Vibrio cholerae Combines Individual and Collective Sensing to Trigger Biofilm Dispersal

Highlights
- Cells in V. cholerae biofilms decide to disperse by combining two sensory mechanisms
- Quorum sensing and RpoS provide information on different environmental parameters
- Integration of both sensory inputs yields robust and optimal dispersal decisions

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In Brief
Triggers and mechanisms of biofilm dispersal are poorly understood. Singh et al. show that biofilm dispersal of Vibrio cholerae is regulated by combining individual and collective cell-level sensing mechanisms, via the RpoS-mediated general stress response and quorum sensing, respectively, for making robust dispersal decisions.
**Vibrio cholerae** Combines Individual and Collective Sensing to Trigger Biofilm Dispersal

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**SUMMARY**

Bacteria can generate benefits for themselves and their kin by living in multicellular, matrix-enclosed communities, termed biofilms, which are fundamental to microbial ecology and the impact bacteria have on the environment, infections, and industry [1–6]. The advantages of the biofilm mode of life include increased stress resistance and access to concentrated nutrient sources [3, 7, 8]. However, there are also costs associated with biofilm growth, including the metabolic burden of biofilm matrix production, increased resource competition, and limited mobility inside the community [9–11]. The decision-making strategies used by bacteria to weigh the costs between remaining in a biofilm or actively dispersing are largely unclear, even though the dispersal transition is a central aspect of the biofilm life cycle and critical for infection transmission [12–14].

Combining information from individual (stress response) and collective (quorum sensing) avenues of sensory input, we show that *Vibrio cholerae* integrates dual sensory inputs to control the dispersal response: cells use the general stress response, which can be induced via starvation, and they also integrate information about the local cell density and molecular transport conditions in the environment via the quorum sensing apparatus. By combining information from individual (stress response) and collective (quorum sensing) avenues of sensory input, biofilm-dwelling bacteria can make robust decisions to disperse from large biofilms under distress, while preventing premature dispersal when biofilm populations are small. These insights into triggers and regulators of biofilm dispersal are a key step toward actively inducing biofilm dispersal for technological and medical applications, and for environmental control of biofilms.

**RESULTS AND DISCUSSION**

The transition from planktonic to biofilm growth of bacteria, which includes sophisticated surface interactions and the involvement of the second messenger c-di-GMP, is increasingly well characterized [3, 4, 10, 15]. By contrast, our understanding of the environmental triggers and molecular mechanisms of dispersal from biofilms back into the planktonic mode is in its early stages [12–14, 16], despite the large technological potential of harnessing biofilm dispersal for controlling biofilm growth.

Intuitively, biofilm-dwelling cells may benefit from a departure when local environmental conditions become hostile, and indeed the inducers of active dispersal that have been identified to date are different kinds of stress. These include the depletion of nutrients, lack of oxygen, pH fluctuations, changes in nitric oxide concentrations, alterations in temperature, and quorum-sensing induction [17–24]. Such environmental cues are likely to influence the production of substances that facilitate departure from the biofilm, such as enzymes that degrade the matrix or degrade the substratum, and/or surfactants that interrupt cell-cell, cell-matrix, and cell-substratum binding [12, 13, 16].

Many central aspects of the biofilm dispersal phenomenon remain unclear. Perhaps most fundamentally: what are the sensory pathways governing dispersal, and how are the sensory inputs integrated to buffer against environmental fluctuations? Here, we target these questions using the human pathogen *Vibrio cholerae*, for which transitions between biofilm and planktonic growth are critical to life in its natural marine habitat and within infected hosts, and for which the dispersal mechanisms are unknown. By investigating which environmental drivers and cellular regulatory responses trigger biofilm dispersal, we discovered that *V. cholerae* tunes biofilm dispersal by integrating information from the individual stress-response and collective quorum-sensing mechanisms to monitor a combination of nutrient quality, mass transport conditions, and community size.

**Characterization of Biofilm Dispersal in V. cholerae**

While cultivating biofilms of wild-type (WT) *V. cholerae* in microfluidic devices with constant flow, we noticed that after flow is halted, mature biofilms rapidly shrink in biomass as cells depart...
from the community (Movie S1). This observation is consistent with prior studies of *V. cholerae* and other model species [13, 18, 23, 25], but it is unclear how or why halting flow causes biofilms to disassemble. Several important environmental parameters can be dramatically altered by changing flow speed. These include altered shear stress applied by the flow itself to the biofilm perimeter, as well as altered solute concentrations in the nearby environment [1, 26]. For example, when the flow is stopped, nutrient levels may be rapidly depleted, while any secreted compounds—which are no longer swept away by flow—can quickly accumulate [26–28].

To better understand the role of solute transport and shear forces in dispersal, we first investigated how changing the flow rate affects the dispersal response. We grew *V. cholerae* WT biofilms in a nutrient medium with flow rate of 0.1 μL/min until they reached a diameter of 20–25 μm, after which the flow rate was held constant, decreased, or increased. The resulting biofilm behavior varied continuously as a function of the change in flow rate through the system. When flow was stopped or sufficiently slowed, dispersal occurred, whereas, if flow rate was increased, biofilms increased in size at a higher rate relative to controls in which flow was held constant (Figure 1A).
results suggest that flow-determined supply or removal of soluble compounds in the medium surrounding the biofilm is involved in regulating biofilm growth and dispersal.

Removal of growth substrates and the addition of nitric oxide have previously been implicated as inducers of biofilm dispersal in other species [16, 18, 19, 22, 23]. In V. cholerae biofilms, we found that removing the carbon source (here, glucose), while leaving the flow rate unchanged, resulted in a strong biofilm dispersal response (Figure 1B). Oxygen removal resulted in a weak but significant dispersal response, and the addition of nitric oxide had no measurable effect compared with controls in which the flow rate and medium composition were left unchanged.

