

# Reprogramming Microbes to Be Pathogen-Seeking Killers

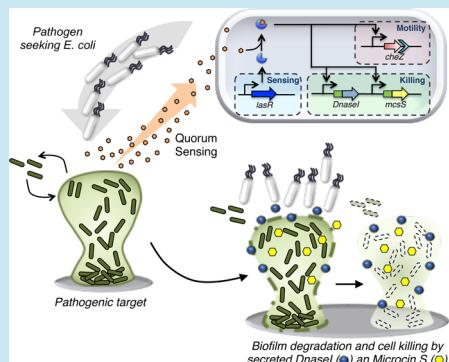
In Young Hwang,<sup>†</sup> Mui Hua Tan,<sup>†</sup> Elvin Koh, Chun Loong Ho, Chueh Loo Poh, and Matthew Wook Chang\*

School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459

Supporting Information

**ABSTRACT:** Recent examples of new genetic circuits that enable cells to acquire biosynthetic capabilities, such as specific pathogen killing, present an attractive therapeutic application of synthetic biology. Herein, we demonstrate a novel genetic circuit that reprograms *Escherichia coli* to specifically recognize, migrate toward, and eradicate both dispersed and biofilm-encased pathogenic *Pseudomonas aeruginosa* cells. The reprogrammed *E. coli* degraded the mature biofilm matrix and killed the latent cells encapsulated within by expressing and secreting the antimicrobial peptide microcin S and the nuclease DNaseI upon the detection of quorum sensing molecules naturally secreted by *P. aeruginosa*. Furthermore, the reprogrammed *E. coli* exhibited directed motility toward the pathogen through regulated expression of CheZ in response to the quorum sensing molecules. By integrating the pathogen-directed motility with the dual antimicrobial activity in *E. coli*, we achieved significantly improved killing activity against planktonic and mature biofilm cells due to target localization, thus creating an active pathogen seeking killer *E. coli*.

**KEYWORDS:** synthetic biology, quorum sensing, *Pseudomonas aeruginosa*, directed motility, biofilm, antimicrobial peptide



Engineering of bacterial cells to fight human pathogens has been gaining wide interest as a novel therapeutic strategy that can offer prophylactic and treatment regimens.<sup>1–5</sup> Recent studies have shown the potential use of commensal bacteria as delivery systems to counteract virulence factors or produce antimicrobial peptides to combat bacterial or viral infections.<sup>6</sup> Despite these advances in the earlier studies demonstrating the specific targeting ability of therapeutic microbes, one notable limitation inherent is that effective antimicrobial activities are highly dependent on the molecular diffusion of produced therapeutic compounds toward the target pathogen. This limitation is equally present in engineered microbes reported in recent studies.<sup>5,7</sup> A potential solution is to engineer microbes to migrate toward the targeting cell upon detection to localize the release of the therapeutic compounds to ensure an effective killing of the pathogen.

Another challenging problem faced in current antimicrobial strategies is the resistance to antimicrobials conferred by the morphological state of the pathogens. A vast majority of microorganisms do not live as a pure culture of single planktonic cells. Rather, they are found as a mixture of planktonic and adherent communities of bacteria known as biofilms.<sup>8</sup> Biofilms are composed of microorganisms encased in extracellular polymeric substances (EPS) such as polysaccharides, nucleic acids, and proteins that can act as a barrier to limit the accessibility of the antimicrobials to the cells and mask their presence from host immune cells, while providing the reservoir of nutrients for the biofilm cells.<sup>8,9</sup> Therefore, targeting both planktonic and biofilm states is important to circumvent the antibiotic resistance conferred by the biofilm matrix and to achieve effective pathogen eradication.

Therefore, in this study, we aimed to engineer *Escherichia coli* to specifically recognize, migrate toward, and eradicate both dispersed and biofilm-encased pathogen cells. The advances of this work are derived from its 2-fold antimicrobial strategy. The first approach addresses both planktonic and biofilm states of pathogens by employing antimicrobial peptide and biofilm degrading enzyme for efficient and sustained killing activity in an autonomous manner. Furthermore, this work undertakes a novel approach of reprogramming chemotaxis response of killer *E. coli* cells to selectively swim toward the pathogen. This adaptable motility augments the activity of secreted molecules by the killer *E. coli* as the source of secretion will be moving closer toward the targeting cells. This motility-assisted localization of *E. coli* toward the pathogen addresses the inherent limitation present in other studies that relies solely on molecular diffusion of the antimicrobial compounds.

To test the improvement of antimicrobial activity of the engineered *E. coli* via pathogen-specific localization, we reprogrammed our cells to detect the model pathogen *Pseudomonas aeruginosa*. We achieved this by introducing a characterized quorum sensing (QS) device, adapted from our previous study.<sup>5</sup> The QS device detects the presence of N-Acyl homoserine lactone (AHL), a quorum sensing molecule produced and secreted by *P. aeruginosa*. The detection is followed by expression of the antimicrobial peptide, microcin S (MccS)<sup>10</sup> and the nuclease DNaseI for cell killing and biofilm

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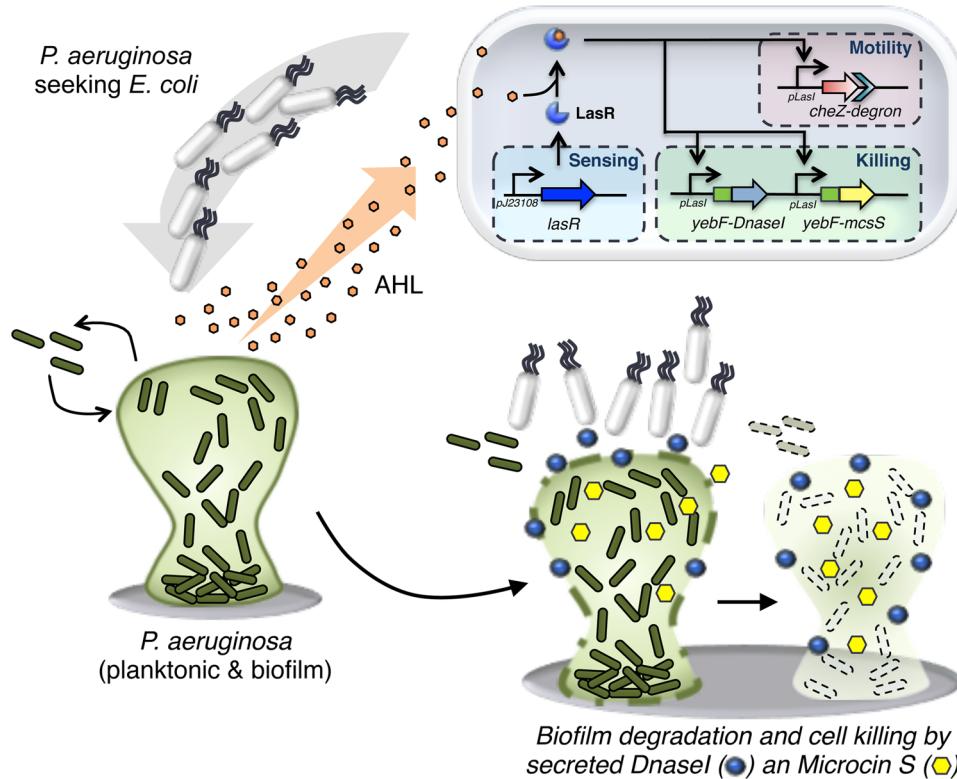


