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2015

Barraud, N., Kjelleberg, S., & Rice, S. A. (2015). Dispersal from microbial biofilms. Microbiology Spectrum, 3(6). doi:10.1128/microbiolspec.MB-0015-2014

https://hdl.handle.net/10356/80430

https://doi.org/10.1128/microbiolspec.MB-0015-2014

Microbiology Spectrum
American Society for Microbiology Press

Dispersal from Microbial Biofilms

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ABSTRACT One common feature of biofilm development is the active dispersal of cells from the mature biofilm, which completes the biofilm life cycle and allows for the subsequent colonization of new habitats. Dispersal is likely to be critical for species survival and appears to be a precisely regulated process that involves a complex network of genes and signal transduction systems. Sophisticated molecular mechanisms control the transition of sessile biofilm cells into dispersal cells and their coordinated detachment and release in the bulk liquid. Dispersal cells appear to be specialized and exhibit a unique phenotype different from biofilm or planktonic bacteria. Further, the dispersal population is characterized by a high level of heterogeneity, reminiscent of, but distinct from, that in the biofilm, which could potentially allow for improved colonization under various environmental conditions.
Here we review recent advances in characterizing the molecular mechanisms that regulate biofilm dispersal events and the impact of dispersal in a broader ecological context.
Several strategies that exploit the mechanisms controlling biofilm dispersal to develop as applications for biofilm control are also presented.

INTRODUCTION

For all organisms the ability to spread and colonize new habitats is crucial to ensure species continuity and prevent extinction (1). In sessile organisms this constraint has led to the evolution of a motile, dispersal phase in their life cycle, which in plants and corals involves the release of differentiated and often phenotypically diverse seeds or propagules. Similarly, sessile microbial biofilms have developed mechanisms to release differentiated, highly motile dispersal cells into the bulk liquid.

Dispersal has profound ecological consequences, allowing biofilm populations to spread and colonize new surfaces as well as to avoid overcrowding and regenerate bacteria in the biofilm core. These benefits represent strong evolutionary pressures for dispersal, propagation, and rejuvenation of biofilms. The ecological drivers of dispersal are complex, because dispersal is both costly (2) and risky when dispersal cells face an uncertain and uncontrolled environment (3). However, the inability to disperse may ultimately lead to the collapse of any sessile biological community through the accumulation of social cheaters, such as those that disturb metabolic cooperation (4), or catastrophic events at one location resulting in either complete loss of the population leading to extinction or in genetic bottlenecks as a consequence of significant population die-off (5).

Sloughing is a passive process of cell loss from the biofilm, which may be a consequence of cell division at the biofilm–bulk liquid interface or shear forces that remove cells. In contrast, active or seeding dispersal is coordinated via regulatory systems and is energy dependent, and dispersal cells exhibit a distinct phenotype. Dispersal relies on a number of cues, both environmental and self-produced signals. Regulatory responses to changes in environmental conditions potentially enhance the chances of successful release of propagules and colonization of new surfaces. In addition, signals and cues from other biological organisms can also influence microbial dispersal. Cells that have

Received: 9 September 2014, **Accepted:** 30 September 2014, **Published:** 20 November 2015

Editors: Mahmoud Ghannoum, Case Western Reserve University, Cleveland, OH; Matthew Parsek, University of Washington, Seattle, WA; Marvin Whiteley, University of Texas at Austin, Austin, TX; and Pranab Mukherjee, Case Western Reserve University, Cleveland, OH

Citation: Barraud N, Kjelleberg S, Rice SA. 2015. Dispersal from microbial biofilms. *Microbiol Spectrum* 3(6):MB-0015-2014. doi:10.1128/microbiolspec.MB-0015-2014.

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dispersed from biofilms exhibit a specialized phenotype that is distinct from that of biofilm and planktonic cells. Moreover, the dispersal subpopulation often comprises a high level of heterogeneity, as a consequence of cell differentiation and phase variation as well as genetic diversification through mutations. The production of such variation in dispersal cells has been hypothesized to increase population fitness, e.g., enhanced colonization and survival due to complementarity and selection effects associated with the formation of such variants.