The strong effect of flow on biofilm dispersal (Figure 1A) could in part also be due to changes in fluid shear stress on the biofilm. The shear stress is largest on the highest points of the biofilm above the substratum, and lowest near the substratum, as a consequence of basic fluid physics. We would therefore expect the outcome of shear-dependent dispersal to vary as a function of the cellular height in biofilms. We tested this prediction by investigating the spatiotemporal dynamics of the biofilm dispersal response at the single-cell level. To perform these measurements, we extended our recently developed biofilm imaging techniques [29] to now also detect cell departure events from a biofilm in an image time series (see STAR Methods). When flow is halted, or when the carbon source is removed while leaving flow intact, we observed that dispersing cells mostly depart from the biofilm’s outer surface (Figures 1C and 1E; see also Figure S1 for an explanation of space-time biofilm heatmaps). However, we found no dependence of dispersal probability on cell height above the substratum (Figure S2), which is also revealed by the 3D visualization of cellular dispersal events (Figures 1D and 1F; Movie S2). As dispersal did not vary with cell height in biofilms, we conclude that changes in shear are not the primary determinant of biofilm dispersal for the range of flow rates we tested; rather, alteration of solute concentrations around the biofilm is the sole plausible cue to trigger biofilm dispersal in our system.

Spatiotemporal measurements of dispersal at high resolution also showed that carbon source removal triggers a significantly faster dispersal response relative to halting flow (Figures 1C and 1E). Finally, we determined that cells primarily depart from the biofilm as individuals; the size of the dispersing cell clusters has a strong peak corresponding to single cells (Figures 1C and 1E).

Dual Regulators of the Dispersal Response

The flow-stop-induced biofilm dispersal response could occur because cells within a biofilm measure the accumulation of certain solutes to detect flow itself, and/or because nutrient sources are gradually depleted after the influx of fresh nutrient medium is halted. To begin untangling these factors, we engineered a translational fusion [30] of the fluorescent protein mRuby3 to RpoS, which regulates the generalized stress response during nutrient limitation [31]. We chose a translational fusion reporter approach because RpoS falls under strong post-transcriptional regulation [32]. Previous work has shown that RpoS is required for the detachment of V. cholerae from mucosal linings in vivo [25, 33]. By monitoring our RpoS reporter in space and time during biofilm growth, we found that stopping the flow or removing glucose resulted in elevated rpoS transcription (Figure S3A) and translation (Figure 2A).

In addition to monitoring the level of soluble nutrients, cells could potentially gain more information about the environment by sensing the accumulation of diffusible compounds that they themselves secrete, whose concentrations convey information about the local rate of fluid flow and community size. Quorum-sensing autoinducer compounds are secreted and sensed by many different bacterial species, and previous work has shown that autoinducer accumulation in biofilms can correlate with the absence of flow [26–28, 34–36]. In addition, autoinducer concentrations are relayed by V. cholerae into biofilm matrix production rates: low autoinducer concentrations (“low cell density” state) induce high expression of biofilm matrix, whereas high autoinducer concentrations (“high cell density” state) cause repression of matrix genes [37].

Given that autoinducer concentration may carry different information about the environment than nutrient levels alone, and given the known connection between autoinducer levels and matrix production rates, we hypothesized that V. cholerae might use quorum sensing to regulate active biofilm dispersal. To investigate this hypothesis, we constructed a translational fusion of the fluorescent protein sfGFP to the quorum-sensing master regulator HapR. Functionality of the HapR-sfGFP fusion was confirmed via qRT-PCR-based measurements of known HapR-targets (Figure S3B). Monitoring HapR-sfGFP levels in space and time revealed that HapR levels substantially increase during biofilm development, even when flow and nutrient supply are not interrupted, though a statistically significant increase of HapR upon flow stoppage is apparent (Figure 2B). Biofilms continued to grow in the presence of high HapR levels, even though matrix production is known to be repressed in the physiological state associated with high autoinducer concentrations.

To understand whether high RpoS levels, high HapR levels, or high levels of both RpoS and HapR are necessary for causing biofilm dispersal, we constructed deletion, complementation, and inducible overexpression strains for hapR, rpoS, or both regulators, which were assessed for their dispersal ability. When glucose was removed from the medium (Figure 3A, red bars), dispersal no longer occurred in ΔhapR, ΔrpoS, and ΔhapRΔrpoS mutants; all three deletion strains instead continued to grow up to a consistent limit, presumably due to the reduced availability of nutrients after glucose-free medium began to enter the growth chambers. When flow was halted (Figure 3A, blue bars), the deletion mutants continued to grow in a manner identical to control conditions in which nutrient flow was uninterrupted (Figure 3A, gray bars), indicating that both regulators must be present for the dispersal response to operate normally.

To confirm that high levels of HapR and/or RpoS induce biofilm dispersal, we constructed WT strains carrying P_lopa/hapR, P_lopa/rpoS, or P_lopa/hapR-rpoS as inducible constructs on a plasmid. These strains were grown as described above, but, instead of triggering dispersal by halting flow or removing glucose from the influent, we induced expression of hapR and/or rpoS by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the influent medium. Induction of hapR alone, rpoS alone, or both genes together triggered biofilm dispersal, with rpoS causing a larger response than hapR (Figure 3B).
The induction of both genes together did not trigger a larger dispersal response than the induction of rpoS alone, because, for the biofilm sizes we investigated, HapR had already accumulated to high levels when the flow-stop or glucose-removal condition was imposed (Figure 2B). Therefore, for the rpoS-inducible strain, HapR levels were naturally high when RpoS production was induced, which permitted the full dispersal response to occur (Figure 3B).

We assessed the contribution of individual components of the quorum-sensing pathway [38] by performing the flow-stop assay with mutants systematically deleted for signal-producing and signal-sensing elements of the quorum sensing circuit (Figure 3C). These experiments demonstrated that production and detection of the species-specific autoinducer CAI-1 has the strongest influence on dispersal induction, while autoinducer AI-2, which is produced by a wide range of bacterial species...
only weakly influences the biofilm dispersal response. Mutants locked in the “low cell density” physiological state (carrying the luxO<sup>D47E</sup> allele) could not disperse, similar to the <i>ΔhapR</i> mutants, but mutants locked in the “high cell density” state (luxO<sup>D47A</sup>) could readily disperse.