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**Figure 1.** Directed chemotaxis-guided motility of *E. coli* upon induction by AHL—reprogrammed ‘Seek and Kill’ system in *E. coli*. The system is divided into three genetic devices: Under LasR-AHL (*N*-Acyl homoserine lactone) responsive promoter, *pLasI*, expression of CheZ (Motility) and secretion tagged proteins (Killing) are induced by the quorum sensing molecule (Sensing). In the presence of a quorum sensing molecule (AHL) from *P. aeruginosa*, reprogrammed *E. coli* cells express CheZ to swim toward the pathogen. Further gene expression is initiated in *E. coli* to secrete antibiofilm (DNaseI) and antimicrobial peptide (MccS) to degrade biofilm and kill planktonic or biofilm-residing cells that are released by the biofilm degradation. pJ23108: constitutive promoter. YebF: secretion tag. *mccS*: gene encoding for microcin S (MccS).

degradation, respectively. Nuclease was chosen to destabilize the biofilm, as several studies had indicated that exogenously added DNases could inhibit biofilm formation, disperse preformed biofilms, and sensitize dormant biofilm bacteria to biocide killing.<sup>11–15</sup> The biofilm degrading activity of DNases can be attributed to the presence of extracellular DNA as a major component of biofilm matrix, especially in *P. aeruginosa*. The dual antimicrobial strategy implemented by MccS and DNaseI coexpression targeting both planktonic and biofilm states of *P. aeruginosa* resulted in a concerted killing activity.

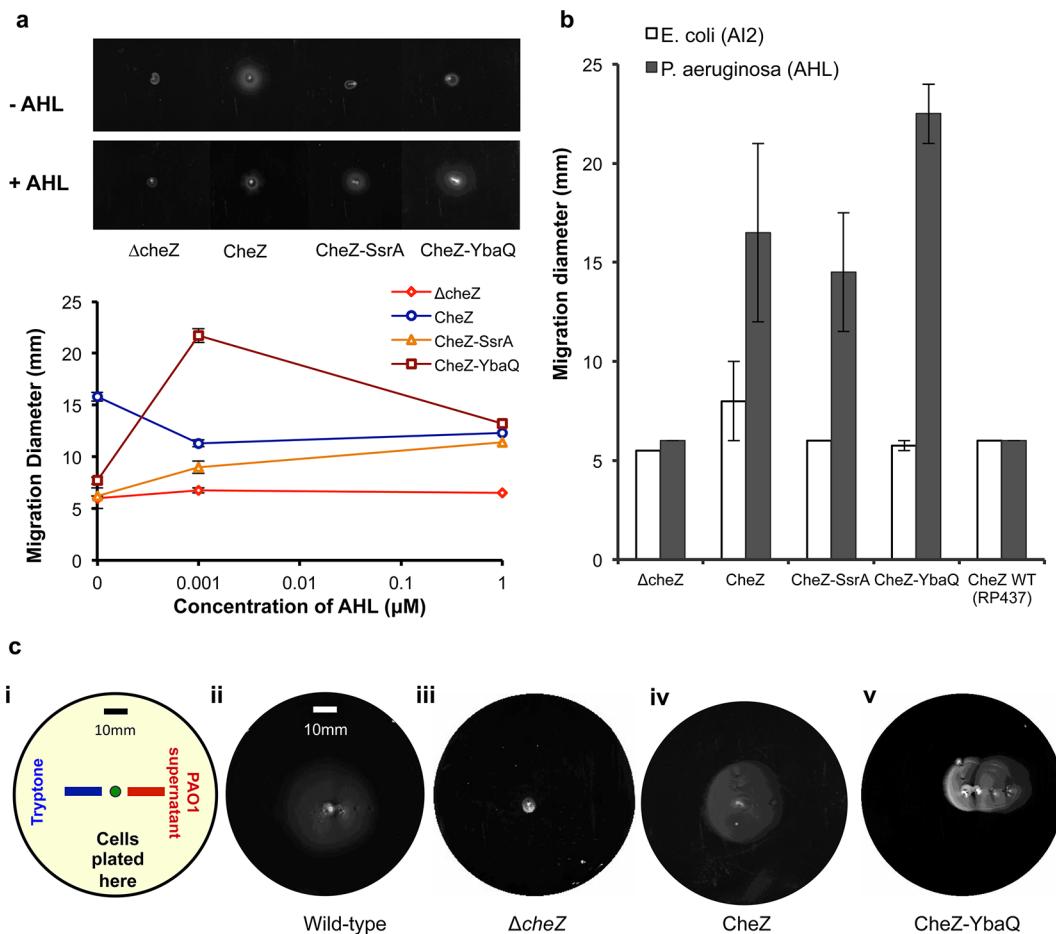
In order to achieve pathogen-specific localization, we employed post-translational modulation of CheZ expression, which enabled the reprogramming of the chemotaxis behavior of *E. coli*. CheZ promotes smooth swimming by acting as an antagonistic phosphatase for CheY, which is a response regulator responsible for inducing cell tumbling when phosphorylated.<sup>16–18</sup> Chemotaxis behavior is sensitive toward the balance of CheZ and CheY ratio;<sup>19</sup> thus, we programmed the chemotaxis of *E. coli* cells by modulating the concentrations of CheZ compared to the innate concentrations of CheY. Previous studies indicated that by controlling CheZ expression, it is possible to reprogram chemotaxis behavior of *cheZ*-deleted strain to swim toward targeted compounds through a process known as pseudotaxis.<sup>20–22</sup> However, implementing pseudotaxis response that is autonomously initiated by pathogenic cells has not yet been reported. Furthermore, regulating CheZ stability as a mechanism to fine-tune the directed motility has not been experimented. In this study, we show that our engineered *E. coli* cells were able to migrate toward *P.*

*aeruginosa* cells and biofilm matrix by sensing the released QS signals. This provides the foundation for exploiting programmable chemotaxis as a novel selective delivery tool for pathogen targeting strategy, thus adding to the arsenal of tools available for engineering therapeutic microbes. Furthermore, this CheZ-mediated *P. aeruginosa* seeking ability of *E. coli* was combined with the cell killing strategy to demonstrate that localization of the engineered *E. coli* to *P. aeruginosa* results in refined antimicrobial and antibiofilm activities.