This article aims to describe features that are common to the dispersal process across species and presents specific examples to define those features. The underlying regulatory and signaling systems are examined in terms of both the extracellular cues and the internal signal transduction cascades that control active dispersal. Further, the consequences of dispersal for the microbial population are addressed. Finally, because dispersal offers unique opportunities to control biofilms, several strategies currently being developed to exploit dispersal pathways and design novel biofilm control measures are described.

THE MOTILE PHASE OF THE BIOFILM LIFE CYCLE

It is now well established that biofilm formation occurs through a series of stages that resemble developmental processes in multicellular eukaryotes. Recent studies have characterized specific regulators involved at each stage of development (6). Initial adhesion to a surface is thus controlled by nutrient availability, surface sensing, and quorum sensing (QS) (7). While flagella-mediated motility may play a role for planktonic bacteria to reach a surface and attach, the initiation of biofilm formation often leads to the loss of flagella (8). Biofilm bacteria are essentially sessile, embedded in a matrix of extracellular polymeric substances (EPSs), with the exception of a subpopulation of cells using surface motility that migrate within the biofilm to form the caps of three-dimensional (3D) microcolonies (9). In the model organism *Pseudomonas aeruginosa*, maturation of the biofilm involves production of specific matrix components and sequentially activated regulatory systems (10). These mechanisms include positive and negative feedback regulations, which are pivotal components of developmental biology. For example, the polysaccharide synthesis locus (Psl) is necessary for *P. aeruginosa* cells to attach onto a surface and subsequently serves to recruit incoming bacteria into the biofilm, establishing a feed-forward loop amplifying biofilm attachment and maturation (11). Over the course of the developmental process, bacteria adopt a succession of different phenotypes. While the biofilm phenotype appears adaptable to changes in environmental conditions, notably through stress responses, a core genetic program takes the biofilm through a series of checkpoints, eventually leading to maturation and dispersal of the biofilm.

During maturation, biofilms establish complex 3D structures comprised of differentiated bacteria, for instance exhibiting different expression of metabolic or EPS genes, and steep nutrient and oxygen (O_2) gradients, rendering the biofilm environment and the bacterial populations highly heterogeneous (12–14). The final stage of biofilm development involves the coordinated release of differentiated, motile, chemotactic cells known as dispersal cells (15). These specialized cells can colonize new surfaces and restart the biofilm life cycle. In several bacteria, biofilm dispersal correlates with the programmed death of a subpopulation of cells in mature microcolonies (16). Surviving cells are then able to escape the biofilm, leaving behind hollow structures in the biofilm. In many species, dispersal typically occurs from mature biofilm microcolonies and is preceded by localized death and lysis of cells in the center of these structures (16–22) (Fig. 1). Bacteria were observed to disperse from larger microcolonies, e.g., with a diameter greater than 40 μm and depth greater than 10 μm, but not from smaller ones (17, 23). This suggests that a specific maturation stage, as defined by the microcolony size and most likely linked to the establishment of specific gradients, e.g., nutrients or O_2, is required to trigger dispersal events. In *P. aeruginosa*, cell lysis has been linked to the activation of a superinfective prophage (16), while in the marine bacterium *Pseudoalteromonas tunicata*, the expression and activity of an autolytic protein caused cell death in mature biofilm microcolonies (24).

It has been shown that dispersal can be prevented by disrupting the cells' ability to generate energy (25), and thus, the surviving cells may benefit from nutrients released from lysed cells, and this energy is used to activate dispersal responses. This is supported by microscopic observations of biofilms showing that mature microcolonies undergo a brief "seething" stage, where cells become highly motile within microcolonies, suggesting that biofilm EPSs have been fully solubilized and that cells have regained motility (17, 26) (Figs. 1 and 2). Dispersal cells then escape by coordinated evacuation from biofilm structures (26), resulting in the hollowing of biofilm microcolony structures leaving behind voids that are typically observed during the dispersal stage for many biofilms. Dispersal cells are characterized by downregulation of