As HapR is a transcription factor, it can enhance the expression of target genes only via recruitment of RNA polymerase and appropriate sigma factors, such as that encoded by <i>rpoS</i> (s<sub>S</sub>). With this in mind, we predicted that HapR and RpoS act synergistically to regulate dispersal and that HapR induction alone cannot promote dispersal in the complete absence of RpoS. To test this hypothesis, we introduced <i>hapR</i> under the control of an inducible promoter into a <i>ΔrpoS</i> background and tested this strain for the ability to disperse after <i>hapR</i> induction. The results demonstrate, as predicted, that strong HapR production alone is not sufficient to induce dispersal (Figure S4). It is important to note that RpoS and HapR have been shown to reciprocally upregulate each other’s expression, suggesting the presence of positive feedback between them for regulation of their joint targets. Understanding whether additional regulators are involved in the decision to disperse from the biofilm is an important topic for future work, along with an investigation of which secreted factors effect the dispersal response.

**Robust and Informed Decisions for Dispersal**

Our results indicate that cells make use of two different, synergistic sensory inputs for regulating biofilm dispersal: cells sense the lack of nutritious molecules, which is relayed via the general stress response regulator RpoS. But cells also sense the accumulation of self-secreted molecules, relayed via HapR, which carries information on the local population density and the mass transport environment.

We next sought to understand the principle of why the <i>V. cholerae</i> dispersal response is governed by two integrated sensory inputs. Given that autoinducer and nutrient concentrations can each be above or below the threshold for HapR and RpoS induction, respectively, there are in principle four potential HapR/RpoS physiological states, which should each result in different biofilm dispersal decisions (Figure 4A), based on the reporter data from Figure 2 and the biomass dispersal data from Figure 3. When biofilms are small, autoinducer retention in the biofilm is low (yielding low HapR levels, Figure 2B), and dispersal will not occur in the presence of nutritious flow (yielding low RpoS levels, Figure 2A). But for such small biofilms, our data from Figure 3 predict that, even if nutrient availability decreases (yielding high RpoS levels), dispersal will not occur. On the other hand, biofilms naturally accumulate autoinducers as they increase in size, leading to induction of HapR (see Figure 2B), which “primes” the group for dispersal in the event of nutrient availability drop [38].
Conclusions
We have shown that biofilm-dwelling V. cholerae cells integrate quorum sensing and nutrient starvation pathways to govern active dispersal with remarkable sophistication. By integrating these two pathways, they are able to optimize the collective decision for biofilm departure while also avoiding the opportunity cost of leaving newly formed biofilm groups before they have had a chance to accumulate significant biomass. Inducing biofilm dispersal, based on the regulators identified in this study, provides a promising approach for controlling and dissolving biofilm populations in industry, environment, and medicine, where they cause substantial costs and life-threatening diseases.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - General plasmids and strains
  - Overexpression strains
  - Complementation strains
  - Mutant strains
  - Reporter strains
  - qRT-PCR
  - Flow chamber biofilm experiments
  - Dispersal experiments
  - HapR and RpoS reporter imaging
  - Microscopy and image analysis
  - RpoS and HapR translational fusions
  - Experimental design
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Model for biofilm size-dependent dispersal

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and two movies and can be found with this article online at https://doi.org/10.1016/j.cub.2017.09.041.

