To utilize biofilms for chemical transformations in biorefineries they need to be controlled and replaced. Previously, we engineered the global regulator Hha and cyclic diguanylate-binding BdcA to create proteins that enable biofilm dispersal. Here we report a biofilm circuit that utilizes these two dispersal proteins along with a population-driven quorum-sensing switch. With this synthetic circuit, in a novel microfluidic device, we form an initial colonizer biofilm, introduce a second cell type (dispersers) into this existing biofilm, form a robust dual-species biofilm and displace the initial colonizer cells in the biofilm with an extracellular signal from the disperser cells. We also remove the disperser biofilm with a chemically induced switch, and the consortial population could tune. Therefore, for the first time, cells have been engineered that are able to displace an existing biofilm and then be removed on command allowing one to control consortial biofilm formation for various applications.
Biofilms are groups of cells at an interface cemented together by polysaccharides, protein, DNA and lipids. Biofilms are related to most bacterial chronic inflammatory and infectious diseases as well as involved in biocorrosion and biofouling in diverse areas. They also may be used for beneficial applications such as bioremediation and hold much potential for chemical transformations in biorefineries. For these applications, compared with monocultures, mixed populations have the advantage of being able to perform more complex transformations (for example, those requiring multiple steps), and they are more resistant to environmental stress. For these reasons, consortia have been heralded as the new frontier in synthetic biology. However, to date, it has not been possible to control consortial biofilm formation.

Based on an understanding of signals and regulatory networks during biofilm development, biofilms have been engineered by manipulating extracellular/intercellular signals and regulators. The first engineered biofilm was a consortium where Bacillus subtilis was engineered to secrete the peptide antimicrobials indoliciolin and bacteriocin to inhibit the growth of sulfate-reducing bacteria and thereby decrease corrosion. Also, the first synthetic signalling circuit to control biofilm formation was developed for Escherichia coli and Pseudomonas fluorescens by manipulating the extracellular concentration of the signal indole produced by E. coli; indole is a biofilm inhibitor for E. coli. In addition, using directed evolution, the quorum-sensing (QS) regulator SdiA was reconfigured to decrease biofilm formation by increasing indole, and the global regulator H-NS was evolved to decrease biofilm formation via prophase excision and cell death. To remove existing biofilms, T7 bacteriophage was engineered to produce dispersin B of Actinobacillus actinomycetemcomitans to disrupt the glycosidic linkages of polymeric β-1,6-N-acetyl-D-glucosamine found in the biofilm matrix during bacteriophage infection. In addition, the global transcriptional regulator Hha of E. coli, which reduces hemolysin production and decreases biofilm formation by increasing indole, and the global regulator H-NS was evolved to decrease biofilm formation via prophase excision and cell death.

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30C12HSL disperses the initial colonizer biofilm. To demonstrate that 30C12HSL disperses biofilms produced by the initial colonizer cells (E. coli hha/pBdcAE50Q-rfp-lasR) by binding LasR and inducing bdcAE50Q, exogenous 30C12HSL at different concentrations was added to biofilms formed by the initial colonizer cells in microfluidic channels. As expected, the initial colonizer biofilms were dispersed upon adding 30C12HSL in a dose-dependent manner (Fig. 3c; Supplementary Fig. S1); near complete biofilm dispersal was obtained at 500 μM of 30C12HSL, and at lower 30C12HSL concentrations, dispersal of the initial colonizer biofilms was reduced (Supplementary Fig. S1). In contrast, there was no dispersal in the absence of 30C12HSL (Supplementary Fig. S1), and the initial colonizer cells formed thick biofilms (10.8 ± 0.6 μm²/μm²) (Fig. 3c). Hence, initial colonizer cells recognize 30C12HSL and this signal may be used to disperse initial colonizer biofilms.

