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Biofilm dispersion

Kendra P. Rumbaugh^{1,2,3}, **Karin Sauer**^{4,5}

¹Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, Texas, 79430, USA.

²Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, Texas, 79430, USA.

³Department of the TTUHSC Surgery Burn Center of Research Excellence, Texas Tech University Health Sciences Center, Lubbock, Texas, 79430, USA.

⁴Department of Biological Sciences, Binghamton University, Binghamton, New York, 13903, USA

⁵Binghamton Biofilm Research Center, Binghamton University, Binghamton, New York, 13903, USA

Abstract

The formation of microbial biofilms enables single planktonic cells to assume a multicellular mode of growth. During dispersion, the final step of the biofilm lifecycle, single cells egress from the biofilm to resume a planktonic lifestyle. As the planktonic state is considered to be more vulnerable to antimicrobial agents and immune responses, dispersion is being considered a promising avenue for biofilm control. In this Review, we discuss conditions that lead to dispersion and the mechanisms by which native and environmental cues contribute to dispersion. We also explore recent findings on the role of matrix degradation in the dispersion process, and the distinct phenotype of dispersed cells. Last, the translational and therapeutic potential of dispersing bacteria during infection will be discussed.

Graphical Abstract

In this Review, Rumbaugh and Sauer discuss the environmental cues and microorganism-derived signals that lead to the biofilm dispersal response, recent findings of matrix-degrading enzymes required for cells to liberate themselves from the biofilm matrix, novel insight into the mechanisms and regulation of dispersal, as well as the implications of these insights for biofilm control effort.

ksauer@binghamton.edu .

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Competing interests

The authors declare no competing interests.

Further links

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INTRODUCTION

Bacteria exhibit two modes of growth: the free-living planktonic mode or the sessile, surface-attached mode within biofilms, which are structured communities encased in a self-produced polymeric matrix¹⁻³. The ability to form a biofilm is not only a common trait of various microorganisms, including lower-order eukaryotes, but has also been recognized as the dominant mode of bacterial growth in nature^{1,3,4}. The formation of biofilms is a developmental process that is initiated by planktonic (free-living) organisms aggregating and/or transitioning to a surface-associated lifestyle, and is completed when cells escape from the biofilm structure in a process referred to as dispersion to return to the single cell, planktonic mode of growth (see below). Although it is now widely accepted that most if not all bacterial species form biofilms in a cyclic process, the first and most detailed information of the biofilm developmental life cycle stems from research with *Pseudomonas aeruginosa*. This developmental process has been described as a sequential, highly regulated process, involving at least five phenotypically distinct stages (Fig. 1a), with each biofilm developmental stage corresponding to unique patterns of protein production and gene expression⁵⁻⁹. Key features relating to the sessile mode of growth include loss of flagella gene expression, production of biofilm matrix components, induction of antibiotic resistance mechanisms (including efflux-pumps even when biofilms were grown in the absence of antibiotics) and increased levels of virulence determinants¹⁰⁻¹². As a consequence, biofilm cells display characteristics and behavior that are distinct from their planktonic counterparts, with the hallmark characteristics including innate resistance to host immune defenses and their increased tolerance to stresses, including starvation, dehydration and antimicrobials. Notably, biofilms have been reported to be 10–1000-times more tolerant to various antibiotics compared to their planktonic counterparts¹³.

These features pose severe consequences in medical and industrial settings. For example, biofilms are frequently found on engineered surfaces where they cause biological fouling (biofouling). Biofouling is associated with materials deterioration such as corrosion, loss in heat-transfer efficiency and mechanical clogging in fluid transport systems (reviewed in Refs.^{14,15}). Marine biofouling is associated with more than a 30% increase in fuel consumption by large sea-going vessels to overcome the viscous drag imposed by fouling organisms on ship hulls¹⁴. Estimates suggest that governments and industry spend more than \$6 billion annually for repairs and preventive maintenance activities that result from biofouling, highlighting the economical challenge of biofilms¹⁴. However, biofilms pose the most daunting challenge to 21st century medical care^{1,16-22}. According to the National Institutes of Health, biofilms are responsible for >80% of microbial infections and >60% of all nosocomial infections, with the Center for Disease Control estimating biofilms to be the etiologic agent in 60% of all chronic infections (program announcements PA-03-047, PA-06-537). Individuals at risk for developing biofilm-related infections include those with medical implants and medical devices, and immunocompromised patients, such as those suffering from cystic fibrosis and diabetic neuropathy^{1,16-22}. In the United States, the annual incidence of biofilm-related infections is 1.96 million cases, causing an estimated 268,000 deaths, with over \$18 billion in direct costs spent on the treatment of these infections²³.

Detachment is believed to be the main mechanism that limits the overall biofilm biomass accumulation and refers to the passive release of biofilm particles due to mechanical or shear stress²⁴. Distinct detachment mechanisms have been recognized based on the shear stress and/or the extent by which biofilm particles are removed by grazing, abrasion, erosion and sloughing²⁵. Grazing refers to loss of biofilm cells by the feeding activity of eukaryotic organisms, whereas abrasion is caused by collisions of biofilm cells with particles from the environment. Erosion and sloughing refer to loss of biofilm cells by fluid frictional forces, with erosion removing small portions of the biofilm and sloughing removing intact pieces of biofilm or the entire biofilm. In contrast to detachment, dispersion is an active event in which sessile, matrix-encased biofilm cells actively escape from the biofilm, leaving behind eroded biofilms and biofilms having central voids (Fig. 1b)^{5,26–28}. Dispersion is generally characterized as the terminal stage of biofilm development (Fig. 1), often referred to as seeding dispersal²⁹, as dispersion is assumed to lead to the translocation of bacteria to new sites for colonization.

Relative to the initiation of biofilm formation, the mechanism or mechanisms by which bacteria disperse from the biofilm have received little attention until recently. However, given that dispersion coincides with biofilm bacteria converting to a planktonic mode of growth that is more vulnerable to antimicrobial agents and immune responses, dispersion is now being considered a promising avenue for biofilm control. Dispersion is a rapidly emerging field of biofilm research, as evidenced by the growing numbers of publications and the growing interest by pharmaceutical and start-up companies pursuing anti-biofilm products.

In this Review, we summarize our current knowledge of biofilm dispersion, including the environmental cues and microorganism-derived signals that lead to the dispersal response, recent findings of matrix-degrading enzymes required for cells to liberate themselves from the biofilm matrix, and novel insight into the mechanisms and regulation of dispersal. In addition, we discuss consequences of escape to dispersed cells and the host as well as implications of these insights for biofilm control efforts. Although we primarily focus on the dispersion response of the Gram-negative pathogen *P. aeruginosa* as the best-characterized dispersion system to date, examples from other microorganisms are included and parallels are drawn when possible. It is important to note that much of what will be discussed in this Review originated from *in vitro* studies that were conducted under a limited number of environmental conditions (for example, experiments carried out under flowing or semi-batch conditions) with a much smaller subset of biofilm experiments having been conducted using animal models. Thus, caution should be used when considering the *in vivo* or clinical implications of biofilm mechanisms that have only been studied *in vitro*.

The biofilm lifestyle

Biofilm formation is generally considered a cyclic process comprising phenotypically distinct stages. Despite the diversity of biofilm-forming species and biofilm architectures, numerous studies using single-species have revealed biofilm formation to coincide with distinct developmental stages and general features regardless of species^{5,30}. For one, biofilm formation is initiated with surface attachment by a few planktonic cells, which occurs in two

stages: reversible and irreversible attachment. Whereas reversible attachment enables first surface contact via electrostatic and hydrophobic interactions, the interaction is unstable, with cells frequently returning to the liquid phase (Fig. 1a). During this stage, first surface contact by rod-shaped flagellated cells is primarily mediated via the flagellum³¹. In addition, pili (such as type 1 pili and type IV pili), curli fibers and antigen 43 have been shown to mediate attachment³¹. Non-motile Gram-positive bacteria make surface contact via pili and adhesins³¹. Examples of adhesins include SagA and Acm of *Enterococcus faecium*, and Ace, Esp and *Enterococcal* biofilm pili (Ebp) of *E. faecalis*³¹. Microscopic observations suggest that once cells are irreversibly attached they cease to move and initiate matrix production, probably to ‘cement’ themselves to the surface or to each other. This is followed by clonal growth into more complex multicellular structures during the maturation stages of biofilm development^{31,32}. Various mature biofilm structures have been reported, including unstructured and overall flat biofilms, and mushroom-like or pillar-like structures referred to as microcolonies or macrocolonies that are interspersed with fluid-filled channels (Fig. 1a). However, most of these structures are only seen in environmental biofilms or biofilms grown *in vitro*. By contrast, available evidence from *in vivo*-grown biofilms and imaging of clinical biopsies indicate much smaller and less differentiated structures or aggregates³³.

Being in a biofilm enables microorganisms to colonize competitive niches and survive stressful environments³⁴. For example, compared to planktonic cells, being in a community improves the chances of survival when challenged by nutrient deprivation, dehydration, pH changes, bacteriophages or predators³⁵. This protective effect is mostly due to the polymeric matrix that encases the community, which not only provides structural support but traps nutrients from the environment for metabolic utilization by the resident bacteria, efficiently retains water through hydrogen-bond interactions, and provides a protective, bunker-like shield for the biofilm residents². During infection the biofilm provides protection from the immune system and from antimicrobial agents including biocides, oxidizing agents and antibiotics³⁵. Current thinking is that resilience against antimicrobial agents observed in biofilm communities is the combined result of different mechanisms, including starvation-induced slow growth and reduced metabolic rates^{36–40}, the presence of persister cells^{41–45}, and the sequestration and restricted penetration of antimicrobials through the matrix^{46–56}. Recent evidence further suggests that microbial biofilm communities resist the action of antimicrobial agents by expressing genes that encode various xenobiotic efflux and export systems^{12,57,58}, activating the stringent response (signaled by the alarmone guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) that modulate transcription of a third of all genes in the cell)⁴⁰, or expressing ribosome hibernation factors¹¹. Dispersion is an active event in which sessile, matrix-encased biofilm cells convert en-masse to the planktonic mode of growth, apparent by single cells actively escaping the biofilm. This process can leave behind eroded biofilms with central voids^{5,26–28}, or cavities within the biofilm structure formed by the evacuation of bacteria located in the center of mature biofilm (Fig. 1b)^{26,29,59}. Dispersion is often referred to as the final stage of biofilm development, as it marks the departure from the biofilm and the transition of formerly sessile cells to the planktonic mode of growth, which facilitates a new cycle of biofilm development at new sites of colonization²⁹(Fig. 1).