## RESULTS AND DISCUSSION

The overall scheme of the strategic approach for this study is divided into 3 modules, which are outlined in Figure 1. The sensitivity of the quorum sensing (QS) device (*sensing module*) to recognize QS molecule (*N*-Acyl homoserine lactone; AHL) secreted by planktonic and biofilm *P. aeruginosa* cells and inducing the downstream expression is established (Supporting Information (SI) Figure 1). Therefore, in the presence of *P. aeruginosa*, the *motility* and *killing* modules are activated. The AHL-responsive chemotaxis allows *E. coli* to swim up the concentration gradient of AHL, thereby localizing the cells closer to *P. aeruginosa* (*motility module*). Furthermore, the production and secretion of antimicrobial peptide (MccS) and antibiofilm enzyme (DNaseI) mediate ‘2-hit killing’ by targeting both planktonic and biofilm states of *P. aeruginosa* (*killing module*).

**Directed Chemotactic Motility of *E. coli* toward *Pseudomonas aeruginosa* PAO1.** When *E. coli* lacks *cheZ*, an integral member of the chemotaxis signaling pathway (strain

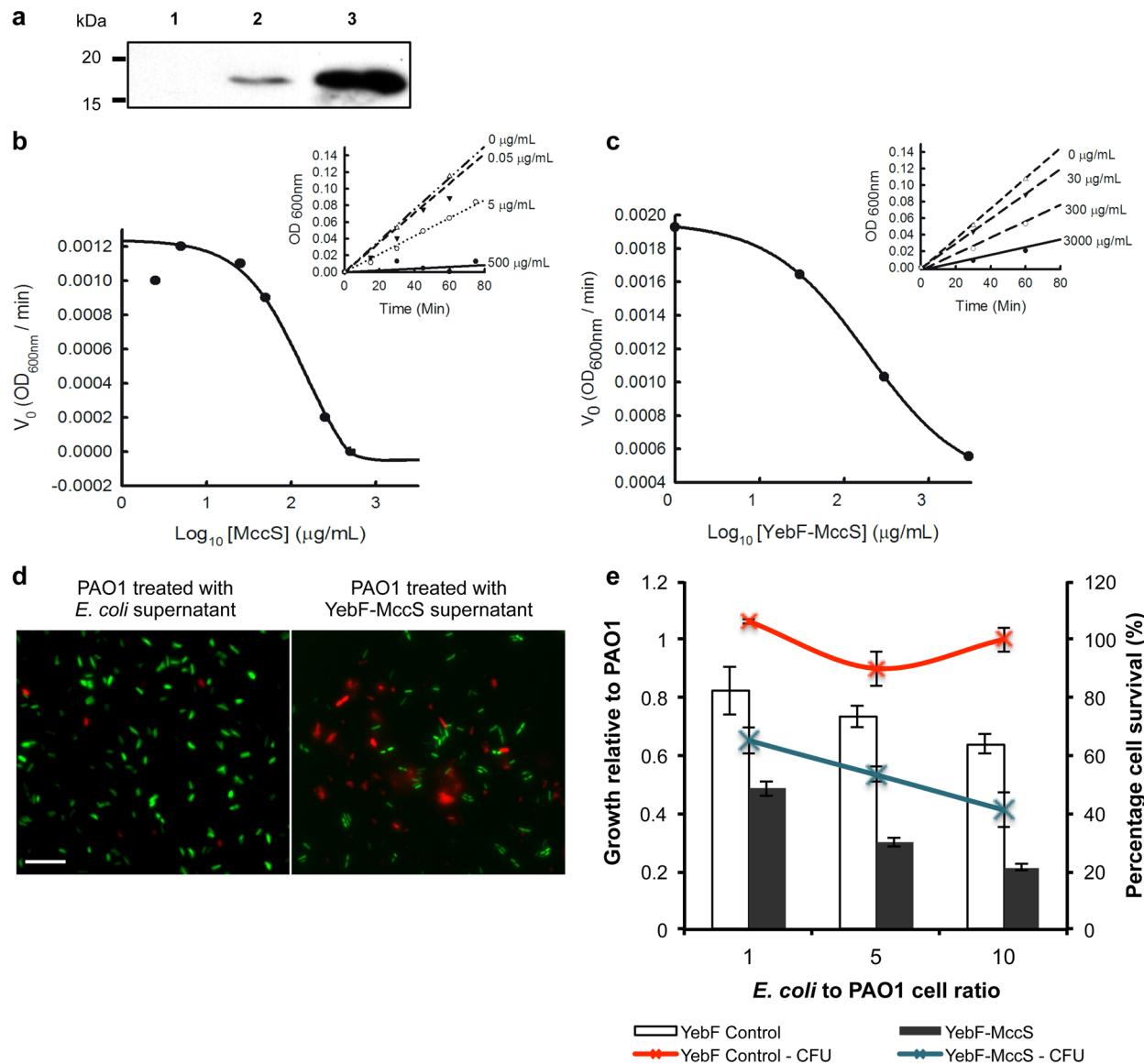


**Figure 2.** Directed chemotaxis-guided motility of *E. coli* upon induction by AHL. (a) Migration of  $\Delta\text{cheZ}$  cells expressing various CheZ variants on semisolid media in the absence or presence of AHL (1 nM). The graph shows the average migration diameter of the *cheZ*-reconstituted cells as a function of AHL concentration when the cells were cultured at 30 °C for 16 h. (b) Migration of CheZ variants expressing cells in the presence of supernatant collected from *P. aeruginosa* PAO1 or *E. coli* cell cultures in exponential growth phase. The *CheZ* deleted strain ( $\Delta\text{cheZ}$ ; UU2685) expressing pLasI-CheZ, pLasI-CheZ-SsrA, and pLasI-CheZ-YbaQ were compared to the *CheZ* wild-type strain (RP437). (c) Directed cell motility of activated *E. coli* is specific to *P. aeruginosa*. (i) Diagram of plates containing semisolid media spotted with *P. aeruginosa* PAO1 supernatant and tryptone (growth media) as outlined. *E. coli* cells were plated at the center, as shown, and grown for 16 h at 30 °C. (ii–v) Motility of wild-type *E. coli* strain (RP437),  $\Delta\text{cheZ}$  deleted (UU2685), UU2685 expressing CheZ or CheZ-YbaQ.