IPTG removes the disperser biofilm. To demonstrate that IPTG disperses biofilms produced by disperser cells by inducing hha13D6, exogenous IPTG at different concentrations was added to biofilms formed by disperser cells in microfluidic channels. As expected, disperser biofilms were dispersed upon adding IPTG in a dose-dependent manner (Fig. 3d) with near complete biofilm dispersal at 2 mM IPTG (Supplementary Fig. S2); hence, we used 2 mM IPTG in subsequent experiments. In contrast, there was no dispersal in the absence of IPTG (Supplementary Fig. S2). Thus, the disperser cell has active hha13D6 to disperse its own biofilm upon IPTG addition.

Engineered BdcA and Hha are necessary for biofilm dispersal. To confirm that the biofilm dispersal upon addition of 30C12HSL and IPTG is the result of production of the engineered biofilm dispersal proteins, we performed dispersal experiments of initial colonizer cells that lack bdcAE50Q and disperser cells that lack hha13D6. As expected, initial colonizer biofilms formed without bdcAE50Q (via E. coli hha/pPRFP-lasR) did not disperse in the presence of 30C12HSL (Fig. 4a), while initial colonizer biofilms formed with bdcAE50Q dispersed with 30C12HSL (Supplementary Fig. S1). Similarly, disperser biofilms formed without hha13D6 (via E. coli hha/pGFP-lasI) did not disperse upon addition of IPTG (Fig. 4b), while disperser biofilms formed with hha13D6 dispersed with IPTG (Supplementary Fig. S2). Hence, BdcAE50Q and Hha13D6 are necessary to disperse the initial colonizer and disperser biofilms, respectively. Taken together, both disperser and initial colonizer cells were constructed to allow us to manipulate biofilm dispersal using a population-driven switch.

Disperser cells displace initial colonizer biofilms. Having verified the disperser and initial colonizer cell elements of the μBE signalling circuit, we combined both cell types to form a consortial
should induce dispersal of initial colonizer biofilms by switching disperser biofilms mature. Then, the 3 to LasR when the concentration of 3 cells synthesize 3 biofilm consortium (Fig. 5a; Supplementary Movie 1). As disperser cells were added to the initial colonizer biofilms for 5 h to form the biofilm, the initial colonizer cells. First, robust biofilms of initial colonizer were developed for 9 h after seeding, and then disperser cells were added so the small number of remaining colonizer cells are not able to form a biofilm while the disperser cells are being removed.

Fine-tuning consortial populations using the araBAD promoter. To fine-tune the consortial composition, a plasmid with the biofilm and investigated whether the disperser cells could displace the initial colonizer cells. First, robust biofilms of initial colonizer cells were developed for 9 h after seeding, and then disperser cells were added to the initial colonizer biofilms for 5 h to form the biofilm consortium (Fig. 5a; Supplementary Movie 1). As disperser cells synthesize 3OC12HSL constitutively, 3OC12HSL should bind to LasR when the concentration of 3OC12HSL is increased as the disperser biofilms mature. Then, the 3OC12HSL + LasR complex should induce dispersal of initial colonizer biofilms by switching on bdcAE50Q under control of the lasI promoter. As expected, the initial colonizer biofilms were displaced from the surface as the disperser cells grew (Fig. 5a; Supplementary Movie 1). After 44 h, 80% of the maximum initial colonizer biofilm formed was removed (Fig. 5a,b; Supplementary Movie 1). The displacement of the initial colonizer cells by the disperser cells was accomplished by the production of 3OC12HSL from the disperser biofilms, not by shear force, as the disperser biofilms that lack LasI did not reduce initial colonizer biofilms; that is, both no LasI and LasI + via E. coli hha/pGFP-lasI) with 2 mM of IPTG for 10 h. Robust biofilms of initial colonizer or disperser cells were developed for 9 h in each microchamber. Scale bar indicates 20 μm. Three independent cultures were tested, and representative images are shown.

The second key element of our design was the removal of the disperser biofilm; we found, we could remove the disperser biofilm by inducing Hha13D6 with IPTG (Fig. 5a). After 62 h (18 h with 2 mM IPTG), 92% of the maximum disperser biofilm was removed (Fig. 5b). Note that the 3OC12HSL signal is still made while IPTG is added so the small number of remaining colonizer cells are not able to form a biofilm while the disperser cells are being removed.

