU of } P. \text{ aeruginosa treated with WT } E. \text{ coli at time } t} \quad (2)$$

Live and dead fluorescent microscopy

Overnight cultures of In7 and pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) were diluted in LB and harvested at an OD₆₀₀ of 0.5 and 1.0, respectively. 3OC₁₂HSL from In 7 was obtained after passing In7 culture through a filter membrane (0.22 μm) and the sterile filtrate was used to induce expression of engineered system by mixing it with pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) in 1:1 mixing ratio to a total volume of 2 ml. The resultant culture was grown for 3 h and filtered with a similar membrane to obtain sterile S5 filtrate. The filtrate was mixed with In7 at OD₆₀₀ of 1.0 in 1:1 mixing ratio to a total volume of 2 ml and incubated for 3 h. One microliter of the final culture was stained with bacterial viability kit (Invitrogen) according to the manufacturer's instruction and analyzed with a fluorescent microscope (Zeiss Axio Scope A1).

Biofilm inhibition assay

P. aeruginosa (In7/PAO1 with pAWG1-1) conferred with chloramphenicol resistance was mixed with pTetR-LasR-pLuxR-S5-pLuxR-E7 (Top10) in the ratio 1:4 to obtain a mixed culture with an overall cell density of 1.0E8 cfu/ml in 6 ml of MHB. The resultant mixture was transferred to the wells of a polystyrene microtiter plate (Iwaki) in

aliquots of 1 ml each and grown at 37°C and 150 rpm. After 18 h of growth, biofilm on the microtiter plate was rinsed and recovered in fresh MHB by sonication and quantified by CFU count on chloramphenicol-selective plate (100 $\mu\text{g/ml}$). The same procedures were repeated for In7 treated with pTetR-LasR-pLuxR-S5 (Top10) and pTetR-LasR-pLuxR-E7 (Top10), and PAO1 treated with pTetR-LasR-pLuxR-S5-pLuxR-E7 as negative controls. Percentage survival of *P. aeruginosa* biofilm was determined as follows:

Percentage biofilm survival

$$= \frac{\text{CFU of } P. \text{ aeruginosa biofilm in treated sample} \times 100}{\text{CFU of } P. \text{ aeruginosa biofilm in treated with WT } E. \text{ coli}} \quad (3)$$

Confocal microscopy of biofilm

Mixed bacteria cultures of *P. aeruginosa* (In7 with pMRP9-1) and engineered *E. coli* systems were grown in MHB in 50 ml tubes containing sterile glass slide. Biofilm developed on the glass slides after 18 h of growth was rinsed in PBS, dried, and visualized by confocal laser scanning microscopy (Zeiss LSM 510). Collected Z-stack biofilm images were reconstructed using Zeiss 2.5D software.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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