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

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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 containments 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, \textit{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 \textit{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 \textit{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 \textit{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 explored \textit{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 \textit{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 \textit{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 \textit{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 \textit{P. aeruginosa} clinical isolates through membrane damage but is ineffective against \textit{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 \textit{E. coli} chassis.

Further, to enable our engineered microbes to produce pyocin S5 only in response to the presence of \textit{P. aeruginosa}, we exploited the quorum sensing mechanisms of \textit{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 \textit{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 \textit{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 \textit{P. aeruginosa}; (ii) produce pyocin S5 upon the detection; and (iii) lyse the \textit{E. coli} cells by E7 lysis protein so that the produced pyocin S5 is released from the cells, leading to the killing of \textit{P. aeruginosa}. In this paper, we demonstrate that our engineered \textit{E. coli} effectively senses and kills \textit{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 \textit{P. aeruginosa}. The \textit{tetR} promoter, which is constitutively on, produces a transcriptional factor, LasR, that binds to AHL 3OC12HSL. The \textit{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 \textit{luxR} promoter, activates the killing and lysing devices, leading to the production of pyocin S5 and lysis E7 proteins within the \textit{E. coli} chassis. Upon reaching a threshold concentration, the lysis E7 protein perforates membrane of the \textit{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
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 below 1.0E-6 M (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 E. coli 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 min. 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.

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, we exposed 3OC12HSL to the engineered E. coli cultures 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
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 ~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).
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 PA01 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 honeycombed 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
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 μm. Z-stack: 40 μm. 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 Xbal/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 verified 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 0.05 CFU/ml under the same conditions. 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, 1.0E-8, 1.0E-6, and 1.0E-4 M]) as a function of input 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),

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

Equation 1 models GFP synthesis rate (y) as a function of input concentration of 3OC12HSL (C12). 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

P. aeruginosa was 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 ln7.

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 x 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 instructions. 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 ln7 and PA01 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 ln7 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 ln7 was also harvested at OD600 of 1.0 after redilution. The culture was diluted 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 ln7 and PA01 using a method described before (Choi et al., 2006). Overnight cultures of P. aeruginosa (ln7/PA01 with pMRP9-1), pTetR-LasR-pLuxR-S5 (Top10), pTetR-LasR-pLuxR-S5-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 ln7 or PA01 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 3 h. The same procedures were repeated for pTetR-LasR-pLuxR-E7 (Top10) and pTetR-LasR-pLuxR-E7 (Top10) as negative controls. Percentage survival of planktonic P. aeruginosa was determined as follows:

Percentage cell survival

\[
\text{Percentage cell survival} = \left( \frac{\text{CFU of P. aeruginosa in treated sample at time } t}{\text{CFU of P. aeruginosa treated with WT E. coli at time } t} \times 100 \right)
\]

Live and dead fluorescent microscopy

Overnight cultures of ln7 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 ln7 was obtained after passing ln7 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 ln7 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 (ln7/PA01 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 microtier plate was rinsed and recovered in fresh MHB by sonication and quantified by CFU count on chloramphenicol-selective plate (100 μg/ml). The same procedures were repeated for ln7 treated with pTetR-LasR-pLuxR-S5 (Top10) and pTetR-LasR-pLuxR-E7 (Top10), and PA01 treated with pTetR-LasR-pLuxR-S5-pLuxR-E7 as negative controls. Percentage survival of P. aeruginosa biofilm was determined as follows:

Percentage biofilm survival

\[
\text{Percentage biofilm survival} = \left( \frac{\text{CFU of P. aeruginosa biofilm in treated sample} \times 100}{\text{CFU of P. aeruginosa biofilm in treated with WT E. coli}} \right)
\]

Confocal microscopy of biofilm

Mixed bacteria cultures of P. aeruginosa (ln7 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.

References


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