Cues and signals of biofilm dispersal

Although dispersion enables dissemination, it also leaves the former biofilm residents vulnerable. Dispersed cells face the loss of multiple fitness advantages, including drug tolerance^{5,60}. So why leave? Simply put, being in a biofilm is not always advantageous. Bacteria residing at different locations within the biofilm structure experience concentration gradients of nutrient resources, oxygen and waste products (such as acids produced by fermentation in oxygen-depleted zones) as well as extracellular signaling molecules^{11,34,61}. Transcriptome analyses of drip reactor-grown *P. aeruginosa* biofilms and *Escherichia coli* colony biofilms confirmed that biofilm cells respond to these gradients by inducing various stress responses (Fig. 2). This was apparent by the presence of high mRNA levels of genes linked to hypoxia (or long-term anoxia), which is indicative of oxygen deprivation, increased expression of RpoS -regulated genes, which is indicative of general stress and stationary phase conditions, and increased expression levels of genes that are linked to nutrient stress and slow growth^{10,11,34} (Fig. 2a). As the biofilm grows in size, these chemical gradients grow steeper, which results in increased non-uniform access to resources by the resident community and leads to the formation of subpopulations^{11,34}. In addition, continued biofilm growth coincides with increased cellular crowding that further exacerbates chemical gradients and leads to nutritional competition^{34,61}. Thus, it is not surprising that the formation of chemical gradients within biofilms have been proposed as the driving force of dispersion^{10,34,61,62}.

But what exactly is it about these steep chemical gradients that lead residents of the sessile community to leave their protective environment? Is there a tipping point at which resuming a solitary lifestyle outweighs the benefits of being in a biofilm and if so, what tips the scale? Two types of dispersal triggers that induce active dispersion have been elucidated: native and environmentally induced. Although native dispersion occurs in response to self-synthesized signaling molecules or cues that are likely to be the result of steep gradients within the biofilm, environmentally induced dispersion occurs in response to sensing factors that are present in the surrounding environment or the changing conditions of the surrounding environment (Table 1).

Native dispersion inducers and inducing conditions.

Native dispersion of biofilms grown in flow cells has been reported to be initiated by bacteria that are located in the center of mature biofilms. Cells coordinately evacuate the biofilm structure, probably through small openings in the biofilm matrix, and enter the surrounding environment, leaving behind large transparent cavities, or hollow structures that consist of non-motile cells^{26,29,59}. These observations highlight an important characteristic of the dispersion response: dispersion rarely involves the entire biofilm (Fig. 1). Instead, only cells in select areas within a biofilm will disperse or show signs of dispersion events²⁹. The specific subpopulation that disperses from the center of biofilms has been linked to the diameter and thickness of the biofilm (but not to biofilm age). Microscopic observations of biofilms grown in flow cells under continuous flow conditions indicated that dispersion events in the biofilm interior were only seen within biofilms that exceeded a minimum diameter of 40 μm and an overall biofilm height of 10 μm ^{28,29}. The biofilm diameter

was found to increase with increasing flow rate, which suggests a relationship between dispersion and medium flow, with the latter affecting the transport of various growth resources into the biofilm structure, including nutrients, oxygen and extracellular signaling molecules. Thus transport, which determines the steepness of the chemical gradient, rather than size of the biofilm, is the determining factor of whether biofilms initiate a dispersion response²⁸. This also suggests that an inducer is responsible for initiating biofilm dispersion, and such an inducer would be removed by diffusive and advective transport when the biofilm is small but accumulate as the biofilm grows in size.

One such inducer was identified in *P. aeruginosa* as the fatty acid signaling molecule cis-2-decenoic acid (cis-DA) (Table 1). Fatty acid signals are involved in intra-species, inter-species and cross-kingdom communication, and they are capable of regulating bacterial growth, virulence, motility, polymer production, biofilm development, and biofilm dispersion and persistence⁶³. Cis-DA is no exception, as this fatty acid signaling molecule induces biofilm dispersion by a range of Gram-negative and Gram-positive bacteria and yeast⁵⁹. Additional fatty acid signaling molecules include *cis*-11-methyl-2-dodecenoic acid (DSF), capable of disaggregating small aggregates such as flocs by *Xanthomonas campestris*⁶⁴, and the *Burkholderia cenocepacia* cis-2-dodecenoic acid (BDSF) that has been shown to induce dispersion of *Francisella novicida* biofilms⁶⁵.

Moreover, oxygen and pyruvate have also been identified as inducers of native dispersion. There are several supportive observations of oxygen depletion as a possible trigger of dispersion. For example, dispersion is initiated in cells grown in biofilms upon cessation of flow^{48,66–69}, dispersion originates deep within biofilm structures in areas that are subject to steep oxygen gradients (Fig. 2b). In addition, cells experiencing oxygen-limiting and electron-rich conditions^{70–72}, referred to as ‘reductive stress’ (that is, too many NADH/ electrons and not enough O₂) undergo dispersal⁷³.

Some microorganisms can cope with oxygen depletion and reductive stress by using an inorganic molecule other than oxygen such as sulfate or nitrate as a final electron acceptor for anaerobic cellular respiration. However, in the absence of alternative anaerobic respiratory pathways or alternative terminal electron acceptors, species like *P. aeruginosa* initiate fermentative processes, including pyruvate fermentation^{74,75}. The pyruvate in *P. aeruginosa* PA14 biofilms that enable pyruvate fermentation was shown to be self-produced and released by metabolically active cells exposed to oxygen, using a pyocyanin-dependent mechanism⁷⁶. Thus, pyruvate is likely to be produced mostly in the periphery of biofilms. The role of pyruvate in biofilm formation was supported by the addition of 10 mM pyruvate to the growth medium of biofilms grown in semi-batch conditions in microtiter plates, which resulted in a substantial increase in biofilm biomass accumulation by *P. aeruginosa*, whereas continuous addition of pyruvate dehydrogenase to deplete pyruvate prevented the formation of three-dimensional biofilm structures⁷². By contrast, enzymatic depletion of pyruvate from mature *P. aeruginosa* biofilms induced dispersion⁷³, which suggests pyruvate availability, and thus the ability to sustain anaerobic survival, is a switch that inversely regulates biofilm formation and biofilm dispersion.

Pyruvate availability also affects biofilm formation and dispersion by *Staphylococcus aureus*, but the mechanism responsible has not yet been elucidated⁷³. Pyruvate formate lyase has been reported to have a role in *S. aureus* biofilm formation, by functioning as a formate supplier for metabolic processes during anaerobiosis, with formate oxidation correlating with the generation of NADH/H⁷⁷. These findings not only point at redox stress being a driving force of dispersion, but also at dispersion being a reversal of biofilm formation. Dispersion being a reversion of biofilm formation is further supported by the role of iron. One study⁷⁸ demonstrated the formation of structured *P. aeruginosa* biofilms to be favored in media with low iron availability, as increasing iron concentrations resulted in increasingly unstructured biofilms. By contrast, *P. aeruginosa* biofilms have been reported to undergo a dispersion response when challenged with free iron *in vitro*⁷⁹.

Dispersion is also associated with increased cell death^{32,80}. Cell lysis has been discussed as a result of localized anaerobic regions within the biofilm, apparent by biofilms predominantly exhibiting gene expression profiles consistent with anaerobic growth^{11,81}, and coinciding with fermentative⁸² or anaerobic respiratory pathways, including denitrification. Anaerobic denitrification in the interior of biofilm microcolonies generates nitric oxide (NO) that, if not reduced by NO reductase to nitrous oxide, can react with superoxide to generate the cell-toxic radical ONOO⁻³² (Fig. 2b). ONOO⁻ causes cellular damage, bacteriophage induction and cell lysis^{32,83}, with the latter resulting in the release of degradative enzymes (not thought to be transported across the cell membrane), that contribute to the enzyme-mediated breakdown of the biofilm matrix and/or loosening of the biofilm matrix^{32,80}. In addition, cell lysis generates nutrients for growth^{32,80} (Fig. 2b). There is also evidence that prophages influence the pattern of cell death and lysis seen in biofilms. For *P. aeruginosa*, prophage-mediated cell lysis was first described for strain PAO1, which displayed characteristic voids in the center of mature microcolonies of flow cell-grown biofilms⁸³. Cell death in the center of these microcolonies was attributed to lysis by prophages that are closely related to the filamentous phage Pf1 and reside within the genome of PAO1. Deletion of this prophage from PAO1 resulted in microcolonies devoid of hollow centers containing lysed cells. It is proposed that prophage-mediated cell death is an important mechanism of differentiation inside microcolonies that facilitates dispersal of a subpopulation of surviving cells. Prophages have since been described to contribute to microcolony maturation and stability, dispersal, virulence and the propagation of morphotypic variants⁸⁴⁻⁸⁶.