UU2685,  $\Delta\text{cheZ}$ ), the cells tumble incessantly and are essentially nonmotile.<sup>16</sup> With this  $\Delta\text{cheZ}$  strain, we first addressed whether PAO1-dependent motility could be re-established by expressing CheZ in response to AHL. Therefore, *cheZ* gene was introduced under the control of a LasR-AHL activator responsive promoter, pLasI, and expressed in UU2685. As prior studies reported that overexpression of CheZ abolishes chemotaxis,<sup>16,19</sup> a range of expression level required for motility needed to be carefully regulated. To this end, a degron was employed to destabilize CheZ to reduce basal activity and broaden the responsive range of inducer concentration. Degrons are short amino acid sequences that are specifically degraded by the ClpXP or ClpAP complexes, resulting in an efficient degradation of the fused protein.<sup>23,24</sup> The level of destabilization was first characterized with GFP (SI Figure 2) and the degron sequence (SsrA or YbaQ) was subsequently fused to the C-terminus of CheZ. Addition of the degron to CheZ resulted in a tight regulation on basal expression, while demonstrating specific motility upon AHL induction (Figure 2a). The specificity of this motility was further confirmed by the preferential cell motility in the presence of PAO1 supernatant (AHL) over *E. coli* supernatant

(AI-2) (Figure 2b and SI Figure 3). Furthermore, directed motility was demonstrated when the populations of CheZ-YbaQ expressing cells migrated toward the PAO1 supernatant (Figure 2c). Therefore, these results show that the reprogrammed chemotactic feature enabled the population of the cells to migrate up an AHL concentration, hence toward PAO1.

**Extracellular Secretion and Characterization of Microcin S Against PAO1.** Microcin S (MccS) was selected, as it is a recently identified antimicrobial peptide that has shown a killing efficiency against a wide range of Gram-negative microbes.<sup>10</sup> MccS is a class II microcin, which is characteristically small sized, requiring no further post-translational modification for activity.<sup>25</sup> With a broad range of killing activity and known immunity gene, it was an ideal candidate to be exploited and tested against PAO1 cells. The bactericidal activity of MccS against PAO1 was demonstrated by incubating OD<sub>600</sub> 0.1 of PAO1 with a range of concentrations of purified MccS (Figure 3a, b). For each growth assay, the change in OD<sub>600</sub> during exponential growth phase was compared to the corresponding change in the control (Figure 3b). For the first

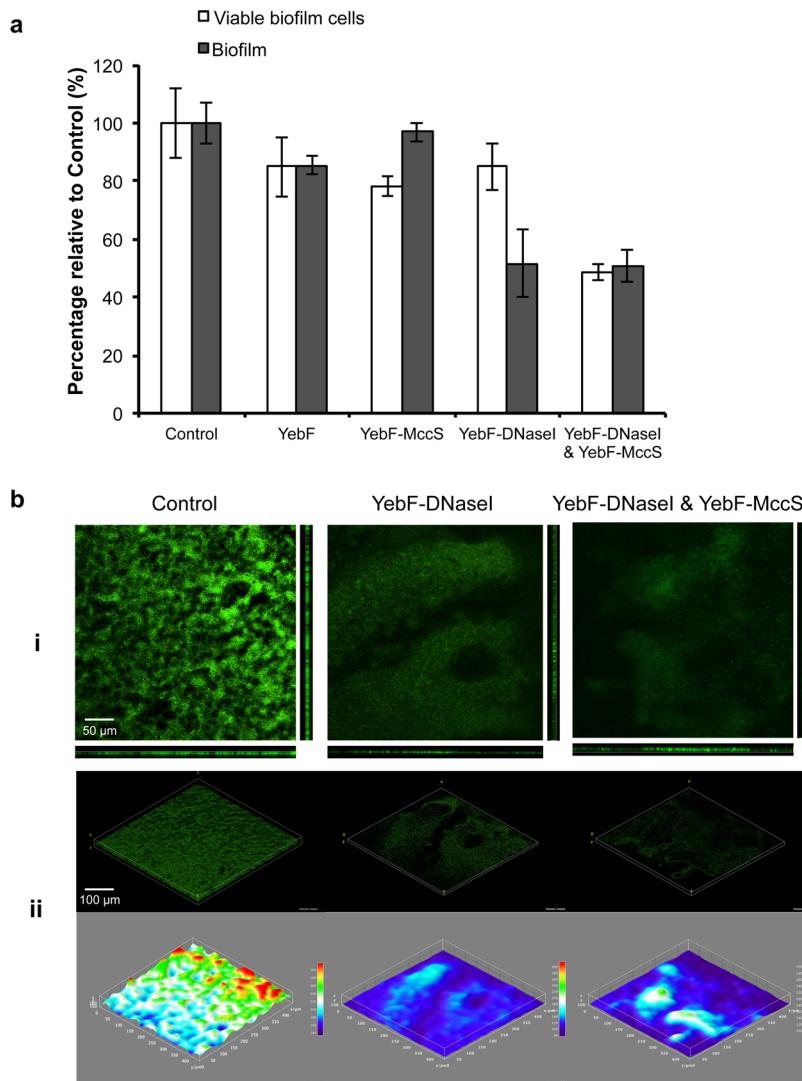


**Figure 3.** Analysis of antimicrobial activity of Microcin S (MccS) against *P. aeruginosa*. (a) Expression and purification of MccS. Lane 1: Untreated UU2685 harboring pBbE8k-pLasI-MccSH<sub>6</sub> plasmid. Lane 2: Transformed UU2685 with AHL induction. Lane 3: Purified MccS. (b) Various concentrations of purified MccS were tested against PAO1 and the effect on growth rate was compared. \*Inset graph: OD value was normalized to the start of the exponential phase. (c) Extracellular expression of YebF-MccS fusion protein after 3 h of induction with 1 μM AHL. The YebF-MccS fusion protein was concentrated using molecular weight cutoff centrifuge filtration and assayed for its activity against PAO1. Resulting protein concentration of extracellular medium containing secreted YebF-MccS was quantified using the Bradford assay and corresponding effect on cell growth was assayed. Half maximal inhibitory concentration ( $IC_{50}$ ) of YebF-MccS against PAO1 cells was determined. (See SI Figure 4 for the full growth curve.) Control (0 μg/mL) is extracellular medium without AHL induction. (d) PAO1 cells expressing GFP after treatment with YebF-MccS were stained with PI dye to determine dead cells. The bar represents 10 μm. (e) YebF-MccS expressing *E. coli* was cocultured with GFP expressing PAO1 cells at the given relative cellular ratio, and the resulting effect on growth rate and cell viability was evaluated. The growth relative to control after 12 h of incubation was calculated by taking the arbitrary GFP fluorescence intensity of cocultured sample relative to untreated PAO1 with GFP expression. YebF control refers to *E. coli* expressing the YebF protein.

time, we demonstrate that the purified peptide is highly active against PAO1 with an  $IC_{50}$  of 14.7 μg/mL (Figure 3b).