Fine-tuning consortial populations using the araBAD promoter. To fine-tune the consortial composition, a plasmid with the

![Figure 2](image1.jpg) **Figure 2** | Plasmid maps of the disperser plasmid and the initial colonizer plasmid that are used to create the μBE circuit. (a) pHha13D6-gfp-lasl with hha13D6 under control of the T5-lac promoter, and gfp and lasl under control of the constitutive CP25 promoter. (b) pBdcAE50Q-rfp-lasR with bdcAE50Q under control of the lasl promoter, and rfp and lasR under control of the constitutive CP25 promoter. cat encodes chloramphenicol acetyltransferase, lacI q encodes a repressor mutant of the lac operator and rrnB-T1 indicates the rrnB T1 transcription termination sequence.

![Figure 3](image2.jpg) **Figure 3** | Biomass of initial colonizer and disperser biofilms. (a) Biomass of initial colonizer biofilms (BdcA50Q + and LasR +; E. coli hha/pBdcAE50Q-rfp-lasl) with 500 μM of 3OC12HSL for 10 h. (b) Biomass of disperser biofilms (Hha13D6 + and Lasl +; E. coli hha/pHha13D6-gfp-lasl) with 2 mM of IPTG for 10 h. (c) Biomass after 19 h for the initial colonizer biofilms with different concentrations of 3OC12HSL (0, 71, 143, 214, 286, 357, 429 and 500 μM for 10 h). (d) Biomass after 19 h for the disperser biofilms with different concentrations of IPTG (0, 0.3, 0.6, 0.9, 1.1, 1.4, 1.7 and 2.0 mM for 10 h). Robust biofilms at 9 h were formed by seeding the initial colonizer or the disperser cells into microchambers for (a), (b), (c) and (d). Three independent cultures were tested, and error bars indicate the s.d. from two different positions in one of the three sets. Biomass was determined by COMSTAT analysis.

![Figure 4](image3.jpg) **Figure 4** | Biofilms formed by cells that lack their respective biofilm dispersal proteins. (a) Initial colonizer biofilms that lack BdcA (BdcAE50Q − and LasR + via E. coli hha/pCP25-lasl) with 500 μM of 3OC12HSL for 10 h. (b) Disperser biofilms that lack Hha (Hha13D6 − and Lasl + via E. coli hha/pGFP-lasl) with 2 mM of IPTG for 10 h. Robust biofilms of initial colonizer or disperser cells were developed for 9 h in each microchamber. Scale bar indicates 20 μm. Three independent cultures were tested, and representative images are shown.
Figure 5 | Dispersal of dual-species biofilms using quorum sensing. (a) An initial colonizer biofilm (red, BdcAE50Q+ and LasR+; E. coli hha/pBdcAE50Q-rfp-lasR) was developed for 9 h, then disperser cells (green, Hha13D6+ and LasI+; E. coli hha/pHha13D6-gfp-lasI) were seeded for 5 h to form both initial colonizer and disperser biofilms. After 44 h, 2 mM of IPTG was added for an additional 18 h to remove the disperser biofilm. (b) Biomass of the initial colonizer (BdcAE50Q+ and LasR+) and disperser (Hha13D6+ and LasI+) biofilms as determined by COMSTAT analysis. (c) Initial colonizer biofilms (BdcAE50Q+ and LasR+) was developed for 9 h, then control disperser cells that lack LasI (green, Hha13D6+ and LasI–; E. coli hha/pHha13D6-gfp) were seeded for 5 h to form both initial colonizer and control disperser biofilms. After 44 h, 2 mM of IPTG was introduced for an additional 20 h to try to disperse the control disperser biofilms, which lack lasI. (d) Biomass of the initial colonizer (BdcAE50Q+ and LasR+) and the no LasI disperser control (Hha13D6+ and LasI–) biofilms as determined by COMSTAT analysis. Scale bar indicates 20 μm. Three independent cultures were tested, and representative images are shown for (a) and (c). Error bars indicate the s.d. from two different positions in one of the three sets for (b) and (d).