Although the mechanism or mechanisms by which (many of) these cues or factors induce dispersion is not fully understood, many of these either exacerbate or eliminate chemical gradients present in biofilms depending on their concentration in the biofilm interior (Fig. 2). For instance, cell lysis may contribute to the nutrient support of surviving cells and the release of enzymes capable of degrading matrix components. Moreover, the sudden availability of nutrients may contribute to the modulation of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) levels (. Likewise, NO has been linked to the reduction of c-di-GMP levels (see below, Fig. 2b). Given that these chemical gradients are the driving forces of biofilm formation by affecting gene expression and protein production, including the modulation of various stress responses^{10,34,61}, it is apparent that some dispersion inducers function by reversing the biofilm developmental process. Moreover,

dispersion is a localized event. As such, dispersion is a response by a subpopulation or subpopulations within the biofilm. For instance, cells in the interior of a *P. aeruginosa* biofilm convert into a motile phenotype and disperse, while non-dispersing biofilm cells are non-motile, primarily found in the biofilm periphery, and create a ‘wall phenotype’²⁹. Although it is unclear whether biofilms differentiate into subpopulations prior to (or during) the dispersion response, it seems that the development of dispersing subpopulations is likely to coincide with biofilm cells experiencing a multitude (or a specific combination) of chemical gradients (Fig. 2). Taken together, biofilm subpopulations capable of dispersing are likely to locate within larger, more mature biofilms, and they are characterized by hypoxia, experience cell death, free radicals and other cues that predispose the cells for dispersion.

Environmentally induced dispersion and inducers.

Dispersion in response to exposure to factors present in the surrounding environment or the changing conditions of the surrounding environment is referred to as environmentally induced dispersion. This is in contrast to native dispersion that occurs in response to signaling molecules or cues that are synthesized by the resident biofilm cells. Similar to native dispersion which is initiated in the biofilm interior, dispersion events induced by environmental factors also result in void formation. However, voids induced by environmental factors look more like eroded biofilms composed of a non-motile layer of cells that make up the base of the biofilm prior to dispersion (Fig. 1b). Similar to native dispersion, environmentally induced dispersion rarely involves the entire biofilm (Fig. 1), with no more than 80% of the biomass being removed upon induction of dispersion⁸⁷. Environmentally induced dispersion cues and factors include NO, cis-DA, iron, induction of starvation inducing conditions (oxygen and/or nutrients), and a step-increase of various nutrients including sugars and amino acids (Table 1). The similarity between conditions and cues initiating environmentally induced and native dispersion is striking, which suggests similarities in dispersion cue sensing and dispersal mechanism or mechanisms between the two active dispersion responses. Moreover, environmentally induced dispersion inducers are not limited to a single species. For example, NO has been demonstrated to disperse single-species and mixed-species biofilms of clinically and industrially relevant microorganisms⁸⁸. Similarly, cis-DA was found to disperse a range of Gram-negative and Gram-positive bacteria and yeast⁵⁹, which suggests that cis-DA may not only be capable of dispersing single species but also mixed species biofilms.

Dispersal mechanisms

Although little is known about native dispersion, the mechanism of dispersion in response to various environmental stimuli including nutrient cues, NO and others (Table 1) has been relatively well characterized in *P. aeruginosa*. Sensing of dispersion cues is accomplished by a membrane-associated protein complex. Cue sensing and relay of signal is accomplished via a series of post-transcriptional modifications that ultimately result in an overall reduction in the levels of the intracellular signaling molecule c-di-GMP. Low c-di-GMP levels in turn, are likely to contribute to the phenotypes of dispersed cells associated with the planktonic mode of growth, including motility, susceptibility and enhanced phagocytosis.

Moreover, it is now apparent that dispersed cells increase expression of genes that assist in the degradation of the polymeric matrix that shields and stabilizes the biofilm structure.

Modulation of c-di-GMP levels.

Although most research investigating the role of c-di-GMP has focused on Gram-negative bacteria, recent findings suggest that c-di-GMP has similar roles in Gram-positive bacteria; however, targets and regulatory mechanisms may differ⁸⁹. Specifically, the intracellular signaling molecule c-di-GMP is now recognized as a near-ubiquitous second messenger that coordinates diverse aspects of bacterial growth and behavior, including motility, virulence, cell cycle progression and biofilm formation (reviewed in Ref.⁹⁰). High levels of c-di-GMP foster the sessile lifestyle, whereas low levels enhance the planktonic mode of growth. Levels of c-di-GMP are modulated by the opposing activities of two sets of enzymes, diguanylate cyclases (DGCs), which generate c-di-GMP from two molecules of GTP, and phosphodiesterases (PDEs), which degrade c-di-GMP⁹⁰.

Although high c-di-GMP levels are generally associated with the biofilm mode of growth, recent findings suggest levels of c-di-GMP are not uniform throughout biofilms (Fig. 2c). Using a GFP reporter for which the fluorescence intensity is directly proportional to the concentration of intracellular c-di-GMP⁹¹, a striking difference in c-di-GMP concentrations was seen across the biofilm structure. c-di-GMP was localized in relatively high amounts at the outer boundary of large, mature biofilms, whereas smaller, less developed biofilms showed a more uniform distribution of c-di-GMP⁹². Whether the c-di-GMP gradients noted in biofilms predispose subpopulations of biofilm cells to disperse is not known. However, several reports indicate that dispersed Gram-negative cells that have escaped the biofilm structure are characterized by reduced c-di-GMP levels compared with biofilm cells^{93–96}. Moreover, dispersed cells display increased expression of PDEs and increased PDE activity^{48,93,97–99}.

In turn, low c-di-GMP levels in dispersed cells enhance phenotypes associated with the planktonic mode of growth, including motility. For example, in *P. aeruginosa*, motility is regulated by the major flagellar gene regulator FleQ, a c-di-GMP-responsive transcriptional regulator that enhances motility but represses Pel polysaccharide production at low c-di-GMP levels¹⁰⁰. Moreover, low c-di-GMP levels affect susceptibility to antibiotics, apparent by clinical *P. aeruginosa* isolates from the cystic fibrosis lung displaying aggregative phenotypes in liquid (that is, a phenotype indicative of high c-di-GMP levels) and displaying 2–8-fold higher minimal inhibitory concentration (MIC) values to several antibiotics than revertants of this aggregative phenotype that displayed low c-di-GMP levels^{101–105}. Moreover, dispersed cells exhibit reduced production of proteins involved in resistance mediated by cationic antimicrobial peptides including colistin¹⁰⁶ and tobramycin⁵⁸.

Dispersion cue sensing.

Based on our current understanding, low c-di-GMP levels are a result of dispersion cue sensing (Fig. 3, see above). In *P. aeruginosa*, dispersion cue sensing involves the chemotaxis-like MCP homolog, BdlA, the c-di-GMP PDEs DipA, RbdA, MucR and NbdA, and the DGCs GcbA and NicD. These proteins form a membrane-associated complex that perceives

and relays dispersion cues to promote the modulation of c-di-GMP levels¹⁰⁷, apparent by an initial increase in c-di-GMP levels followed by a sharp decrease¹⁰⁷. The DGC NicD and the PDE NbdA contribute to dispersion in a cue-specific manner, with NbdA sensing NO⁹⁷ and NicD sensing nutrient cues¹⁰⁷ (Fig. 3). By contrast, BdlA and the PDEs DipA and RbdA are central to the dispersion response regardless of the cue that is being sensed (Fig. 3). Sensing of dispersion cues results in the activation of BdlA by non-processive cleavage, with active BdlA recruiting and activating two PDEs, RbdA and DipA, ultimately resulting in the reduction of c-di-GMP levels^{97,107,108}. In addition, fatty acid signaling contributing to native dispersion has been linked to the decrease of intracellular c-di-GMP levels¹⁰⁹.

Dispersion induced by fatty acids such as cis-DA has also been linked to the modulation of c-di-GMP^{64,110}. Previous work on *X. campestris* demonstrated that fatty acid sensing requires a two-component system, RpfC–RpfG, whereas in *B. cenocepacia* fatty acids directly bind to the receptor protein RpfR, with binding stimulating the PDE activity of RpfR¹¹¹. Although detailed investigations of the dispersion response by Gram-positive bacteria are limited¹¹², it is likely that c-di-GMP has a similar role in the dispersion response by Gram-positive bacteria, albeit signaling may involve a new conformation of c-di-GMP and if so, likely novel receptors and novel binding motifs for c-di-GMP relative to Gram-negative bacteria¹¹³.

Disintegration of the biofilm matrix.

Events subsequent to cue sensing and reduction of c-di-GMP levels are less defined but support the notion that dispersion coincides with the disintegration of the biofilm matrix. Although the matrix composition is highly diverse amongst bacterial species, it is generally composed of extracellular polysaccharides, proteinaceous factors such as carbohydrate-binding proteins, adhesins, or amyloid fibers and extracellular DNA (eDNA) (Box 1; reviewed in Ref.¹¹⁴). The matrix, which accounts for ~90% of the biofilm biomass, is a viscoelastic structure that not only tethers cells within the biofilm and functions as a protective shield, but also functions as a scaffold for the three-dimensional biofilm structure¹¹⁴.

Matrix composition has been extensively investigated in *P. aeruginosa* and found to be composed of the Psl, Pel and alginate exopolysaccharides, eDNA and matrix stabilizing proteins such as the adhesin CdrA¹¹⁴ (Fig. 4a). Alginate is a linear unbranched polysaccharide that contains different amounts of (1→4′)-linked β-d-mannuronic acid and α-l-guluronic acid residues, Psl is a neutral pentasaccharide subunit that contains mannose, rhamnose and glucose, and Pel is a glucose-rich cellulose-like polysaccharide that can cross-link to eDNA. The level of these polysaccharides within the matrix varies across *P. aeruginosa* strains. Given that dispersion coincides with cells evacuating the protective biofilm environment, it is not surprising that dispersion has been linked to the increased expression and production of matrix-degrading enzymes. Specifically, NO- and nutrient-induced dispersion have been linked to increased expression of genes encoding endonucleases such as *endA*¹¹⁵ (EndA degrades DNA present in the matrix) and glycoside hydrolases such as *pslG* and *pelA*¹¹⁶ (PslG and PelA degrade Psl and Pel matrix polysaccharides¹¹⁶), as well as the increased production of matrix degrading enzymes,

compared to planktonic and biofilm cells⁹⁶. Below we summarize the events that lead to the enzymatic disintegration of the biofilm matrix in response to dispersion cue sensing.