AUTHOR CONTRIBUTIONS

C.D.N. and K.D. conceived the study and designed the experiments. P.K.S., S.B., and L.V. generated strains and performed experiments. R.H. developed and performed image analysis. P.K.S., S.B., R.H., H.J., and K.D. analyzed the data. R.H. made the figures. All authors interpreted the data and critically discussed the results. P.K.S., C.D.N., and K.D. wrote the manuscript with the help of all authors.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| E. coli ΔlacU169 (ΔlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir | Invitrogen | S17 |
| E. coli mcrAΔ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15ΔlacX74deoRecA1 araD139Δ(ara-leu)7697 galU galK rpsL endA1 nupG | Invitrogen | TOP10 |
| V. cholerae C6706 (O1 El Tor, Sm^r)/ Wild type | This work | KDV201 |
| V. cholerae C6706 ΔcqsA ΔcqsS | This work | KDV47 |
| V. cholerae C6706 ΔhapR | This work | KDV48 |
| V. cholerae C6706 ΔluxO | This work | KDV51 |
| V. cholerae C6706 ΔcqsS | This work | KDV52 |
| V. cholerae C6706 luxO^DATA | This work | KDV53 |
| V. cholerae C6706 luxO^DATE | This work | KDV54 |
| V. cholerae C6706 ΔcqsA | This work | KDV56 |
| V. cholerae C6706 contains plasmid pNUT542 | This work | KDV296 |
| V. cholerae C6706 hapR::10aa-sfGFP | This work | KDV425 |
| V. cholerae C6706 lacZ,P_ tac-sfgfp::lacZ | This work | KDV428 |
| V. cholerae C6706 ΔhapR, lacZ,P_ tac-sfgfp::lacZ | This work | KDV433 |
| V. cholerae C6706 ΔluxO, lacZ,P_ tac-sfgfp::lacZ | This work | KDV435 |
| V. cholerae C6706 luxO^DATA, lacZ,P_ tac-sfgfp::lacZ | This work | KDV439 |
| V. cholerae C6706 luxO^DATE, lacZ,P_ tac-sfgfp::lacZ | This work | KDV441 |
| V. cholerae C6706 ΔcqsA ΔluxS, lacZ,P_ tac-sfgfp::lacZ | This work | KDV443 |
| V. cholerae C6706 ΔcqsA, lacZ,P_ tac-sfgfp::lacZ | This work | KDV445 |
| V. cholerae C6706 hapR::10aa-sfGFP lacZ,P_ tac-mRuby2::lacZ | This work | KDV459 |
| V. cholerae C6706 ΔluxPQ, lacZ,P_ tac-sfgfp::lacZ | This work | KDV488 |
| V. cholerae C6706 ΔcqsS ΔluxPQ, lacZ,P_ tac-sfgfp::lacZ | This work | KDV510 |
| V. cholerae C6706 lacZ,P_ tac-sfgfp::lacZ contains pNUT641 plasmid | This work | KDV515 |
| V. cholerae C6706 contains pNUT894 plasmid | This work | KDV537 |
| V. cholerae C6706 ΔropS, lacZ,P_ tac-sfgfp::lacZ | This work | KDV571 |
| V. cholerae C6706 ΔropS, lacZ,P_ tac-sfgfp::lacZ contains pNUT968 plasmid | This work | KDV574 |
| V. cholerae C6706 ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT970 plasmid | This work | KDV575 |
| V. cholerae C6706 hapR::10aa-sfGFP, lacZ,P_ tac-mRuby2::lacZ contains pNUT641 plasmid | This work | KDV581 |
| V. cholerae C6706 ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT641 plasmid | This work | KDV583 |
| V. cholerae C6706 ΔropS, lacZ,P_ tac-sfgfp::lacZ contains pNUT641 plasmid | This work | KDV584 |
| V. cholerae C6706 lacZ,P_ tac-sfgfp::lacZ contains pNUT968 plasmid | This work | KDV586 |
| V. cholerae C6706 lacZ,P_ tac-sfgfp::lacZ contains pNUT970 plasmid | This work | KDV587 |
| V. cholerae C6706 ΔropS, ΔhapR, lacZ,P_ tac-sfgfp::lacZ | This work | KDV591 |
| V. cholerae C6706 ΔcqsS, lacZ,P_ tac-sfgfp::lacZ | This work | KDV710 |
| V. cholerae C6706 ΔhapR, lacZ,P_ tac-sfgfp::lacZ, lacZ,P_ hapR::hopR::lacZ | This work | KDV750 |
| V. cholerae C6706 ΔropS, lacZ,P_ tac-sfgfp::lacZ, lacZ,P_ mpo::hopS::lacZ | This work | KDV753 |
| V. cholerae C6706 lacZ,P_ tac-sfgfp::lacZ contains pNUT1076 plasmid | This work | KDV756 |
| V. cholerae C6706 ΔropS, ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT1076 plasmid | This work | KDV758 |
| V. cholerae C6706 ΔropS, ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT641 plasmid | This work | KDV765 |
| V. cholerae C6706 ΔropS, ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT968 plasmid | This work | KDV766 |
| V. cholerae C6706 ΔropS, ΔhapR, lacZ,P_ tac-sfgfp::lacZ contains pNUT970 plasmid | This work | KDV767 |
| V. cholerae C6706 ropS::4aa-mRuby3 | This work | KDV901 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Knut Drescher (k.drescher@mpi-marburg.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Standard molecular biology techniques were applied to construct plasmids and strains [45]. Restriction enzymes and DNA polymerase enzymes were purchased from New England Biolabs. All V. cholerae strains used in this study are derivatives of the wild-type V. cholera O1 biovar El Tor strain C6706. V. cholerae deletion mutations were engineered using the pKAS32 suicide vector harbored in E. coli S17-1 pir. All overexpression constructs were cloned into a low copy number plasmid with a pSC101 origin of...
replication with a gentamycin resistance cassette, or inserted at the lacZ site with the help of the suicide plasmid pKAS32 [43]. All strains except those containing hapR and rpoS reporters contain the fluorescent protein-encoding locus sfGfp, constitutively expressed under the control of the Ptac promoter. Plasmid clones were first constructed in E. coli strain Top10 and then mated into V. cholerae using an E. coli strain harboring the conjugation plasmid pRK600. All strains were grown in LB medium supplemented with appropriate antibiotics at 37 °C for normal growth. Biofilm experiments with V. cholerae were performed in M9 minimal medium, supplemented with 2 mM MgSO4, 100 mM CaCl2, MEM vitamins, 0.5% glucose, and 15 mM triethanolamine (pH 7.1). 1 mM IPTG and 250 nM nitric oxide (NO) were also added into the media whenever needed. Detailed strain and plasmid and oligos lists are provided in key resource table.

**METHOD DETAILS**

**General plasmids and strains**

Plasmid pNUT325 was constructed by ligating the superfolder green fluorescent gene (sfGfp), a pSC101* origin of replication, and a gentamyacin resistance gene. The pSC101* origin of replication (ori) was replaced by the p15a origin of replication in pNUT325 to generate pNUT330. Plasmid pNUT542 was constructed by ligating a commercially synthesized Ptac promoter in front of the sfGfp gene into pNUT325, amplified with the oligonucleotides kdo622/kdo642. Plasmid pNUT641 was created by self-ligating the amplification product generated from pNUT542 with oligos kdo368/kdo622. Plasmid pNUT480 was constructed by ligating 1 kb upstream and downstream DNA sequences of the lacZ gene together with P_{tac}-sfGfp into the plasmid pNUT144 [8]. Strains KDV428,433,435,439,441,445 and 571 were generated by inserting the P_{tac}-sfGfp cassette at the lacZ locus into the strains KDV201,48,51,55,56 and 752, respectively.

**Overexpression strains**

Overexpression plasmids pNUT968 and pNUT970 were made by amplification of the V. cholerae rpoS gene (oligos kdo1217/kdo1218) and the hapR gene (oligos kdo1120/kdo1216), respectively, and ligating them into pNUT542, amplified with oligos kdo1212/kdo1216. Plasmid pNUT1076 was also based on the pNUT542 backbone where both rpoS and hapR genes were transcriptionally joined using the sewing PCR method, using oligos kdo1217/kdo1344. Overexpression strains KDV586,587,756 (respectively) were generated by conjugating plasmids pNUT968,970,1076 into strain KDV428. HapR overexpression in rpoS deletion background strain (KDV916) was generated by conjugating plasmid pNUT970 into KDV571.