Subsequently, N-terminal fusion of MccS to YebF was implemented to facilitate extracellular secretion of the peptide. YebF is a small, soluble endogenous protein, which can carry fusion proteins in their active states to the medium, as early as 3 h after induced expression.<sup>26</sup> The activity of YebF-MccS in extracellular medium after 3 h induction remained active against PAO1 cells (Figure 3c). The extracellular medium was collected and concentrated using molecular weight cut off (MWCO) filtration unit to selectively collect proteins within 5

kDa to 30 kDa range as the estimated YebF-MccS was approximately 25 kDa. The activity of the YebF-MccS in the extracellular medium was demonstrated against PAO1 (Figure 3d). Approximate  $IC_{50}$  of the secreted protein was 188 μg/mL, which indicates that the addition of the YebF to MccS resulted in approximately 10-fold increase in  $IC_{50}$  value. As the extracellular medium was concentrated and selected based on molecular weight only, this value is potentially lower if YebF-MccS is purified further. In addition, although the secretion tag used, YebF, is reported to retain the activity of the fused protein, it is possible to hinder the antimicrobial activity of



**Figure 4.** Analysis of antibiofilm activity of DNaseI against PAO1. (a) The mature biofilm was incubated with the engineered *E. coli* cells for 16 h, and the resulting biofilm was stained with crystal violet and quantified by taking absorbance reading at 595 nm. Viable biofilm cells were also determined by performing CFU counting after 16 h incubation of mature biofilm with *E. coli* cells. (b) Antibiofilm activity of DNaseI was observed under confocal laser scanning microscopy (CLSM). (i) Pseudomonas biofilm with green fluorescence was grown on 8-well chambered glass slide for 48 h, which was subsequently treated with the engineered *E. coli* for 16 h, and visualized under CLSM. Scale bar represents 50 µm. (ii) Images were reconstructed from biofilm Z-stacks using Image J. Scale bar represents 100 µm.

MccS. Cell killing was verified by LIVE/DEAD cell viability assay, where PAO1 cells treated with YebF-MccS showed significant proportion of cells stained with PI dye indicating cell death, whereas the cells treated with the control supernatant were mostly stained with SYTO 9 dye, which denotes that most cells are viable. Therefore, the extracellular medium containing secreted YebF-MccS remained active and caused significant inhibition against PAO1 cell growth.

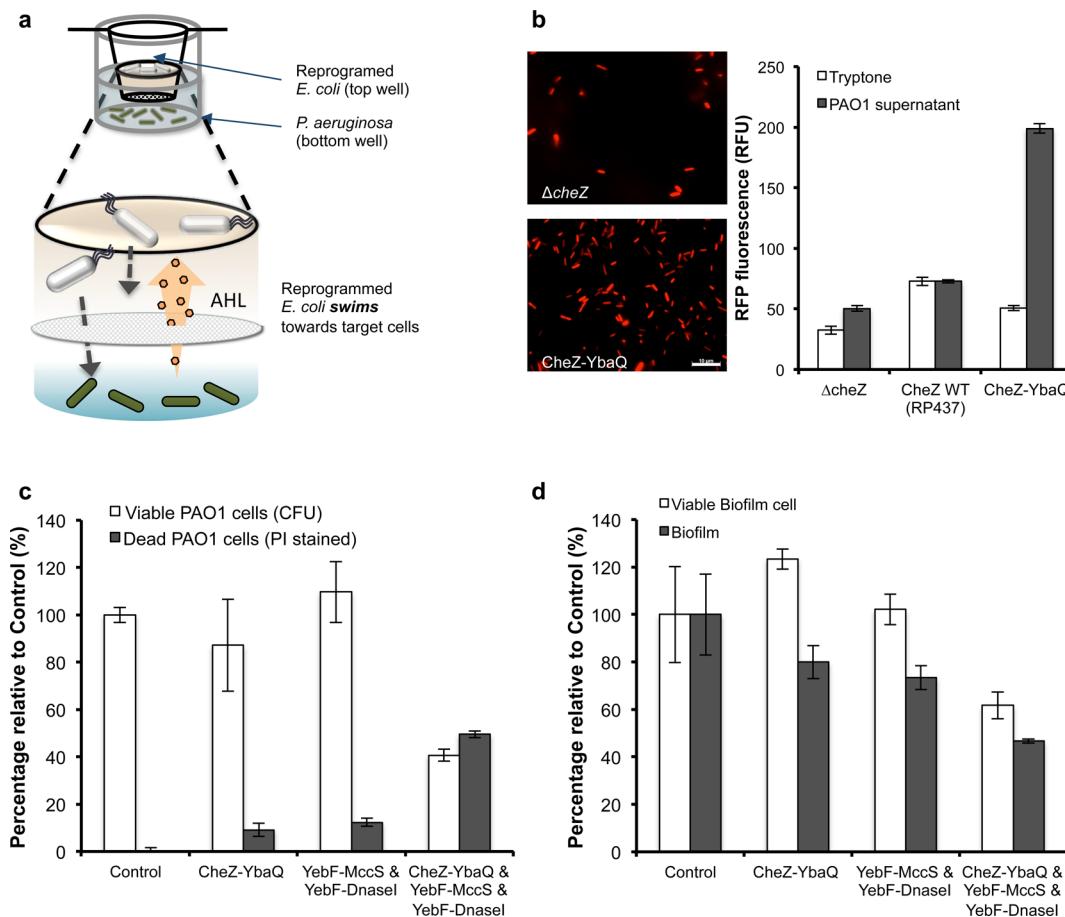
**Cellular ‘Sense and Killing’ Against PAO1 Cells.** To further verify whether our killer cells could autonomously sense the presence of PAO1 cells to initiate cell killing, coculture of the YebF-MccS secreting *E. coli* with PAO1 was set up. PAO1 constitutively expressing GFP was cocultured with *E. coli* at the indicated starting cell ratio based on OD<sub>600</sub> value. After 12 h, the resulting GFP fluorescence and viable PAO1 cells were measured. Significant reduction in growth rate and cell viability of PAO1 was achieved at equivalent starting OD<sub>600</sub> of *E. coli* cells (Figure 3e). When converted to actual cell number (SI

Figure 5), this ratio demonstrates that our engineered *E. coli* cells show inhibitory ratio (IR<sub>50</sub>) of 3 *E. coli* cells per PAO1 cell.