CdrA is a c-di-GMP regulated adhesin that reinforces the biofilm matrix, probably by tethering Psl^{117,118}. High c-di-GMP levels have been linked to a cell surface localization of CdrA, whereas low c-di-GMP levels coincide with the release of CdrA from the outer membrane via cleavage by the periplasmic cysteine protease LapG. The first evidence of CdrA contributing to dispersion was inferred by the finding that *lapG* mutants displayed increased attachment to plastic and glass surfaces¹¹⁷, whereas a *cdrA* deletion mutant displayed decreased attachment relative to the parental strain upon extended incubation. The role of CdrA in dispersion has recently been confirmed by the observation that a *cdrA* deletion mutant was 'hyper'-dispersive relative to *P. aeruginosa* strains with an inactivated *lapG*, which exhibited reduced dispersion¹¹⁵. These findings suggest that the presence of the surface-associated matrix protein CdrA impedes the dispersion response and that CdrA release, and thus untethering of the matrix, is likely to be a first step that enables matrix degradation. (Fig. 4b)

A similar mechanism was noted to contribute to dispersion by *P. putida* and *P. fluorescens*. In these two species, the adhesin LapA, similar to CdrA, is localized at the outer membrane at high c-di-GMP levels, but the adhesin is cleaved from the cell surface by the protease LapG in the presence of low levels of c-di-GMP¹¹⁹. Starvation-induced dispersal of *P. putida* biofilms was dependent on LapG-dependent proteolytic cleavage of LapA^{66,94}. Similarly, LapG-dependent proteolytic cleavage of LapA at low c-di-GMP levels was found to contribute to phosphate limitation-induced dispersal of *P. fluorescens* biofilms¹¹⁹. However, it is likely that other proteases in addition to LapG have a role. This is supported by CdrA release being sensitive to self-produced proteases other than LapG¹²⁰, proteinase K being capable of disassembling *P. putida* biofilms⁹⁴, and the finding that lowering the c-di-GMP level in *P. aeruginosa* by overexpressing the phosphodiesterase PA2133 was not sufficient to induce dispersion¹¹⁶.

It is now widely acknowledged that eDNA is a ubiquitous and pivotal structural component of biofilms by diverse group of microorganisms, including *P. aeruginosa*, uropathogenic *coli*, nontypeable *Haemophilus influenzae* and *Staphylococcus epidermidis*^{121,122}. As a structural component, eDNA may also have a role in dispersion (Fig. 4c). Although exogenously added DNAses were shown to be effective in disassembling young *P. aeruginosa* biofilms¹²³ and biofilms produced by *P. putida*, *S. aureus*, *Shewanella oneidensis* and *Bacillus licheniformis*^{124–127}, DNAses were mostly ineffective in disassembling mature biofilms. The difference in the efficacy of DNase to disassemble biofilm structures is likely to be due to the fact that eDNA being predominately found in the interior of mature biofilms (Figs. 4a, 4c). However, dispersed *P. aeruginosa* cells exhibit increased expression of genes encoding the secreted nucleases EndA, EddA and EddB, which are not only capable of degrading eDNA present in the biofilm matrix but are essential for the dispersion response¹¹⁵. Dispersed cells also have increased expression of *pelA* but not *pslG*¹¹⁶, which encode glycoside hydrolases, with purified PelA and PslG having been shown to degrade Pel and Psl, respectively, and contribute to biofilm disassembly^{128–130}. Interestingly, overexpression of *pelA* but not *pslG* expression resulted in dispersion of *P.*

aeruginosa biofilms¹¹⁶, and *pslG*-induced dispersion was only detectable in the absence of CdrA¹¹⁶.

Polysaccharide degradation has been linked to dispersion of biofilms of various Gram-positive and Gram-negative bacteria, including *Aggregatibacter actinomycetemcomitans*, *Aggregatibacter pleuropneumoniae*, *E. coli* and *S. epidermidis*, harbor linear polymers of N-acetyl-D-glucosamines referred to as polysaccharide intercellular adhesin (PIA); poly-N-acetyl glucosamine (PNAG, β -(1–6)-linked N-acetylglucosamine), and polysaccharide adhesin (PGA, β -1,6-N-acetyl-D-glucosamine)(Fig. 4d). The enzyme responsible for the degradation has been identified as dispersin B, a secreted glycoside hydrolase^{131–133} (Box 1). Moreover, the *E. coli* CsrA protein has been shown to repress the synthesis of PGA, , thus aiding in dispersion³¹. Likewise, the *agr* system of *S. aureus* that contributes to the expression of matrix polysaccharides and an extracellular protease that degrades proteins such as adhesins, has been shown to contribute to dispersal¹³⁴.

These findings indicate that dispersion coincides with the expression of genes encoding matrix-degrading enzymes, and requires several matrix-degradative enzymes that work together to degrade multiple matrix components, including eDNA, polysaccharides and adhesins (Fig. 4). It is of interest to note that the regulatory mechanisms driving matrix degradation are not well understood. More specifically, it is unclear whether matrix degradation is the direct result of c-di-GMP modulation or if additional factors or regulatory proteins are required to induce the expression of genes encoding matrix-degrading enzymes. Although it has been suggested that dispersed cells can be generated by simply reducing the intracellular c-di-GMP content through modulation of PDEs¹³⁵, recent evidence suggests that molecular mechanisms regulating the dispersion response are likely to be more complex, requiring both c-di-GMP-dependent and -independent processes^{58,116}. This is supported by recent findings of dispersion being induced upon overproduction of the endonuclease 1 EndA 115, and the glycosyl hydrolase PelA 116, whereas the adhesin CdrA was found to impede the dispersion response 116. *endA*, *pelA*, and *cdrA* are directly regulated by AmrZ, the central regulator of biofilm formation by *P. aeruginosa*^{136–138}. Given the contribution of CdrA and matrix-degrading proteins in the *P. aeruginosa* dispersion response, it is likely that the central regulator of biofilm formation AmrZ has a role in the regulation of the dispersion response.

Escape and consequences

Although it is widely assumed that dispersed cells return to their planktonic state, studies with many different microbial species have shown that dispersed cells display distinct phenotypes. A common hallmark of dispersed cells is their high level of phenotypic heterogeneity¹³⁹. This is not surprising considering that cells within the biofilm itself have high mutation frequency, presumably due to the close association of a high concentration of cells, as well as the adaptive pressure of different microenvironments present within the biofilm¹⁴⁰. Dispersing cells with diverse phenotypes into the environment are likely to represent an important ecological adaptation mechanism that increases the chance some cells will survive and create new biofilm communities.

Many reports characterize dispersed cells as having phenotypes somewhere between that of biofilm cells and planktonic cells, but distinct from both^{57,87,139,141–143}, and the heterogeneity within the dispersed subpopulation can be vast. For example, *Klebsiella pneumoniae* cells that spontaneously detached due to shear stress in a flow-cell biofilm displayed transcriptomes that were distinct from planktonic cells and sessile biofilm cells¹⁴⁴. Cells representing five different physiological states were documented (that is, exponential planktonic growth, stationary planktonic growth, a 7 hr-old biofilm, a 13 hour-old biofilm and a dispersed biofilm), and 40 distinct transcriptional signatures were reported. Although the transcriptomes of dispersed cells resembled those of cells in a 7 hr-old biofilm more than the other cell subpopulations, they still displayed distinct transcriptional profiles. The most representative clusters of orthologous group (COG) affiliations of dispersed cells were translation, ribosomal structure and biogenesis and amino acid transport and metabolism, which suggests that these cells possess distinct metabolic capabilities compared to both biofilm and planktonic cells.

Dispersed cells are typically more motile, virulent and adherent, display different metabolic signatures and altered antimicrobial susceptibilities compared to their biofilm and planktonic counterparts (see below)^{57,87,141–143,145}. Although these phenotypes can be short-lived as the dispersed cells transition to a more planktonic phenotype over time, this suggest that detached or dispersed cells are primed for recolonization at new sites^{57,142}. For example, RNA sequencing analysis of *Candida albicans* grown in flow-cells demonstrated that the dispersed yeast cells exhibited enhanced adhesion, invasion and biofilm formation in comparison to their age-matched planktonic counterparts¹⁴². The dispersed cells also seemed to be transcriptionally reprogrammed to adopt different metabolic capabilities, which enables them to acquire specific nutrients and amino acids, and to metabolize alternative carbon sources in comparison to their biofilm-associated counterparts, despite being grown in the same nutritional environment. This reprogramming was manifested in the significant up-regulation of select high-affinity transporters and increased expression of genes involved in gluconeogenesis in dispersed versus biofilm-associated cells¹⁴². In another study, dispersal of *P. aeruginosa* was induced by the reduction of intracellular c-di-GMP, and transcriptomic analysis was performed on the resulting cell subpopulations⁵⁷. Planktonic *P. aeruginosa* cells exhibited higher expression levels of genes involved in quorum sensing compared to biofilm and dispersed cells. Not surprisingly, biofilm cells exhibited higher expression of the genes involved in extracellular matrix synthesis, and lower expression of the genes involved in motility and chemotaxis compared with planktonic and dispersed cells. By contrast, dispersed cells exhibited higher expression of virulence genes and lower expression of the genes involved in iron uptake in comparison to planktonic cells and biofilm cells.