**Complementation strains**

Complementation strains KDV750 and KDV753 were generated by using plasmids pNUT1058 and pNUT1060, respectively. Plasmids pNUT1058 and pNUT1060 were constructed by ligating the amplified hapR locus with its native promoter (using oligos kdo1323/kdo1324) and the rpoS locus together with its native promoter (using oligos kdo1218/kdo1325) into plasmid pNUT480, respectively. Plasmids pNUT1058 and pNUT1060 were conjugated into strain KDV201 to generate complementation strains KDV750 and KDV753.

**Mutant strains**

To generate the ΔluxPQ strain (KDV488), the 1kb flanking regions of luxPQ operon were amplified with oligos kdo902/kdo904 and kdo903/kdo905, and the fused PCR product was amplified using kdo902/kdo903. The PCR product was ligated into plasmid pNUT144 (a variant of pKAS32 harboring a kanamycin resistance cassette [8]. The resulting plasmid pNUT727 was conjugated into strain KDV428, to generate the ΔluxPQ deletion mutant, following the selection protocol described earlier [43]. Finally, cells containing correct mutation were screened by PCR.

**Reporter strains**

For mRuby2-based transcriptional reporter plasmid pNUT894, the promoter sequence of rpoS was amplified with oligonucleotides kdo1112/kdo1113 and cloned in front of mRuby2 in the plasmid pNUT883. The reporter plasmid pNUT894 was conjugated into KDV201 to generate KDV537.

For construction of a HapR-10aa-sfGFP translational fusion at the hapR native locus, the hapR-10aa-sfGfp sequence was constructed in a pNUT144 plasmid backbone. Two 1 kb DNA fragments upstream (including hapR) and downstream of hapR were separately amplified using oligos kdo842/kdo849 and kdo846/kdo847, respectively. The sfGfp gene was amplified from the pNUT480 plasmid template using oligos kdo845/kdo848. All three PCR fragments were joined by sewing PCR and finally amplified by oligos kdo842/kdo847. The final PCR product was ligated into pNUT144 at appropriate restriction sites. The resulting plasmid, pNUT688, was mated into V. cholerae for allelic substitution, yielding strain KDV425. Strains KDV459 was generated by inserting the P_{tac}-mRuby2 cassette at the lacZ locus into the strains KDV425. A control plasmid pNUT641 was conjugated into KDV459 to generate KDV581.

For construction of a RpoS-4aa-mRuby3 translational fusion at the rpoS native locus, the same strategy was used as for the HapR-10aa-sfGFP translational fusion. The 4aa linker was taken from an analogous RpoS-mCherry fluorescent protein fusion that was previously used and tested in E. coli [30]. The rpoS-4aa-mRuby3 sequence was constructed in a pNUT144 plasmid backbone as follows. Two 1 kb DNA fragments upstream (including rpoS) and downstream of rpoS were separately amplified using the Ptac promoter in front of the sfGfp gene into plasmid pNUT325, amplified with the oligonucleotides kdo842/kdo849 and kdo846/kdo847, respectively. The sfGfp gene was amplified from the pNUT480 plasmid template using oligos kdo845/kdo848. All three PCR fragments were joined by sewing PCR and finally amplified by oligos kdo842/kdo847. The final PCR product was ligated into pNUT144 at appropriate restriction sites. The resulting plasmid, pNUT688, was mated into V. cholerae for allelic substitution, yielding strain KDV425. Strains KDV459 was generated by inserting the P_{tac}-rpoS-mRuby3 cassette at the lacZ locus into the strains KDV425. A control plasmid pNUT641 was conjugated into KDV459 to generate KDV581.
Strain KDV425 was grown in LB medium at 37°C. To confirm the functionality of HapR-10aa-sfGFP translational fusion, measurements of the relative mRNA levels were performed by qRT-PCR. Plasmid pNUT542 was conjugated into KDV901 to generate strain KDV903.

**qRT-PCR**

To confirm the functionality of HapR-10aa-sfGFP translational fusion, measurements of the relative mRNA levels were performed. Strain KDV425 was grown in LB medium at 37°C under shaking conditions. Overnight culture was back-diluted into fresh LB, and samples of 2 mL culture were collected when the optical density at 600 nm (OD600) of the culture was 0.1 and 2, representing the "low cell density" and "high cell density" states, respectively. Total RNA was isolated using the QIAGEN RNA isolation kit. 5-10 ng of the isolated total RNA and qRT-PCR specific primers were mixed together with Kapa RT mix and Kapa SYBR FAST qPCR Master mix, supplied in the KAPA SYBR FAST one-step qRT-PCR kit (Kapa Biosystems) in a single PCR tube for each gene. A CFX384 Touch Real-Time PCR Detection System (Bio-Rad) and the Sybr Green mix (Kapa Biosystems) were used for real-time PCR measurements according to the supplier’s protocol. Primers used for qPCR are listed in Table S1 of reference [46].

**Flow chamber biofilm experiments**

*V. cholerae* biofilms were grown in microfluidic flow chambers as described earlier [29] (chamber dimensions: 500 μm wide, 100 μm high and 7 mm long) made from poly(dimethylsiloxane) (PDMS) that were bonded to glass coverslips using an oxygen plasma. The microfluidic design contained four or eight separate channels on each coverslip. The manufacturing process of these microfluidic channels guarantees highly reproducible channel dimensions and surface properties in the channels. The channels were imaged on an inverted microscope, through the coverslip at the bottom of the channels. Each channel was inoculated with freshly diluted cultures from *V. cholerae* strains, which were grown overnight at 37°C in liquid LB medium under shaking conditions. Following inoculation of the channels, the cells were given 1 hr to attach to the glass surface of the channel, before a flow of 100 μL/min M9 was initiated for 45 s to wash away non-adherent cells and to remove LB growth medium from the channels. The flow rate was then set to 0.1 μL/min until the end of the experiments. Flow rates were controlled using a high-precision syringe pump (Harvard Apparatus).