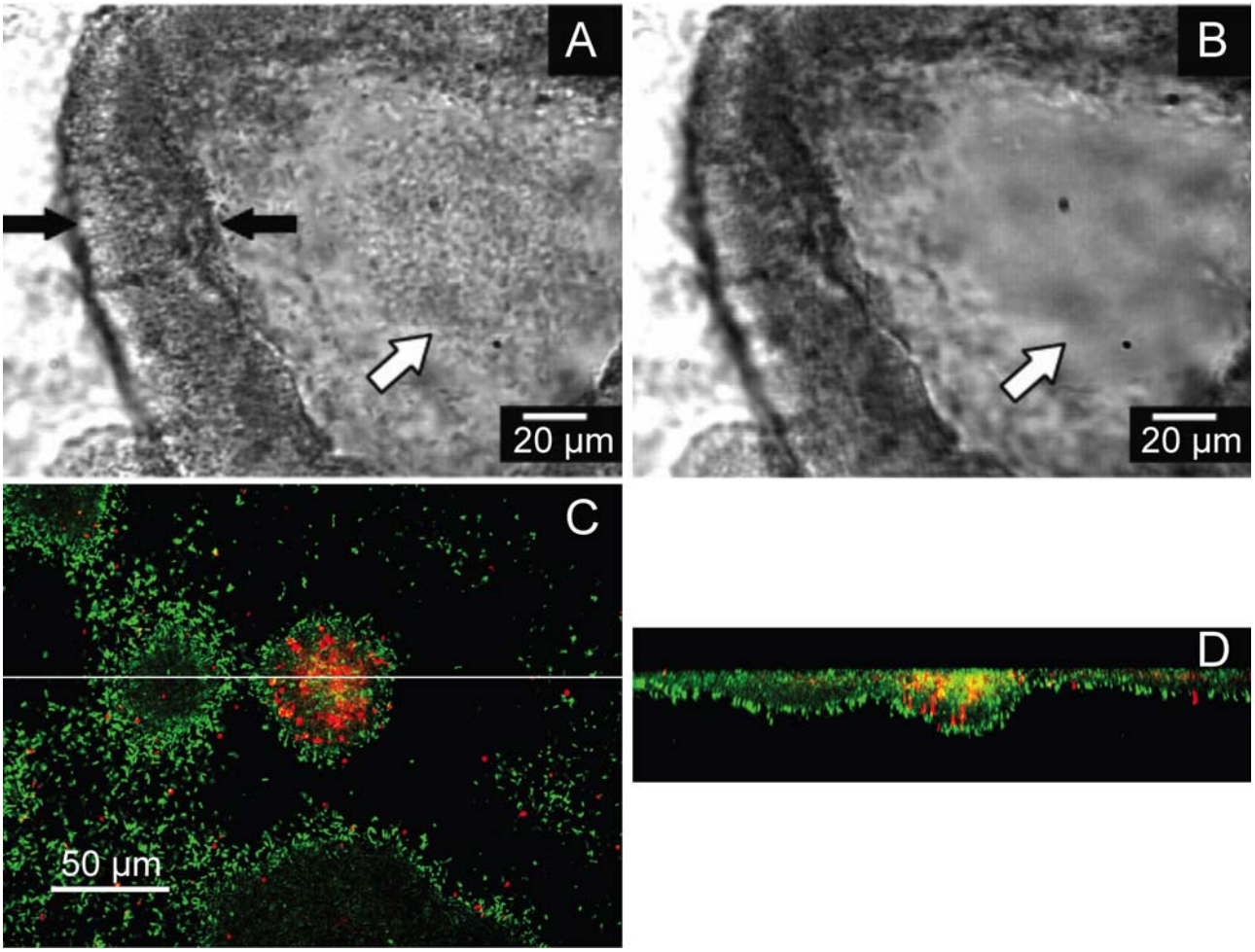


FIGURE 1 Microscopic images of biofilm microcolonies during seeding dispersal. (A-B) Motile cells appear in mature biofilm microcolonies. (A) Single frame of a mature microcolony. (B) Picture showing the average of 30 frames captured over a 1-second period. The highly motile cells "average" out and appear blurred in the center of the microcolony, demonstrating the extent of the motile region (white arrow in panels A and B). The sessile "wall" region is indicated by the black arrows in panel A (taken from reference 26, with permission from the publisher). (C) Live/dead staining of a 7-day-old biofilm reveals patterns of cell death inside biofilm structures that occur simultaneously with biofilm dispersal, as indicated by the formation of hollow biofilm structures. Live cells are green and dead cells are red (adapted from reference 56, copyright © American Society for Microbiology). (D) XZ cross-view of the biofilm in panel C (XY view) at the location indicated by the white line. doi:10.1128/microbiolspec.MB-0015-2014.f1

genes that encode for the sessile biofilm phenotype, such as exopolysaccharides and fimbriae, and upregulation of genes encoding factors important for the motile lifestyle, including flagella and chemotaxis (27–30).