It is intuitive that dispersed cells express high levels of genes encoding virulence factors and genes important for motility and adhesion as they leave the protective environment of the biofilm and enter a much harsher environment, and the consequence to the host could mean a subsequently worse infection. In a *C. albicans* jugular vein catheter mouse model, genetic induction of dispersal from biofilms growing in the lumen of catheters resulted in enhanced disseminated infection, whereas repressing dispersal resulted in a 15-fold decrease in *C. albicans* infection of distal organs¹⁴². Likewise, dispersed *P. aeruginosa* cells have

also displayed enhanced virulence *in vivo*. It was shown that *P. aeruginosa* cells that have been dispersed from *in vitro* grown biofilms by reduction of intracellular c-di-GMP killed significantly more macrophages and *C. elegans* than their planktonic counterparts⁵⁷. Moreover, dispersing *P. aeruginosa* cells from a chronic wound infection with glycoside hydrolases caused systemic spread of the bacteria and death by sepsis in a mouse model¹⁴⁶ (Fig. 5). Notably, these three examples include two different kingdoms of microorganisms, three different dispersal methods and completely different *in vivo* models, yet all the dispersed cells displayed enhanced virulence. Although clearly more studies are needed, existing data suggest that some dispersal phenotypes, such as heightened virulence, are conserved regardless of dispersal method, and the consequences of dispersal on the host or environment must be thoroughly considered.

Biofilm control

It has been proposed that removing cells from the protective shield of a biofilm will render them more susceptible to antimicrobials and the host immune response. Given our current understanding about the mechanisms of dispersal and the broad phenotypes dispersed cells can adopt, one would not necessarily predict that dispersion would be an effective method for biofilm control. It is possible that dispersed cells would be more tolerant to antimicrobials than MIC testing on planktonic cells would predict, be highly virulent and adept at forming new biofilms at other locations. Thus, dispersing cells, without the capability of efficiently killing them, could result in a substantially larger ecological problem or more deadly infection. However, despite these perils, dispersal agents have gained traction over the past decade as a viable therapeutic option, and many published studies have demonstrated proof-of-principal for this strategy.

With a fundamental understanding of what constitutes the extracellular polymeric substance (EPS) of biofilms, enzymes that degrade these components gained enthusiasm early on as potential dispersal agents. Dozens of enzymes, including many nucleases, glycoside hydrolases and proteases, have been tested for their ability to degrade biofilms and disperse cells (reviewed in Ref.¹⁴⁷). As microorganisms themselves produce and use these enzymes for biofilm degradation and dispersal, their exploitation is an intuitive strategy. Most dispersal attempts have been early-stage, pre-clinical studies, which applied purified enzymes to *in vitro*-grown biofilms and determined the reduction in biomass or percentage of cells dispersed. Biofilms formed by various bacteria and fungi were shown to be susceptible to degradation and some enzymes have also demonstrated efficacy on polymicrobial biofilms (reviewed in Ref.¹⁴⁷).

Although previous studies have widely affirmed the potential of EPS-degrading enzymes to disrupt biofilms *in vitro*, far fewer have examined the efficacy of dispersal agents *in vivo*. Glycoside hydrolases represent one class of EPS-degrading enzymes that have moved into animal studies and the commercialization pipeline. The most well studied of these enzymes is dispersin B from *A. actinomycetemcomitans*. Dispersin B degrades PNAG by hydrolyzing $\beta(1,6)$ glycosidic linkages. This enzyme is effective at disrupting the biofilms of multiple Gram-negative and Gram-positive bacterial species, including many pathogens^{132,148–151}. Dispersin B has also shown efficacy in some *in vivo* models^{152–154} and commercialization

of the agent is being pursued, although currently only for the treatment of animals. Effective treatment with other glycoside hydrolases, including amylase and cellulase, has also been demonstrated *in vivo* in recent reports^{128,146}, which supports the rationale for their use in treating of biofilm-associated infections.

As investigators have learned more about what chemical cues lead to dispersal they have exploited these processes for therapeutic purposes. The standouts in this category are cis-DA acid and NO^{63,155}. As indicated above, cis-DA acid is a fatty acid signal that prevents biofilm development and induces biofilm dispersion for a number of microorganisms *in vitro*^{59,156–158}. Moreover, cis-DA has also shown some promise in a mouse model of periprosthetic joint infection¹⁵⁹. NO is a ubiquitously produced biological signaling molecule that induces biofilm dispersal for a number of different microorganisms *in vitro* (reviewed in Ref.¹⁵⁵); however, it has also shown efficacy in a number of animal models. For example, applying NO-releasing chitosan dressings to methicillin-resistant *Staphylococcus aureus* -infected wounds in mice resulted in enhanced biofilm dispersal, wound size reduction, epithelialization rates and collagen deposition in comparison to control groups¹⁶⁰. A liposomal formulation of an NO donor, applied as a topical sinus wash, effectively reduced *S. aureus* biofilms in a sheep model of rhinosinusitis¹⁶¹, and NO-releasing catheters have shown effective at reducing biofilm burden *in vivo*^{162,163}. Excitingly, NO-based therapeutic approaches have entered human clinical trials. Interventional studies testing the efficacy of NO to treat recalcitrant chronic rhinosinusitis, chronic non-healing wounds, prevent urinary tract infections, and reduce antibiotic tolerance in patients with cystic fibrosis have all entered clinical trials.

In many ways a biofilm is analogous to a cancerous tumor. Biofilms are tolerant and difficult to eradicate and have the potential to metastasize to other locations. As with cancer, the goal is to kill the cells in the biofilm with minimal collateral damage to the host. There are many potential options to chemically induce, enzymatically induce or mechanically break up biofilm and cause dispersion, but the successful agents will cause low levels of collateral damage, lack toxicity and demonstrate successful clinical trials. Importantly, biofilm dispersal agents have the capacity to potentiate the efficacy of antimicrobials. Ideally, they would be used as adjunctive agents with broad application for many biofilm-related infections, and because most are not bactericidal they are unlikely to cause resistance.

Conclusions

Dispersion is an active event in which sessile, matrix-encased biofilm cells convert en-masse to the planktonic mode of growth, apparent by single cells that are actively escaping the biofilm. The active escape from the protective biofilm environment seems to be driven by steepening chemical concentration gradients of nutrient resources, oxygen and waste products over the course of biofilm development that lead to the accumulation of molecules that are not only capable of inducing dispersion (that is, NO) but furthermore contribute to conditions that foster the formation of subpopulations in the interior of biofilms. These subpopulations are either more susceptible to dispersion cues or are being exposed to dispersion conditions to egress from the biofilm. The review of native and

environmental dispersion cues suggests not only a striking similarity between cues initiating environmentally induced and native dispersion, but also similarities in cue sensing and the pathways between the two active dispersion responses. Based on current knowledge, the dispersion response involves dispersion cue sensing which subsequently results in an overall reduction of the c-di-GMP pool. Although subsequent events are less defined, dispersion coincides with the enzymatic disintegration of the biofilm matrix and subsequent egress from the biofilm. The dispersed subpopulation is typically more motile, virulent and adherent, displays different metabolic signatures and altered antimicrobial susceptibilities, which has resulted in dispersed cells being considered phenotypically between that of biofilm cells and planktonic cells, but distinct from both.

Based on a number of recent studies summarized here, it is apparent that dispersion has the potential to be exploited for therapeutic purposes to treat biofilm-related infections. However, although we have gained a deeper knowledge of the dispersion response in recent years, there remain numerous gaps in our knowledge. For instance, how is the sensing of dispersion cues linked to matrix degradation and subsequent dispersion? Is the relay simply based on the modulation of c-di-GMP or are additional non-ci-di-GMP regulated mechanisms involved? Given that dispersion in *P. aeruginosa* is linked to motility and matrix degradation, it is likely that FleQ is involved. Likewise, AmrZ, the central regulator of biofilm formation by *P. aeruginosa*, may have a role as the genes encoding endonucleases such as *endA* and glycoside hydrolases such *pelA* and *cdrA*, are directly controlled by AmrZ. Additionally, we know little about how the biofilm environment influences subpopulations to disperse. Is it possible that these subpopulations are already primed to disperse and if so, what pushes them over the edge to leave the biofilm? And what regulatory mechanisms contribute to the distinct phenotypes displayed by dispersed cells? When considering dispersion as a treatment strategy, we also know little about how efficient the dispersion response has to be for it to be an effective therapeutic agent, or whether enzymatic disassembly the biofilm structure is as effective as dispersion. But more importantly, our current understanding of dispersion is based on experimental evidence obtained under limited *in vitro* conditions with few microorganisms grown as single species biofilms. To close these gaps in our knowledge, it will not only be necessary to explore dispersion by a larger number of mono-species and mixed-species biofilm pathogens, but also to expand dispersion research from basic to translational studies, by exploring dispersion and dispersion inducers in pre-clinical and clinical studies.

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GLOSSARY

Abrasion

Loss of biofilm cells by collisions with particles from the environment; form of detachment

Dispersion

Generally characterized as the terminal stage of biofilm development, dispersion is an active regulated event during which cells actively escape from the biofilm, leaving behind eroded biofilms and biofilms having central voids. Often referred to as seeding dispersal or dispersal²⁹, as dispersion is assumed to lead to the translocation of bacteria to new sites for colonization.

Disassembly

Describes the egress from biofilms and/or the disintegration of the biofilm structure in response to exogenously added matrix-degrading enzymes. The term is frequently used when it is unclear whether the released cell subpopulation retains the biofilm phenotype or adopts the dispersal phenotype.

Detachment

Characterized as the passive release or loss of biofilm cells or biofilm particles due to mechanical, physical or frictional forces. Another term used to refer to detachment is dissolution.

Dissemination

The translocation of dispersed biofilm cells to new sites.

Diffusive and advective transport

Processes that move nutrients, waste, gases or other compounds through the biofilm and the surrounding environment. Advection refers to transport of compounds with fluid flow whereas diffusion eliminates sharp discontinuities of compounds through the action of random motions.

Native dispersion (also referred to as native dispersion)

Refers to dispersion in response to self-synthesized signaling molecules or cues that are likely to be the result of steep gradients within the biofilm.

Quorum sensing

also referred to as cell-to-cell signaling, refers to the regulation of gene expression in response to the production and release of chemical signal molecules called autoinducers that increase in concentration as a function of cell density.