**Dispersal experiments**

All dispersal experiments were performed in flow chambers as described above. For flow-stop experiments, once biofilms reached a well-defined size (20-25 μm diameter for all experiments, except for Figure 4), the acquisition of an image time series was initiated, and then the flow was stopped for 3 hr and 45 min. While flow was halted, images were taken at 1 hr intervals. Floating cells that resulted from biofilm detachment and dispersal were washed away by restarting the flow at a rate of 10 μL/min for 15 min, exactly 3 hr and 45 min after the flow was stopped. Final time point images were taken after washing was completed (Movie S1). Biofilm biomass changes were measured as described in the section on image analysis below. In every experiment, one channel was left with constant flow as a handling control, to confirm that in these conditions biofilms did not disperse.

For glucose removal experiments, biofilms were grown as described above; at the start of the dispersal assay, the original syringes containing M9+glucose were exchanged with syringes containing M9 without glucose. Flow continued for 4 hr while imaging at 1 hr intervals. As a handling control, the original syringes containing M9+glucose were exchanged for new syringes also containing M9+glucose. The change in biofilm biomass from the time at which the medium was changed to the end of the 4 hr measuring period was measured.

For dispersal assays involving overexpression strains, biofilms were first grown as above in media containing the corresponding antibiotic for plasmid maintenance. After biofilms were grown up to the well-defined size (20-25 μm diameter), the media-providing syringes were exchanged to syringes containing M9+glucose+antibiotic-IPTG (1 mM) immediately before imaging was started. One channel for each strain was imaged without adding the inducer (IPTG) in each run of the experiment, as a control. In addition, a channel containing biofilms of a *V. cholerae* strain (KD583) containing the empty vector (pNUT641) was imaged in the presence of IPTG, as a separate control.

**HapR and RpoS reporter imaging**

Biofilms of strains KD581 (hapR::10aa-sfGFP, P_tac-mRuby2) and KD537 (pRpoS-mRuby2, P_tac-sfgfp) and KD903 (pRpoS: 4aa-mRuby3, containing plasmid pNUT542) were separately grown under constant flow (0.1 μL/min) in flow channels, as described above. After 6-7 hr incubation at room temperature, flow channels containing microcolonies (cell clusters of approximately 100-200 cells) were installed on the confocal microscope for time-lapse imaging. Growth of the biofilms was monitored over time by confocal imaging and flow-stop or glucose removal was applied once biofilms were sufficiently large (20 μm diameter), as performed during the dispersal assay in the previous sections.
Microscopy and image analysis
Most of our biofilm dispersal imaging were performed on a Nikon Ti-E inverted microscope configured for widefield epifluorescence imaging using a 40x air objective. The sfGFP protein, produced constitutively by our strains, was excited at 488 nm, and fluorescence emission was captured between 500 and 530 nm.

To track single cell dispersal events (Movie S2) or for spatiotemporal measurements of different reporters, biofilms were imaged with a Yokogawa CSU confocal spinning disk unit mounted on a Nikon Ti-E inverted microscope using a 100x oil NA 1.35 objective (Olympus) by exciting fluorescence with a 488 nm laser (for sfGFP) and a 552 nm laser (mRuby2). The hardware was controlled by Micro-Manager [48], or by NIS Elements (Nikon). Images were captured on both microscopes by an Andor iXon EMCCD camera, cooled to −90°C. Images were acquired at very low excitation light intensities with 60 ms exposure time while amplifying the readout using the EM-gain of the camera. A Nikon PFS hardware autofocus was used to correct focus drift. For single cell tracking an additional 2x lens was placed between the CSU and the Nikon Ti-E side port to increase the magnification. Image stacks were acquired at a spatial resolution of 63.2 nm (xy-plane) and 400 nm (along z) every 10 min.

To track cellular dispersal events at the single cell level, we monitored all cells in the biofilm individually and checked for the presence/absence of each cell from one time point to the next in order to identify discrete cell departure events at high resolution. Prior to cell detection, image stacks were registered to correct for small drifts in all spatial dimensions and up-sampled along the z-axis to obtain equal voxel side lengths. Then, cells were identified using MATLAB similarly as described by Drescher et al. [29], but with significant improvements. In this case segmentation was performed employing 3D edge detection and not by thresholding of 2D planes. Clumped structures were individualized by watersheding and/or k-means clustering. To identify the volume of dispersing cell clusters, cell tracking was performed. In successive image stacks cells were related by maximizing the spatial overlap. Between two 3D image frames (Δt = 10 min) individual cell movement was normally below the average cell width, except for dispersing cells. In case a cell was present at a specific location in frame i but not in frame i+1, this cell was labeled in frame i to be dispersing within the next 10 min. All connected dispersing cells were labeled as one cluster and the cluster size distribution was determined for each frame. To determine where in the biofilm dispersal events occur, the change in local biomass was calculated by splitting the biofilm into a 3D grid with grid-side length 1.3 μm. For each dissected grid cube, the occupied volume (corresponding to the local biomass) was calculated. In addition, the distance d_surf of each grid cube to the biofilm surface was estimated. Finally, a distribution function was calculated returning the normalized change in biomass for biofilm regions close to the surface (small values for d_surf) to the inner biofilm core (large values for d_surf).

For 3D-visualization the Paraview software was used [49]. For spatiotemporal monitoring of different reporters, the acquired image stacks were processed differently. As the data was acquired at lower spatiotemporal resolution, whole biofilms were segmented and analyzed using the method we introduced in Besharova et al. [50]. Briefly, the biofilm was segmented by 3-level thresholding according to Otsu’s method [51] based on a constitutive fluorescence marker and dissected into small cubes.