#### Antibiofilm Activity of DNaseI Against PAO1 Biofilm.

Our reprogrammed cells were also designed to detect and target biofilm matrix of PAO1 by secreting antibiofilm nuclease, bovine pancreatic DNaseI. When the YebF-DNaseI transformants were cocultured with *P. aeruginosa* mature biofilm, the expression was sufficiently induced and consistent detachment of biofilm was observed (Figure 4a). The extent of biofilm detachment was not affected when the cell was expressing both YebF-DNaseI and YebF-MccS. When the viable biofilm cells were counted, only cells expressing both proteins significantly reduced biofilm mass as well as viable biofilm cells. This result indicates that our reprogrammed cells exerted significant biofilm degradation and cell killing that are autonomously induced in the presence of PAO1 cells and mature biofilm.

***P. aeruginosa* Targeting Motile *E. coli* with ‘2-Hit Killing’ System—Seek and Kill.** The results shown in Figures 2 to 4 demonstrate successful reprogramming of



**Figure 5.** Testing the final construct for efficient QS-mediated motility with biofilm-disrupting and cell killing—Seek and Kill system. (a) Schematic depicts AHL-directed cell motility of the reprogrammed *E. coli* (from the inverted transwell insert) toward the PAO1 cells initially seeded on the bottom of the transwell apparatus. Due to the diffused AHL across the insert, the activated *E. coli* begins to swim vertically toward PAO1 due to AHL-induced CheZ expression. Subsequently, the expression of MccS and DNaseI for secretion mediates cell killing and disrupts the biofilm matrix. (b) Specific motility of reprogrammed *E. coli* (*pLasI-CheZ-YbaQ*) across the transwell shown by microscopic images and a graph of RFP fluorescence collected from the bottom well. (c) The viability of PAO1 cells was measured after 16 h of incubation with reprogrammed *E. coli* on the transwell insert. A LIVE/DEAD BacLight kit was also used to determine cell death. The proportions of PI stained (dead) compared to SYTO9 stained (all) cells treated with *E. coli* expressing CheZ with MccS and DNaseI (*pLasI-CheZ-YbaQ* and *McsI-pLasI-YebF-MccS<sub>H6</sub>-YebF-DNaseI*) compared to nonmotile MccS and DNaseI were compared (*McsI-pLasI-YebF-MccS<sub>H6</sub>-YebF DNaseI*). (d) The transwell insert with reprogrammed *E. coli* was seeded on the transwell containing mature PAO1 biofilm. After 16 h of incubation, the resulting biofilm and viable biofilm cells were determined using crystal violet staining and CFU counting, respectively.

chemotaxis toward PAO1 (*motility* module) and the efficacy of MccS and DNaseI secreting cells (*killing* module) against PAO1 cells in a coculture system. Hence, the two modules were integrated with QS device to create the ‘Seek and Kill’ system in *E. coli* against PAO1. In Figure 1, we depict the assembled system<sup>27</sup> comprising sensing and motile killer *E. coli* cells that (i) detect QS molecules emanating from PAO1 cells in planktonic and biofilm states, (ii) migrate toward PAO1 cells/biofilm while also expressing antimicrobial and antibiofilm enzymes, and (iii) mediate biofilm disruption and cell killing. In Figure 5a, we have placed *E. coli* with integrated ‘Seek and Kill’ system (CheZ-YbaQ, YebF-MccS, and YebF-DNaseI) at the top compartment of a transwell apparatus with agar medium. We first checked for specific bacterial migration toward the targeted pathogen by adding culture supernatant of PAO1 cells by measuring RFP fluorescence (Figure 5b) and OD<sub>600</sub> (SI Figure 6) after 16 h incubation (time used to observe directed motility and both ‘2-hit killing’ activities of the engineered *E. coli*). Specific migration of *E. coli* was observed with CheZ-YbaQ expressing cells in the presence of PAO1 culture

supernatant, while other controls have shown basal level of migration.

Once the PAO1 responsive bacterial migration was established in this assay, our engineered *E. coli* with the integrated system was tested against PAO1 cells and biofilm. The resulting viability of PAO1 cells was measured by antibiotic-selective colony forming unit (CFU) counting. The *E. coli* with the integrated system showed approximately 60% reduction in survival cells while others with either *motility* (CheZ-YbaQ) or *killing* (YebF-MccS and YebF-DNaseI) module alone resulted in insignificant reduction in PAO1 cell survival (Figure 5c). This result was complemented with the highest percentage of dead cells (~50%) obtained from the Syto9-PI staining. Therefore, with the fully integrated motility and killing system, the cells were able to achieve approximately 4-fold higher cell killing activity. Furthermore, when our integrated ‘Seek and Kill’ *E. coli* was tested against PAO1 biofilm, 60% reduction in PAO1 biofilm relative to the control was observed (Figure 5d). Furthermore, a complementary 40% reduction in viable PAO1 biofilm cells was observed by our

'Seek and Kill' system. Taken together, our integrated system could respond to both planktonic and mature biofilm PAO1 and exhibit cell migration and localization that accentuate cell killing activities of our engineered *E. coli*, which effectively resulted in reduction in PAO1 cells and biofilm matrix.

**Discussion.** In summary, we engineered *E. coli* to be able to integrate inputting signals from a target pathogen (quorum sensing molecules) into a programmed response, which comprises of production and secretion of antimicrobial peptide and antibiofilm enzyme, and directed migration toward pathogenic *P. aeruginosa* cells for closer localization for concerted killing activity.

The biofilm formation plays a role in the pathogenesis of many chronic infections due to its resistance to conventional antimicrobial agents and host defenses. The strategy employed in this study addressed this by utilizing antibiofilm enzyme (DNaseI), thus reducing the resistance provided by the biofilm structure. Concurrently, coexpression of antimicrobial peptide (MccS) by our engineered *E. coli* cells further eliminated the released dormant, resistant cells from the biofilm structure. The killing activity of MccS against *P. aeruginosa* was verified for the first time in this study. While it is highly effective, neither the mechanism of action nor the resistance conferred by the immunity gene (*mcsI*) is clear. However, based on the PI staining of *P. aeruginosa* cells after treatment, MccS can be postulated to possess membrane-permeabilizing property, similar to other members of the class II microcin family.<sup>28–31</sup> Further characterization of MccS activity will shed light in fully exploiting this effective antimicrobial peptide against *P. aeruginosa* and other pathogenic strains. Nonetheless, as the activities of both DNaseI and MccS are not confined to *P. aeruginosa*, this combination is potentially effective in targeting biofilms in general, as the composition of biofilms is unlikely to be of homogeneous population. The implementation of this dual targeting strategy in our engineered *E. coli* cell is an advanced approach that can potentially mediate broad-spectrum killing activity against pathogens. Although *P. aeruginosa* was used as a model pathogen to evaluate the activity of our engineered *E. coli* with the dual targeting system, this approach can be adapted to target other pathogens.