RpoS

also referred to as *sigma-38* (σ_{38}), primary regulator of stationary phase genes and central regulator of the general stress response

Shear

strain in the structure of a substance produced by pressure

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Textbox 1.**Effectors leading to biofilm disassembly rather than dispersion**

Biofilms disperse via several mechanisms, and this commonly involves extensive regulatory and metabolic changes that a biofilm undergoes to enable the evacuation of dispersed cells. Moreover, dispersion coincides with extensive structural changes, apparent by the substantial loss of biofilm biomass, indicating that a key aspect of the dispersion response is the partial breakdown of the biofilm matrix composed of extracellular DNA (eDNA), polysaccharides and proteins.

In recent years, several effectors have been described that target the biofilm matrix. The effectors that are capable of matrix dissolution often have enzymatic activity and include polysaccharide-degrading enzymes, chitinases, proteases and nucleases (reviewed in Ref.¹²⁷). These enzymes are similar to enzymes that are produced by the dispersing biofilm subpopulation. However, although the production of dispersal-induced matrix-degrading enzymes are the result of regulatory and metabolic changes that are induced during the dispersion response, these enzymes can be exogenously added to biofilms to induce a passive biofilm disassembly without inducing regulatory and metabolic changes by the resident biofilm population. Thus, induced biofilm disassembly may result in an egressing cell population that retains the biofilm phenotype rather than adopts the dispersal phenotype. Notable phenotypes associated with dispersion but likely not during biofilm disassembly include increased susceptibility to antimicrobials, shift towards an acute virulence phenotype, and susceptibility to immune cells. Moreover, biofilm disassembly may coincide with the release of cell aggregates rather than single planktonic cells.

One such effector is dispersin B, a poly-*N*-acetylglucosaminidase capable of degrading matrix polysaccharides^{131–133}. Originally identified in a transposon screen of *Aggregatibacter actinomycetemcomitans* for genes that are essential for dispersal, dispersin B was later found to induce biofilm disassembly in a range of bacterial species, including *Staphylococcus epidermidis*, when exogenously added to biofilms^{131–133}. Additional glycoside hydrolases, enzymes that hydrolyze the glycosidic linkages between two or more carbohydrates¹⁷⁵, have been demonstrated to disassemble biofilms. Exposure of purified *P. aeruginosa* glycoside hydrolases PelA and PslG that are not only required for the synthesis their respective exopolysaccharides Pel and Psl but also contribute to their degradation¹²⁹, has been shown to disassemble established *P. aeruginosa* biofilms¹²⁹. Likewise, a cocktail of glycoside hydrolases (cellulase, α -amylase) that target glycosidic linkages commonly seen within the exopolysaccharides secreted by a wide range of pathogens including β -1,4 bond present in cellulose, and the α -1,4 bond in amylose, has been shown to lead to the disassembly of *S. aureus* and *P. aeruginosa* monoculture and co-culture biofilms¹⁷⁶. In addition to eDNA being a source of nutrients, phosphorus and nitrogen¹⁷⁷, eDNA was described in 2002 to have a role in biofilms¹²³. Since then, eDNA has been shown to be deposited on the stalk of biofilms enabling the formation of mushroom-shaped microcolonies, to enhance biofilm formation probably by enabling direct or indirect interactions with the bacterial cell surface, and to cross-link matrix components¹⁷⁸. Given eDNA is a structural element of

the biofilm matrix, eDNA degradation by DNase I has likewise been shown to result in the release of large amounts of biomass in various biofilm-forming species, including *P. putida*, *S. aureus*, *S. oneidensis* and *Bacillus licheniformis*¹²⁷. However, DNase treatment leading to biofilm disassembly seems to be limited to young, less structured biofilms¹²³, probably due to mature biofilms harboring increasing amounts of matrix material other than eDNA, and eDNA being more centrally located within the biofilm structure.

In addition to matrix-degrading enzymes, amphipathic molecules that reduce surface tension have been shown to induce biofilm disassembly. For instance, exogenous addition of rhamnolipids triggers detachment by *P. aeruginosa* biofilms, whereas the biosurfactant putisolvins induces detachment by *P. putida* biofilms detachment¹²⁷. It is of interest to note that rhamnolipids failed to disassemble filamentous biofilms by *S. marcescens* str. MG1¹⁶⁹.

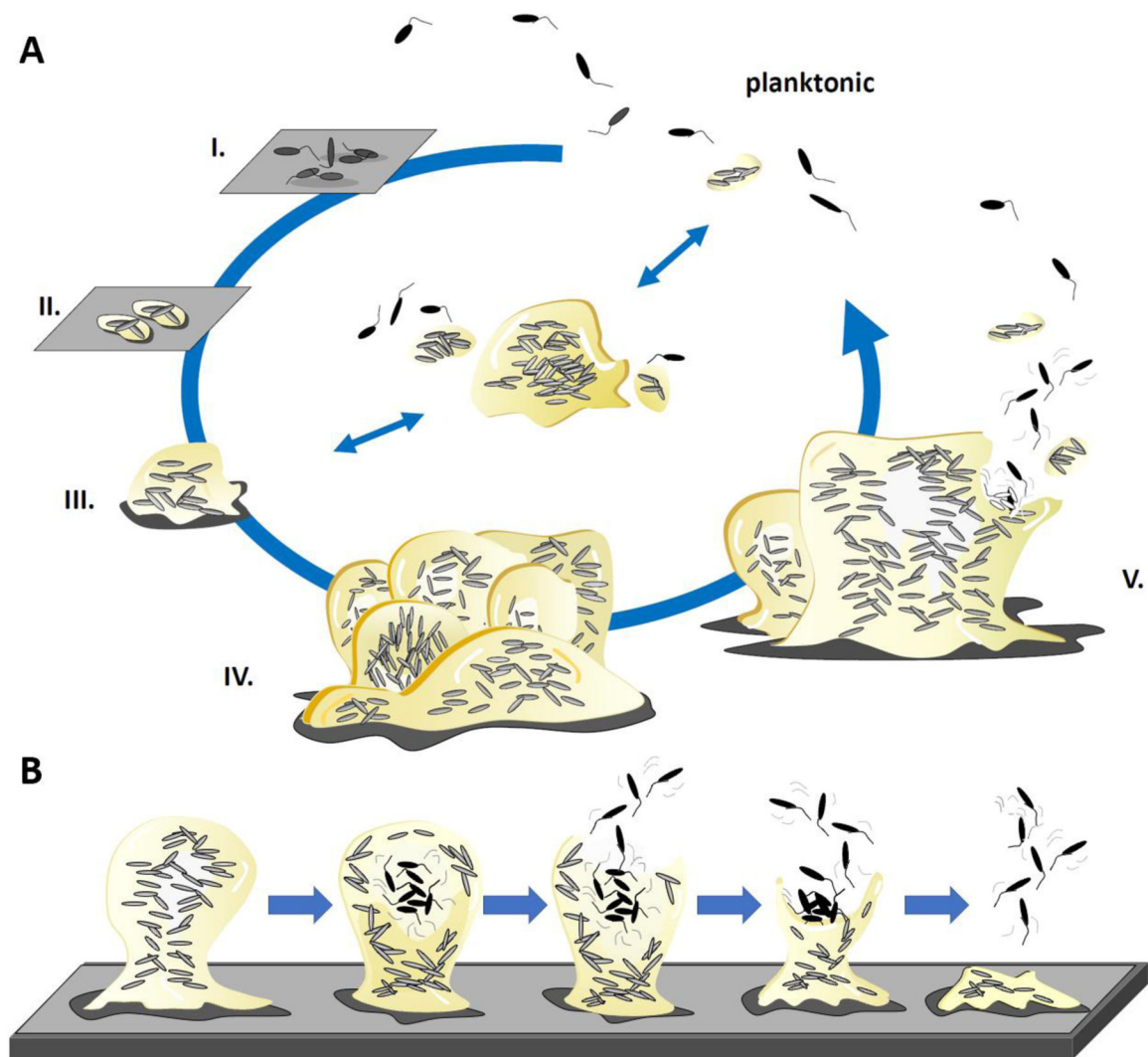


Figure 1: Biofilm formation and dispersion.

a) The schematic is based on the *in vitro* analysis of single species *Pseudomonas aeruginosa* biofilms. The formation of biofilms is a cyclic process that occurs in a stage-specific and progressive manner. The process is initiated following surface contact by single planktonic cells. Several developmental steps are discernible as reversible attachment (step I), irreversible attachment (step II) and biofilm maturation (steps III-IV)^{6,26}. During the initial event in biofilm development, bacteria attach to substratum at the cell pole or via the flagellum (step I). Cells are cemented to the substratum and form nascent cell clusters with all cells in contact with the substratum. Transition to this stage coincides with loss of flagella gene expression and the production of biofilm matrix components (step II). Cell clusters mature and are several cells thick, embedded in the extracellular polymeric substances matrix (step III). Biofilms fully mature, which is apparent by clusters and microcolonies having reached maximum thickness (step IV). The biofilm life cycle comes full circle when biofilms disperse (step V). This stage is characterized by cells evacuating the interior portions of cell clusters, forming void spaces. Recent reports describe the formation of aggregates that exhibit biofilm-like features in the absence of surfaces

(middle panel)¹⁷⁴. Based on *in vivo* observations, these aggregates can attach to surfaces or disperse¹⁷⁴ (indicated by the arrows). However, it is unclear if aggregate formation requires the same biofilm formation pathways and dispersion events. **b)** The schematic shows the discernable events that lead to biofilm dispersion. During dispersion, the inside of a biofilm becomes fluid, and cells within this zone begin to show signs of agitation and movement. Subsequently, cells escape the biofilm via a disruption in the microcolony wall through which cells evacuate, entering the bulk liquid as single bacteria. This leaves behind biofilms with central voids. If the dispersion response is extensive, the biofilm structure may further erode.