For each cube the average reporter fluorescence intensity and the relative location inside the biofilm (in terms of the distance from the biofilm’s center of mass projected to the surface of the substrate, d_CM) was determined. Finally, the distributions of cube reporter intensities were investigated. For each biofilm, the spatiotemporal expression levels were summarized in a heatmap as illustrated in Figure S1. The average biofilm surface was obtained from the average biofilm volume by assuming a semi-spherical shape.

RpoS and HapR translational fusions
Two different amino acid spacers were used for making RpoS and HapR translational fusions.

- Nucleotide sequence of the 10aa linker between HapR and sfGFP: TCAGGAACGGAATCACGGCATAGG taken from reference [52].
- Nucleotide sequence of the 4aa linker between RpoS and mRuby3: AGCGATTTTATG taken from reference [30].

Experimental design
All experiments were performed in n independent replicas, which were treated identically and were analyzed identically. Randomization or blinding of replicas do not apply to our data. The number of bacteria in each biofilm that was analyzed varied with biofilm size so that the sample size per replica is variable. No data were excluded from the study.

QUANTIFICATION AND STATISTICAL ANALYSIS
To test for statistical significance, heatmaps of different biofilms were averaged and compared with an averaged heatmap of non-treated control biofilms. After the “glucose removal”- or “flow stop”-condition was triggered, a one-tailed non-parametric Wilcoxon rank sum test was performed for each heatmap colour-tile (which is representing a local average across different biofilms), to test for a change in expression levels. Tiles which represent a significant change (p<0.01, where p is the statistical p-value) in RpoS or HapR expression are enclosed by a red line. We define a “significant change” in RpoS or HapR expression levels from the control conditions, when there is a probability smaller than 1% that the differences in protein expression levels we measured are due to noise.

AGCGATTTTATG taken from reference [30].

Nucleotide sequence of the 10aa linker between HapR and sfGFP: TCAGGAACGGAATCACGGCATAGG taken from reference [52].

Nucleotide sequence of the 4aa linker between RpoS and mRuby3: AGCGATTTTATG taken from reference [30].
Model for biofilm size-dependent dispersal

Below is the description of the mathematical model for calculating the critical biofilm size that induces biofilm dispersal after stopping the flow. This model is motivated by the observation that in flow conditions, high HapR levels are already present for moderately-sized biofilms (Figure 2B), so that the critical biofilm size for a dispersal response in the flow stop treatment should correspond to the biofilm size that depletes the nutrients in the surroundings and thereby induces high RpoS levels in the biofilm.

Stopping the flow over V. cholerae biofilms induces dispersal for biofilms with diameter 20-25 μm, as shown in Figure 1A of the main manuscript. The data from Figures 1, 2, and 3 of the main manuscript collectively indicate that for dispersal to occur, both HapR and RpoS levels need to be high. To obtain a prediction of the critical biofilm size that induces dispersal after a stoppage of the flow, we can simply calculate the biofilm size that results in nutrient depletion (and therefore RpoS induction) after flow is halted, as HapR levels are already high for relatively small biofilms (Figure 2).

For the calculation of the biofilm size that leads to nutrient depletion, it is important to take into account that the flow stop treatment lasts for 4 hr, as described in the Method Details section above. If the flow stop treatment would have been infinitely long, any biofilm size would eventually lead to nutrient depletion.

We will first consider the case of a spherical biofilm in an infinite, static bath of M9 nutrient medium, where the limiting nutrient is the concentration C of glucose. In this scenario, the nutrient transport to the biofilm surface is governed by diffusion. For a spherical object with radius R in an infinite static bath, the current of glucose molecules (units: number of glucose molecules per second) that can be taken up by diffusion across the entire spherical surface (assuming that the surface is a perfect absorber/sink for glucose) is

$$I_{\text{diffusive}} = 4\pi D C_{\infty} R,$$  \hspace{1cm} (Equation 1)

where D is the molecular diffusion constant of glucose, and $C_{\infty}$ is the concentration of glucose at infinity [8, 40]. However, the required current of nutrients for normal cellular growth in the biofilm is

$$I_{\text{required}} = \left(\frac{4}{3} \pi R^3\right) \beta \rho,$$  \hspace{1cm} (Equation 2)

which scales with the volume of the biofilm, and depends on the number of glucose molecules consumed per cell per time $\beta$, as well as the cell density $\rho$ (units: number of cells per volume) in the biofilm. Note that $I_{\text{diffusive}} \sim R$, whereas $I_{\text{required}} \sim R^3$, so that as R increases, there will be a biofilm size regime for which $I_{\text{diffusive}} < I_{\text{required}}$ [40]. In such an infinite static bath, nutrient limitation would therefore occur for $R > R_c$, where the critical radius $R_c$ is defined by

$$I_{\text{diffusive}} = I_{\text{required}}.$$  \hspace{1cm} (Equation 3)

To obtain a model for nutrient consumption and nutrient limitation as a function of biofilm size in our flow chamber system, we first assume that biofilms are spherical. Note that in reality, V. cholerae biofilms in our flow chambers are nearly hemispherical [29], so a reasonable assumption is to mirror the flow chamber at the substratum surface (the coverslip surface) and thus consider a flow channel with twice the volume, and a spherical biofilm in the center between the top and the bottom surface of this hypothetical flow chamber.