In addition, it is possible to further refine this system by introducing a regulatory mechanism that allows the cells to respond to pathogens in a dynamic manner. For instance, the current system is composed of multiple modules that are not necessarily required to be expressed concurrently. In establishing an autonomous system, it is worthwhile to pursue more defined QS device that is able to differentially direct biofilm-responsive expression for antibiofilm enzyme, while maintaining a general QS responsive expression for antimicrobial peptide and cellular motility. The differential expression of the modules depending on the morphological states of the pathogen may allow better allocation of metabolic resources for optimal expressions to result in an enhanced antimicrobial activity.

To reinforce the antimicrobial strategy, we have also introduced selective bacterial migration through regulated expression of CheZ in the presence of planktonic and biofilm states of *P. aeruginosa* cells. While previous studies primarily focused on introducing a chemotaxis-guided motility toward new chemicals,<sup>20,22</sup> this study has demonstrated the reprogrammable bacterial migration that is responsive to a dynamic presence of quorum sensing molecules, *N*-(3-oxo-decanoyl)-L-homoserine lactone (3OC12-HSL) and *N*-butyryl-L-HSL

(C4-HSL), prominently produced by planktonic and biofilm *P. aeruginosa* cells, respectively.<sup>32,33</sup> The most notable challenging aspect of utilizing CheZ is the extremely narrow range of expression required to fine-tune the level of phosphorylated CheY (CheY-P) that controls the motor proteins activity.<sup>34</sup> The motor proteins are extremely sensitive to the level of CheY-P, and therefore, the cessation of cell motility is invariably observed when a high concentration of CheZ is expressed in an inducible system.<sup>16,17,19,20,22</sup> This is in part due to an adaptive remodeling of the motor protein composition, which shifts the operating range of chemotactic response to maintain sensitivity and robust adaptation.<sup>35–38</sup> While this study employed an addition of degron to destabilize the CheZ to circumvent excessive accumulation, other regulatory components involved in chemotaxis can be used to modulate CheZ or other motility related genes to enhance cell migration to cater for wider applications.

In this study, we presented the establishment of a conceptual pathogen seeking and subsequent localization of *E. coli* with dual antimicrobial targeting capability as an effective cell killing strategy. However, the target localization can be useful in other potential applications when the motility is further harnessed for sustained optimal performance to cover a more effective range of distance. Furthermore, the autonomous 'Seek and Kill' system demonstrated in this paper can be further applied to other pathogens with sensing of particular signaling molecules, to utilize target-dependent localization to deploy not only toxins but also other enzymes.

## METHODS

**Strains and Growth Conditions.** All bacterial cells were maintained in LB media (BD Difco) with appropriate antibiotic(s) supplemented in all experiments and incubated at 37 °C in a shaker at 225 rpm for all growth experiments. *E. coli* Top10 was used for cloning, while the CheZ was expressed in UU2685. This is a CheZ deletion mutant, (*cheZ* Δ4-211), derived from a wild-type chemotaxis strain, RP437.<sup>39</sup> This was a kind gift from Professor Parkinson, University of Utah. Either GFP reporter plasmid pMRP9-1 or chloramphenicol-resistant plasmid pAWG1.1 was transformed into PAO1 using a method described previously.<sup>40</sup>

**Plasmid Construction.** YebF and CheZ were PCR amplified from *E. coli* MG1655 genome. The codon-optimized sequences of microcin S (*mcsS*), microcin S immunity (*mcsI*) and nuclease (*bpDNaseI*) were synthesized (GeneArt, Germany) and introduced into a Bglbrick vector for expression. The genetic constructs developed in this study were assembled using standard synthetic biology protocols.<sup>41</sup> Plasmids from chemically transformed cells were purified by affinity columns and verified by DNA sequencing. All plasmids used with the relevant genes are summarized in SI Table 1.

**Motility Assays.** Agar medium was prepared (tryptone broth with 0.25% agar) and poured into Petri dishes (85 mm diameter). Prior to pouring, various concentrations of AHL from aqueous stock solution or diluted culture supernatant of PAO1 or *E. coli* was mixed for even distribution to induce expression of CheZ. Diluted cell suspensions from midlog-phase cultures (5 μL, OD<sub>600</sub> 0.5) were applied at the center of the plate and measured the diameter of distance traveled was measured (outer ring).

For spatial localization, the agar medium was prepared, and solutions of AHL (100 nM) or supernatant of PAO1 cell cultures (500-fold diluent of OD<sub>600</sub> 1.0), or tryptone broth

alone, were applied in the pattern shown in Figure 2C by spotting with a micropipet (2  $\mu$ L/5 mm), and the plates were air-dried for 30 min. Again, the diluted cell suspensions (5  $\mu$ L, OD<sub>600</sub> 0.5) were applied at the location shown in Figure 2C, and the plates were dried in air for 15 min and incubated overnight at 30 °C.

#### Protein Secretion, Purification, and Concentration Measurement.

For protein purification, 1 L of *E. coli* cells with the recombinant plasmid pBbE8k-pLasI-MccSH<sub>6</sub> were grown to an OD<sub>600</sub> 0.8, and their expression was induced fully by 1  $\mu$ M exogenous 3-oxo-dodecanoyl-homoserine lactone (3OC<sub>12</sub>HSL, Sigma Aldrich) for 2 h at 37 °C. Subsequently, the cells were pelleted by centrifugation at 6000g for 20 min and homogenized using Emulsiflex-C3 homogenizer (Avestin, Inc.). Purification of microcin S (MccSH<sub>6</sub>) was achieved using nickel affinity columns, and the protein was washed with 50 mM imidazole and eluted with 500 mM imidazole in PBS with 10% glycerol. The eluate was concentrated by ultrafiltration using a molecular mass cutoff membrane (Amicon Ultra-15 Centrifugal Filter Unit, Millipore). As the estimated size of the protein is approximately 12 kDa, the first concentration was done using 30 kDa cutoff membrane where the flow-through was collected and subsequently passed through 5 kDa cutoff membrane. The resulting retentate was collected and the concentration was quantified using Bradford assay for subsequent use. Extracellular medium containing the secreted YebF-MccS was collected after 3 h postinduction with AHL (1  $\mu$ M) and also concentrated using Amicon Ultra centrifugal filter units.