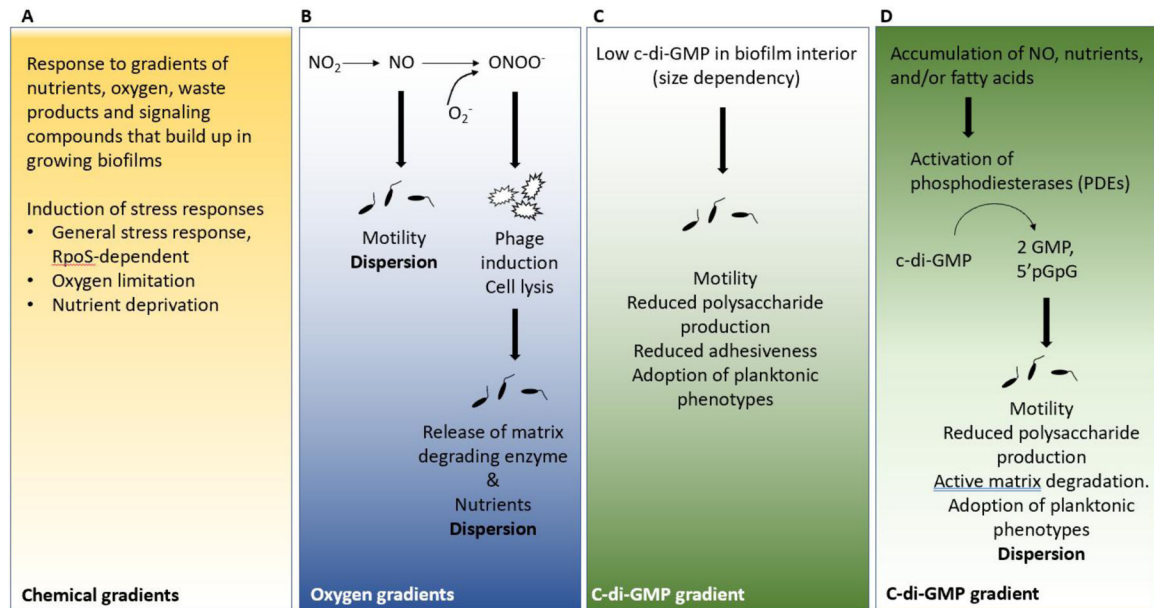


Figure 2: Environmental conditions initiating dispersion.

Each panel indicates various chemical gradients that are likely to be present in biofilms; low concentrations of those chemicals have been linked to dispersion. Moreover, phenotypic changes such as motility or induction of events that are triggered in response to the limited availability of compounds are shown. **a)** Bacteria residing at different locations within the biofilm structure experience concentration gradients of nutrient resources, oxygen, waste products and extracellular signaling molecules. Biofilm cells respond to these gradients by inducing various stress responses, such as the RpoS -dependent general stress response, as well as increased expression of genes involved in the response to oxygen limitation and nutrient deprivation. **b)** Under limiting oxygen conditions, such as found in the interior of the biofilm, anaerobic denitrification leads to the generation of nitric oxide (NO). Exposure to NO is linked to the reduction in cellular bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) levels and thus increased motility and dispersion. Furthermore, NO can react with superoxide to generate the cell-toxic radical ONOO⁻. ONOO⁻ causes cellular damage, bacteriophage induction and cell lysis. Cell lysis results in the release of degradative enzymes that contribute to the enzyme-mediated breakdown of the biofilm matrix and/or loosening of the biofilm matrix. In addition, cell lysis generates nutrients for growth. **c)-d)** Levels of c-di-GMP vary throughout the biofilm, with the lowest levels being detected in the biofilm interior. Low c-di-GMP levels contribute to phenotypes generally associated with the planktonic mode of growth, including increased motility, increased drug susceptibility, but reduced adhesiveness and matrix production. Bacteria residing at different locations within the biofilm structure experience the accumulation of native dispersion cues including NO, cis-DA, and nutrients. Sensing of dispersion cues activates phosphodiesterases (PDEs) capable of degrading c-di-GMP, ultimately resulting in an overall reduction in the levels of c-di-GMP. Phenotypes associated with low c-di-GMP levels include increased motility, reduced adhesiveness, reduced matrix production and dispersion.

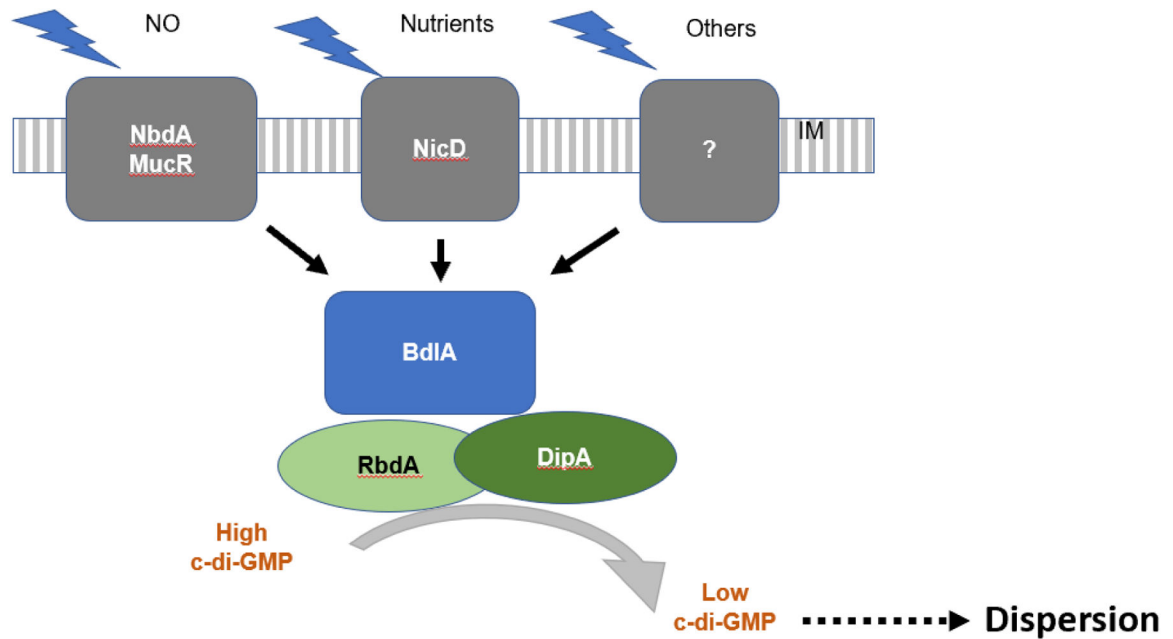


Figure 3: Sensing of dispersion cues.

Model of dispersion cue sensing and modulation of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) levels to induce dispersion. In *Pseudomonas aeruginosa*, dispersion cue sensing involves the chemotaxis-like MCP homolog, BdlA, the c-di-GMP phosphodiesterases (PDEs) DipA, RbdA, MucR and NbdA, and the diguanylate cyclases (DGCs) GcbA and NicD. The membrane-associated PDEs MucR and NbdA and the DGC NicD are involved in perceiving and relaying dispersion cues to promote the modulation of c-di-GMP levels. The DGC NicD and the PDE NbdA contribute to dispersion in a cue-specific manner, with NbdA sensing NO and NicD sensing nutrient cues. Receptors for other dispersion cues such as fatty acids have not yet been elucidated (indicated by the question mark). BdlA and the PDEs DipA and RbdA are central to the dispersion response regardless of the cue that is being sensed and contribute to the overall reduction of c-di-GMP levels post dispersion cue sensing.

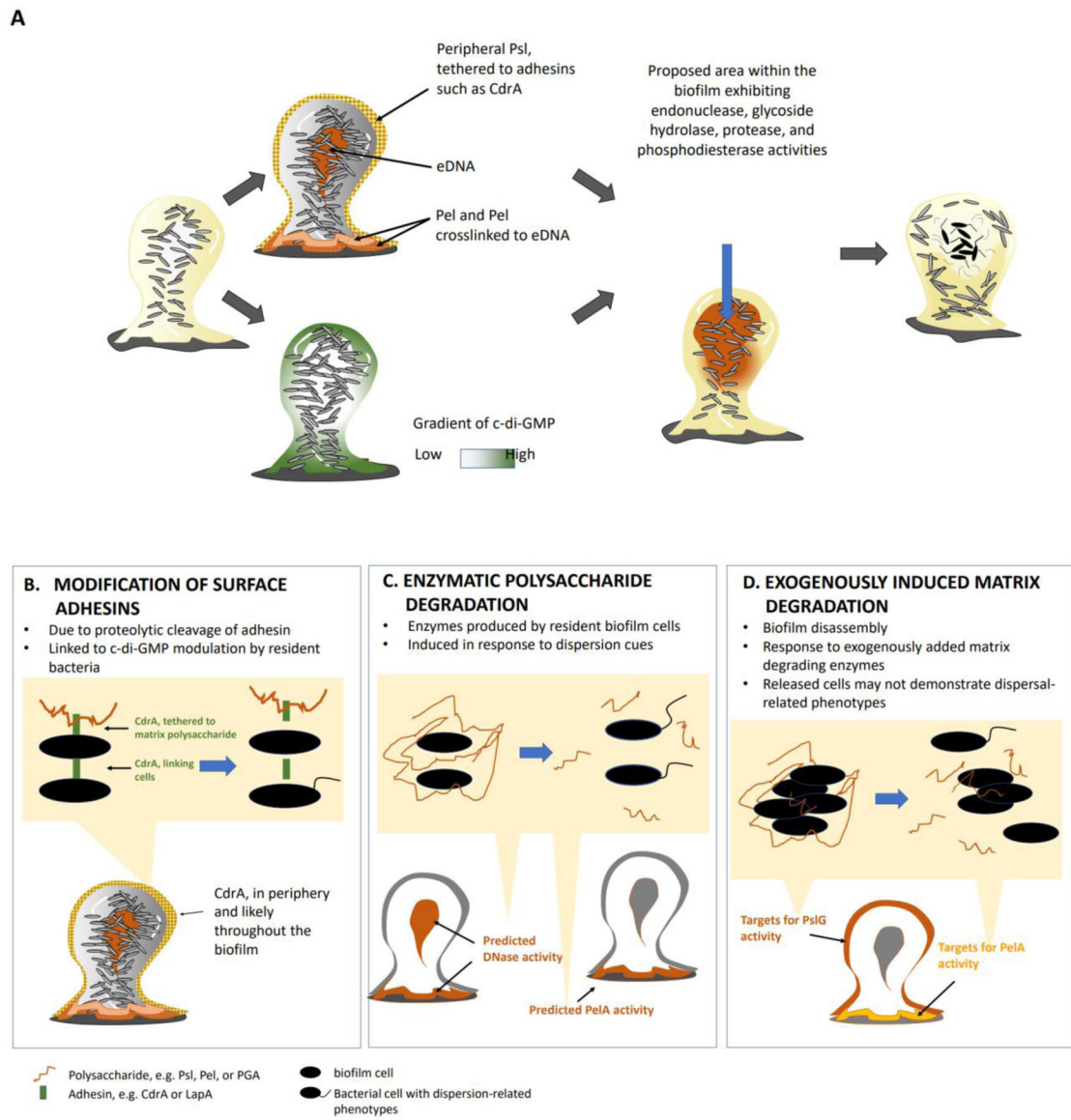


Figure 4. Mechanisms resulting in biofilm dispersal.