Discussion

We developed a synthetic μBE system by combining a QS signaling module with two of our engineered biofilm dispersal proteins. With this synthetic circuit, in a microfluidic channel, we formed an initial colonizer biofilm with cells tagged red, introduced a second cell type (dispersers, tagged green) into this existing biofilm, created a means of communication between the two cell types and formed a robust biofilm with the disperser cells in an existing initial colonizer biofilm. We then displaced the initial colonizer cells in the biofilm with a QS signal from the disperser cells, and removed the disperser cells with a chemically induced switch. Our work demonstrates that biofilms can be formed, that new cells may be engineered to integrate and then replace the initial colonizer biofilm, and that both cell types may be removed, which is a promising strategy for applications requiring different kinds of engineered cells such as creating a biorefinery.

Although some of the biofilms may be dispersed naturally upon changes in environmental conditions (for example, nutrition level and oxygen depletion), it is a significant challenge to remove biofilms as cells in biofilms are cemented in place by the secreted polymer matrix consisting of polysaccharide, protein, DNA and lipids. The matrix holds bacterial cells together and forms a protective barrier that confers resistance to killing by nonspecific and specific host defenses during infection and that confers tolerance to various antimicrobial agents such as disinfectants and antibiotics.
Several biofilm dispersal signals have been identified, including growth rates. This clearly demonstrates that a QS circuit was required. Staphylococcus aureus cannot displace the initial colonizer biofilm based on a difference in growth rates. After 21h, 1% arabinose in tryptone medium was added for an additional 26h to disperse the initial colonizer-2 biofilm. Three independent cultures were tested, and representative images of biofilms from channel 2 at each time point are shown. Scale bar indicates 20μm. (b) Biomass (μm³ μm⁻²) of initial colonizer-2 and disperser biofilms at each time point in channel 2. Error bars indicate the s.d. from two different positions.

Thus, the defensive nature of the biofilm colony makes most biofilms difficult or impossible to eradicate; hence, our demonstration that both the initial colonizer and disperser biofilms may be nearly completely removed is significant.

To preferentially remove one type of cell in a biofilm, our system requires that the second cell type elicits robust growth such that it can attach to the existing biofilm and propagate, that it flourishes, and that it communicates to the other cell type via a QS signal, and that it displaces the existing biofilm without itself being displaced so that it instead forms a strong biofilm. Here, we produced the QS signal in the biofilm itself to remove the initial colonizer cells. As the signal accumulated, the engineered BdcA in the initial colonizer cells reduces c-di-GMP levels, which results in a cascade of events, such as an increase in motility and reduction in adhesion production, that allows the initial colonizer cells to disperse.

As the initial colonizer cells disperse, the disperser cells must form a robust biofilm. After the disperser biofilm is formed, the engineered Hha protein, once induced, causes dispersal by inducing cell lysis. Therefore, our synthetic μBE system provides a useful platform for the removal of existing deleterious biofilms via generating signalling molecules in situ. In addition, as the disperser cells grow more slowly than the initial colonizer ones, the disperser cells cannot displace the initial colonizer biofilm based on a difference in growth rates. This clearly demonstrates that a QS circuit was required to complete this feat of progressive biofilm development/dispersal. As several biofilm dispersal signals have been identified including the auto-inducing peptide of the agr QS system of Staphylococcus aureus, changes in carbon sources, reduction in the concentration of c-di-GMP (as utilized here with BdcA), surfactant, and 2-decenoyl-3-oxoacyl-

Figure 6 | Dispersal of dual-species biofilms using arabinose. (a) Initial colonizer-2 biofilms (E. coli hha/pPBAD-bdcAESQ-rfp-lasR, red) were developed in the microfluidic device for 10h, then disperser cells (E. coli hha/pHha13D6-gfp-lasI, green) were seeded for 5h to form both initial colonizer-2 and disperser biofilms in LB-glucose medium. After 21h, 1% arabinose in tryptone medium was added for an additional 26h to disperse the initial colonizer-2 biofilm. Three independent cultures were tested, and representative images of biofilms from channel 2 at each time point are shown. Scale bar indicates 20μm. (b) Biomass (μm³ μm⁻²) of initial colonizer-2 and disperser biofilms at each time point in channel 2. Error bars indicate the s.d. from two different positions.