**Protein Electrophoresis and Western Blotting.** Proteins were separated on 17% SDS-PAGE gel and then transferred to a nitrocellulose membrane for immunoblotting. Trans-Blot SD Semi-Dry Transfer Cell from BIO-RAD was used to transfer the gel, which was run for 60 min at 150 V in Tris/Glycine electrophoresis buffer. To detect the histidine tag, HRP-linked anti6x His tag antibody (ab1187, Abcam) was used. Chemiluminescence detection system (ECL; Pierce Biotechnology) was used according to manufacturer's instructions for developing the membrane.

**Cell Killing Assay.** Optical density at 600 nm (OD<sub>600</sub>) readings of PAO1 cells were taken using a Biotek Synergy HT Multi-Mode plate reader set to maintain 37 °C at regular intervals over the course of the experiment. Total culture volume of purified MccSH<sub>6</sub> or YebF-MccSH<sub>6</sub> in a well of a 96-clear-bottom microtiter plate (Falcon, Oxnard, CA) was 150  $\mu$ L.

**Coculture Assay.** *P. aeruginosa* PAO1 expressing GFP and corresponding *E. coli* cell cultures were diluted and mixed to a final OD<sub>600</sub> of 0.015 PAO1 and 0.015/0.075/0.150 *E. coli* to setup cell ratio of 1, 5, and 10 respectively. Cell cultures were then incubated at 37 °C with agitation for 12 h and the change in GFP fluorescence (ex: 485 nm, em: 540 nm) was measured as relative fluorescence unit (RFU) using the Biotek Synergy HT Multi-Mode plate reader. In addition, CFU was measured at 12 h time point.

**Detachment of Mature *P. aeruginosa* Biofilm by the Secreted Biofilm Enzyme.** The wells of a 96-well microtiter plate were filled with 150  $\mu$ L of medium containing a single cell suspension of PAO1 cells at OD<sub>600</sub> of 0.05 and incubated at 37 °C for 24 h with pegs of polystyrene microtiter lid (catalog no. 445497; Nunc TSP system) immersed for bacterial biofilm formation. The peg lid was rinsed with PBS and immersed into microtiter plate with *E. coli* expressing YebF-DNaseI with or without YebF-MccS (200  $\mu$ L/well), and the plates were

incubated for 16 h. The peg lids were washed with PBS and the biofilm remaining attached to the surface was stained with crystal violet (0.1% w/v), rewashed under running water, and dried. The amount of biofilm mass was quantified by destaining the biofilms for 20 min with 200  $\mu$ L of 95% ethanol and then the absorbance of the crystal violet solution at OD<sub>595</sub> was measured.

**Biofilm Cell Viability Assay.** Percentage biofilm survival was assayed after 16 h of incubation with *E. coli* cells. The peg lid was rinsed and recovered in fresh LB by sonication and centrifugation (810g, 20 min)<sup>42</sup> and quantified by CFU count on chloramphenicol-selection plate (100  $\mu$ g/mL). The final CFU values were normalized to the control (PAO1 biofilm) and percentage survival after the *E. coli* treatment with respect to the control was graphed.

**Confocal Microscopy of Biofilm.** An overnight subculture of PAO1 with pMRP9-1 was adjusted to an OD<sub>600</sub> of 0.05 and was added to 8-well sterile microscopic chamber slides (SPL Life Sciences) and incubated for 48 h at 37 °C to allow biofilm growth. Subsequently, the PAO1 biofilm developed on the glass slides was treated with *E. coli* expressing YebF-DNaseI with or without YebF-MccS (250  $\mu$ L/well) and further incubated for 16 h. After the incubation, the slide was rinsed in PBS, dried, and visualized by LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany). The collected Z-stack biofilm images were reconstructed using Image J software.

**Transwell Assay to Assess the Final *P. aeruginosa*-Specific 'Seek and Kill' System.** Supernatant (1 mL) of PAO1 cell cultures was added into the bottom compartment of the transwells (3  $\mu$ m porosity; Corning) in 6-well plates.<sup>27</sup> Agar medium was prepared (tryptone broth with 0.25% agar) and poured into the top compartment of transwells with a depth of 5 mm. Diluted cell suspensions from midlog-phase cultures (200  $\mu$ L, OD<sub>600</sub> 0.5) were applied at the center of the media agar. The plates were then incubated statically at 37 °C for 16 h. The relative fluorescence unit (ex: 540 nm; em: 600 nm) of the reprogrammed *E. coli* in the bottom compartment was assessed as a marker of cell motility.

For killing assay, 300  $\mu$ L of PAO1 cell cultures (OD<sub>600</sub> 0.002) was added into the bottom compartment of the transwells in 24 well plates. Agar medium was prepared and poured into the top compartment of transwells with a depth of 5 mm. Diluted cell suspensions of *E. coli* from midlog-phase cultures (100  $\mu$ L, OD<sub>600</sub> 0.5) were applied at the center of the media agar. The plates were then incubated statically at 37 °C for 16 h. The CFU of the PAO1 in the bottom compartment was assessed to reflect killing efficiency by the reprogrammed *E. coli* cells. A complementary killing activity that reflected the percentage of dead PAO1 cells was determined using LIVE/DEAD BacLight kit (Invitrogen) according to manufacturer's instructions. Measurements of the fluorescence were done using a microplate reader with detection of green fluorescence (ex: 485 nm; em: 540 nm) and red fluorescence (ex: 485 nm; em: 645 nm) that indicate live and dead cells, respectively. To assess the efficacy of the reprogrammed *E. coli* on the mature PAO1 biofilm and the viable biofilm cells, 1 mL of PAO1 cells at OD<sub>600</sub> of 0.05 was added into each well and incubated at 37 °C for 24 h for biofilm formation prior to being treated as the setup above at 30 °C for 16 h. The total OD<sub>600</sub> of the *E. coli* introduced was 0.05, 0.1, and 0.225, respectively. Crystal violet staining was conducted on the treated PAO1 at the bottom of the transwell to assess the biofilm disruption of our reprogrammed *E. coli*, while CFU counting was performed to

deduce the number of viable PAO1 biofilm cells remaining after treatment. Untreated PAO1 was used as a control.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supplementary Figures (1–6) and a table of DNA constructs used in the work. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Tel.: +65 6513 8063. Fax: +65 6794 7553. E-mail: matthewchang@ntu.edu.sg.

### Author Contributions

†These authors contributed equally to this work.

### Author Contributions

I.Y.H., M.H.T., and M.W.C. conceived the project. I.Y.H. designed and performed the experiments, together with M.H.T. and E.K. I.Y.H. and M.W.C. conducted data analysis and interpretation and wrote the manuscript. C.L.P. advised on data analysis. M.H.T. and H.C.L assisted in the writing of the manuscript. M.W.C supervised the project.

### Notes

The authors declare no competing financial interest.

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