(a) Model of spatial localization of biofilm matrix components and bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), and proposed biofilm areas with active matrix- and c-di-GMP-degrading activities, that lead to biofilm dispersion. In *Pseudomonas aeruginosa* PAO1, the matrix polysaccharide Psl is localized at the periphery and the base of biofilms. Psl interacts with the adhesin protein CdrA and forms a protective but non-rigid structure in between cells and around the biofilm structure. The Pel polysaccharide is primarily located at the base of the biofilm and crosslinked to extracellular DNA (eDNA). However, eDNA is not limited to the biofilm base but has also been detected in the biofilm interior. Mature biofilms are characterized by low c-di-GMP levels in the interior of the biofilm whereas c-di-GMP levels in immature or less structured biofilms is more uniform. Considering the link between dispersion, low c-di-GMP levels, and matrix degradation, the biofilm periphery and biofilm interior are the most likely to be the locations within

the biofilm that experience increased matrix- and c-di-GMP-degrading activities. Moreover, these locations coincide with observed dispersion events such as void formation and erosion of the biofilm structure. b) Surface adhesins such as CdrA or LapA are cleaved to untether the polysaccharide matrix and break cell-cell interactions. (c) Matrix components are enzymatically degraded (by intrinsic DNases or the hydrolase PelA) in response to native or environmental dispersion cues (nitric oxide, nutrients or cis-2-decenoic acid). d) Biofilm disassembly can be induced by exogenously added matrix-degrading enzymes. As enzymes are exogenously added, matrix degradation is expected to primarily target the periphery of the biofilm structure (see Box 1).

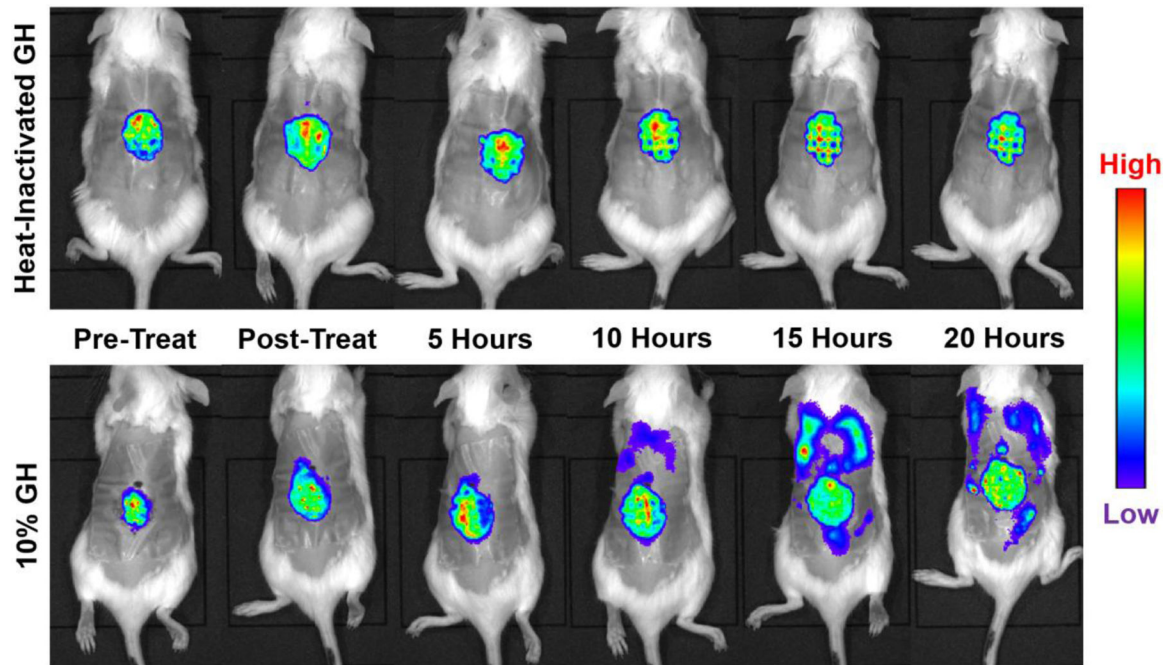


Figure 5. Bacterial dissemination triggered by glycoside hydrolase treatment of *Pseudomonas aeruginosa* biofilms.

In vitro image system (IVIS) imaging shows that treatment of 48-hour-old mouse chronic wounds, infected with bioluminescent *P. aeruginosa*, with α -amylase and cellulase (glycoside hydrolase treatment), resulted in systemic spread of the infection (bottom panels). By contrast, systemic spread was not observed after treatment with heat-inactivated enzymes (top panels). Modified from Ref.¹⁴⁶.

Table 1.

Cues, signals and environmental factors linked to dispersion.

Cues, signals and factors	Species	Effector regulatory system	Source
Fatty acid signaling (DSF and BDSF)	<i>Xanthomonas campestris</i>	DSF and Rpf genes positively control the synthesis of <i>manA</i> -encoded endo- β -1,4-mannanase, which degrades the matrix; DSF negatively affects the expression of <i>xagABC</i> encoding a glycosyl transferase system Induction of low c-di-GMP levels	Correct reference is Davies and Marques, 2009, Ref #59 ^{64,109,164}
Fatty acid signaling (cis-DA)	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Bacillus subtilis</i> , <i>Proteus mirabilis</i> , <i>Staphylococcus aureus</i> , <i>Streptococcus pyogenes</i> , <i>Candida albicans</i>	Similar to DSF and BDSF, cis-DA may induce low c-di-GMP levels, [Au: is this entry specific for <i>Pseudomonas aeruginosa</i> etc? yes, specific for <i>P. aeruginosa</i> but could also be for other strains but has not been explored]	
Oxygen depletion	<i>P. aeruginosa</i>	Dispersion response is dependent on PDE RbdA, probably to induce low c-di-GMP levels	165
Starvation due to cessation of flowing conditions (oxygen or nutrients)	<i>Shewanella oneidensis</i>	Dispersion-inducing conditions are linked to <i>mxdB</i> encoding a putative membrane-associated glycosyl transferase and the probable induction of a PDE to reduce c-di-GMP levels	48
	<i>Pseudomonas putida</i> , <i>P. aeruginosa</i> , <i>P. fluorescens</i>	Dispersion-inducing conditions have been linked to low c-di-GMP levels and the release of the adhesin LapA in biofilms by <i>P. putida</i> and <i>P. fluorescens</i> . Additionally, reports suggest lack of nutrients and cell lysis but not the accumulation of a metabolic product to be contribute to the dispersion response	66-69
	<i>S. aureus</i>	Quorum sensing, Agr-dependent	134
Nutrient availability (glucose, glutamate, succinate and citrate)	<i>P. aeruginosa</i>	Dispersion in response to nutrients is dependent on phosphorylation events, and coincides with a decrease in c-di-GMP and increased PDE activity Factors identified to have a role include BdlA, PDEs DipA and RbdA, and DGCs NicD and GcbA Induction of matrix-degrading enzymes include endonucleases EndA and EddA, and glycosyl hydrolases PeIA and PsIG	87,93,95,107,115,116,166,167
	<i>Acinetobacter sp. S. pneumoniae</i>	Dispersion response is linked to quorum sensing/Interkingdom signaling	168
	<i>Serratia marcescens</i>	dispersion is linked to quorum sensing	169
Nitric oxide	<i>P. aeruginosa</i>	Dispersion in response to nitric oxide coincides with a decrease in c-di-GMP and increased PDE activity Factors identified to have a role include BdlA, PDEs NbdA, MucR, DipA and RbdA Induction of matrix-degrading enzymes include endonucleases EndA and EddB, and glycosyl hydrolases PeIA and PsIG	97,98,115,116,170
	<i>Nitrosomonas europaea</i> Ammonia oxidizer	no effector proteins have been identified	171
Iron	<i>P. aeruginosa</i>	Exposure to elevated iron concentrations repress the expression of certain genes essential for scavenging iron, with scavenging and acquisition of iron being essential for biofilm formation by <i>P. aeruginosa</i> .	79,172
Reduction of c-di-GMP	<i>P. aeruginosa</i>	Dispersion induced by overexpression of the response regulator RcsB which leads to induction of the PDE PvrR	17368
	<i>P. putida</i>	Dispersion induced by overexpression of <i>E. coli</i> -derived PDE YjhH, which might lead to LapG-dependent release of LapA	94

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PDE, phosphodiesterase; DGC, diguanylate cyclase, cis-DA, cis-2 decenoic acid; DSE, cis-11-methyl-2-dodecenoic acid; BDSF, cis-2-dodecenoic acid; c-di-GMP, bis-(3'-5')-cyclic dimeric guanosine monophosphate; DGCs, diguanylate cyclases.