Figure 7 | Population dynamics of initial colonizer-2 and disperser biofilms. (a) A gradient of initial colonizer-2 biofilms (E. coli hha/pPBAD-bdcAESQ-rfp-lasR, red) was developed across channels 1 through 8 in the microfluidic device for 10h such that the maximum initial colonizer-2 biofilm was formed in channel 8, then a uniform concentration of disperser cells (E. coli hha/pHha13D6-gfp-lasI, green) were seeded for 5h in all eight channels to form both initial colonizer-2 and disperser biofilms in LB-glucose medium at different biofilm ratios across the eight channels. After 21h, 1% arabinose in tryptone medium was added to disperse the initial colonizer-2 biofilm. Three independent cultures were tested, and representative images of biofilms at time 21h and 37h are shown. Scale bar indicates 20μm. (b) Biomass (μm³ μm⁻²) of initial colonizer-2 and disperser biofilms at time 21h and 37h in the eight channels. Error bars indicate the s.d. from two different positions.
supplemented with glucose (0.2%), at a turbidity of 600 nm of -0.1. The bacterial suspension was then introduced into the eight biofilm microchambers through the top layer in the PDMS device (Fig. 1b). During this process, the main inlet valve (Fig. 1b) remained closed to prevent cells from entering and forming biofilm in the gradient-mixing channels, and to ensure proper mixing of dispersal signals before they enter the microchambers. The main outlet valves and seeding valves were then closed, and the culture was maintained without flow for 2 h to enable attachment of bacteria to the glass surface (see methods). After 2 h, both main inlet and outlet valves were opened, unattached cells were removed and the attached bacteria were allowed to grow by flowing LB-glucose at 2 µl min⁻¹. After 3 h, the medium was switched from LB-glucose to M9-glucose for 3 h because we found that a sudden depletion of nutrients promoted rapid development of biofilms. The biofilm was then developed for another 3 h by introducing LB-glucose into the chambers in a semi-batch mode (55-min static and 5-min flow). Thus, within 9 h after seeding, a robust and mature biofilm was formed. To disperse the biofilm, LB-glucose and LB-glucose containing a single concentration of the dispersal signal (IPTG for disperser cells and 3c12HSL for initial colonizer cells) was introduced through the two media inlets and allowed to mix in the serpentine gradient-generating channels to form eight concentrations of the dispersal signal in LB-glucose medium. Each stream leaving the diffusive mixer was used to perfuse a specific biofilm microchamber for 10 h.

For dual-species biofilm dispersal experiments using 3c12HSL signalling, initial colonizer cell biofilms were developed uniformly across all eight microchambers for 9 h as for mono-species biofilms. During this 9-h period, unattached initial colonizer cell biofilms were removed from each microchamber using 1% arabinose in tryptone broth solution after 9 h and then developed for another 3 h by introducing LB-glucose into the chambers in a semi-batch mode (55-min static and 5-min flow) for 28–30 h. The static condition ensured biofilm development and build-up of 3c12HSL required for induction of the BdcAE50Q dispersin protein in initial colonizer cells. To remove the disperser cell biofilm, LB-glucose containing 2 mM IPTG was introduced in semi-batch mode for 18 to 20 h. For dual-species biofilm dispersal experiments using arabinose, initial colonizer cell biofilms were developed as a gradient across the eight microchambers for 10 h, then a uniform concentration of disperser cells were seeded for 5 h in all eight channels in LB-glucose medium. After 21 h, 1% arabinose in tryptone medium was added for an additional 26 h to disperse the initial colonizer biofilm.

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Author contributions
T.K.W. and A.J. conceived the experiments with T.K.W. primarily supervising the synthetic biology experiments and A.J. supervising the microfluidic experiments. S.H.H. constructed the strains and performed the assays. M.H. and J.K. performed the microfluidic experiments and evaluated the biofilm compositions, and X.W. evaluated some of the bacterial strains. All authors helped write the manuscript.

Additional information
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