If we now consider the case where we stop the flow over the spherical biofilm in our hypothetical flow chamber geometry, Equation 1 does not apply, as the concentration of glucose at infinity $C_{\infty}$ is not appropriate anymore. Even if we assume that $C_{\infty}$ simply refers to the glucose concentration at the boundaries of the channel (far away from the biofilm), this concentration does not stay constant due to the glucose consumption by the biofilm. We then estimate that the glucose concentration far away from the biofilm changes with time, as $C_0 - I_{\text{required}} t / V$, where $C_0$ is the concentration of glucose in the medium far away from the biofilm before the flow is stopped, $t$ is the time after the flow stoppage, and $V$ is the volume of nutrient medium per biofilm in the channel. Therefore, an estimate for the diffusive current of glucose molecules through the biofilm surface in this flow chamber geometry is

$$I_{\text{diff}} = 4 \pi DR \left(C_0 - \frac{I_{\text{required}} t}{V}\right),$$  \hspace{1cm} (Equation 4)

while the required current of glucose molecules to the biofilm in this geometry remains

$$I_{\text{req}} = \left(\frac{4}{3} \pi R^3\right) \beta \rho.$$  \hspace{1cm} (Equation 5)

Setting $I_{\text{diff}} = I_{\text{req}}$ again defines the critical biofilm radius $R_c$, beyond which nutrient depletion occurs at a time $t$ after flow stoppage in the flow chamber geometry, because after the flow is stopped, glucose can only be transported to the biofilm by diffusion. The equation for $R_c$ can be rearranged to

$$R_c = \left(\frac{4}{3} \pi \frac{I_{\text{req}}}{\beta \rho}ight)^{1/3},$$

which scales with the volume of the biofilm, and depends on the number of glucose molecules consumed per cell per time $\beta$, as well as the cell density $\rho$ (units: number of cells per volume) in the biofilm. Note that $I_{\text{diffusive}} \sim R$, whereas $I_{\text{required}} \sim R^3$, so that as R increases, there will be a biofilm size regime for which $I_{\text{diffusive}} < I_{\text{required}}$ [40]. In such an infinite static bath, nutrient limitation would therefore occur for $R > R_c$, where the critical radius $R_c$ is defined by

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while the required current of glucose molecules to the biofilm in this geometry remains

$$I_{\text{req}} = \left(\frac{4}{3} \pi R^3\right) \beta \rho.$$  \hspace{1cm} (Equation 5)

Setting $I_{\text{diff}} = I_{\text{req}}$ again defines the critical biofilm radius $R_c$, beyond which nutrient depletion occurs at a time $t$ after flow stoppage in the flow chamber geometry, because after the flow is stopped, glucose can only be transported to the biofilm by diffusion. The equation for $R_c$ can be rearranged to

$$R_c = \left(\frac{4}{3} \pi \frac{I_{\text{req}}}{\beta \rho}ight)^{1/3},$$

which scales with the volume of the biofilm, and depends on the number of glucose molecules consumed per cell per time $\beta$, as well as the cell density $\rho$ (units: number of cells per volume) in the biofilm. Note that $I_{\text{diffusive}} \sim R$, whereas $I_{\text{required}} \sim R^3$, so that as R increases, there will be a biofilm size regime for which $I_{\text{diffusive}} < I_{\text{required}}$ [40]. In such an infinite static bath, nutrient limitation would therefore occur for $R > R_c$, where the critical radius $R_c$ is defined by

$$I_{\text{diffusive}} = I_{\text{required}}.$$  \hspace{1cm} (Equation 3)
\[ 0 = R_c^3 + \frac{V}{4\pi d} R_c^2 - \frac{3C_0 V}{4\pi \rho pt} \]  
(Equation 6)

which is a standard cubic equation for \( R_c \). To evaluate this equation and obtain a numerical estimate for \( R_c \), we need to know the experimental parameters \( V, t, D, \beta, C_0, \) and \( \rho \).

From our single-cell resolution imaging data, we can extract the cell density to be \( \rho = 0.25 \) cells/\( \mu m^3 \).

The diffusion constant of glucose in water at room temperature is documented in the literature [53] as \( D = 600 \) \( \mu m^2/s \).

For M9 medium with 0.5% glucose, the glucose concentration can be converted to standard units, giving \( C_0 = 28 \) mM = \( 168 \times 10^5 \) molecules/\( \mu m^3 \).

The glucose uptake rate of \( E. coli \) has also been measured in M9 medium with glucose to be 12.8 g/(g dry weight)/hour [54]. Noting that the dry weight of an \( E. coli \) cell in stationary phase is approximately 180 fg, we can compute that 1 g dry weight corresponds to \( 5.6 \times 10^{12} \) cells [55]. The glucose uptake rate is therefore \( \beta = 1.3 \times 10^8 \) molecules/min/cell. A similar value for the glucose uptake rate can be estimated using a different approach: The generation time of \( E. coli \) in M9 + glucose is approximately 38 ± 1 min [56]. The number of glucose molecules required to double an \( E. coli \) cell is approximately \( 2 \times 10^9 \) [57]. Careful measurements of the biomass yield of \( E. coli \) grown in M9 with glucose show that the standard yield is 0.5 g of \( E. coli \) biomass per 1.0 g of glucose [58]. Taken together these values indicate that \( 4 \times 10^9 \) glucose molecules need to be acquired within the \( \approx 40 \) min division time, yielding an estimate of \( \beta = 1 \times 10^8 \) molecules/min/cell.

To obtain the volume \( V \) of nutrient medium per biofilm in the channel, we note that the flow channels are 500 \( \mu m \) wide, 100 \( \mu m \) high, and 7000 \( \mu m \) long. For the mathematical model described above, we made the assumption of mirroring the channel around the coverslip to consider spherical biofilms that are located in the middle between the top and bottom surfaces of the channel. We can therefore assume that \( V = 500 \mu m \times 200 \mu m \times L \), where \( L \) is the mean spacing between biofilms in the channel, if all biofilms would be aligned on a single line along the length of the channel, which is located at the mid-point of the channel width. Experimentally, the value for \( L \) from our biofilm assays is \( L = 13 \pm 4 \) \( \mu m \).

Noting that \( t = 4 \) hr = \( 1.4 \times 10^4 \) s for our flow stop assays, and using the experimental values for \( V, t, D, \beta, C_0, \) and \( \rho \) described above, we estimate that \( R_c = 9 \) \( \mu m \) for our experiments. A red line indicating this value for the predicted critical biofilm diameter \( (\approx 2 R_c) \), beyond which nutrient limitation is induced by stopping the flow, is shown in Figure 4B of the main text. This estimate for \( R_c \) shows good agreement with the biofilm size that marks the transition from non-dispersing and dispersing biofilms.

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