REGULATION AND COORDINATION IN SEEDING DISPERSAL

The release of dispersal cells from biofilms occurs in a precisely coordinated manner and in response to changes in environmental conditions. Growth in a biofilm can lead to stress; for instance, as the biofilm grows in size, bacteria in the lower layers will experience reduced access to nutrients and electron acceptors available from the bulk liquid interface or will accumulate waste products and toxins. Stress cues including nutrient starvation, either carbon (31, 32), phosphate (33), or iron sources (34), can trigger dispersal responses. Depletion of O_2 (35, 36) and addition of the metabolism inhibitor carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (37)

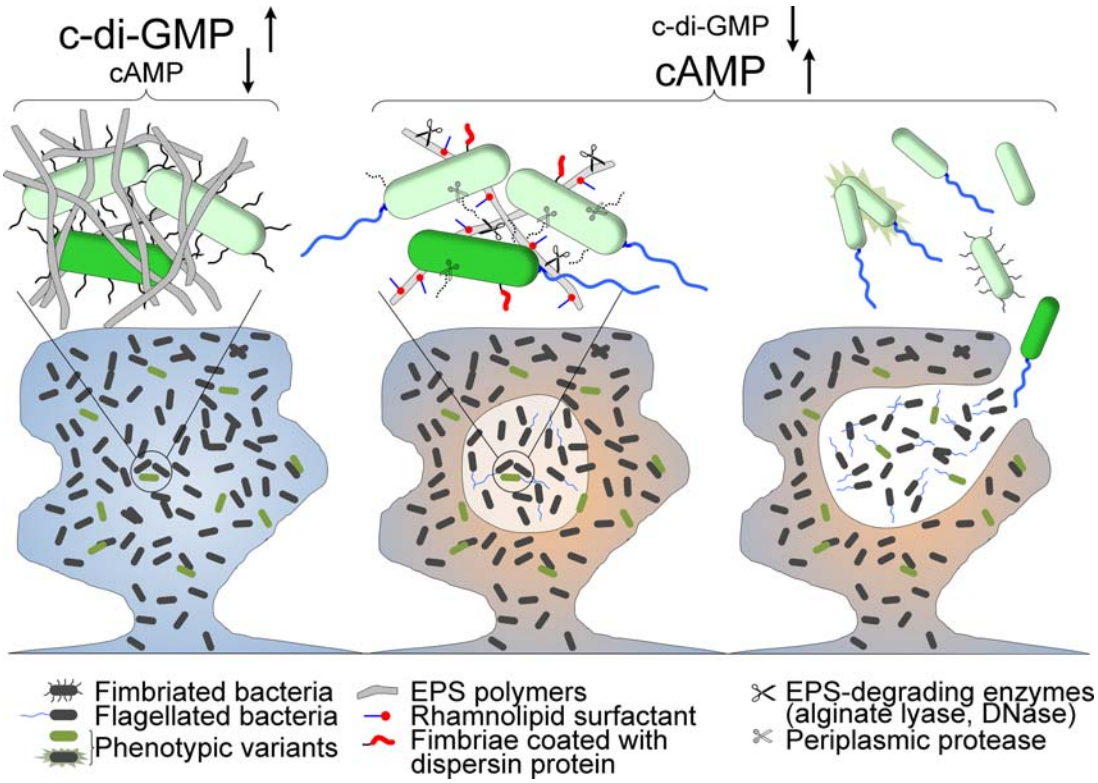


FIGURE 2 Effectors of biofilm dispersal. Bacteria within the center of microcolonies induce a number of mechanisms to degrade and solubilize the biofilm EPS matrix and extracellular appendages such as fimbriae that immobilize cells. When the interior of the microcolony becomes fluid, cells begin to show signs of motility, and a breach is made in the microcolony wall through which dispersal cells are released. doi:10.1128/microbiolspec.MB-0015-2014.f2

were also found to induce dispersal. In mature biofilms, dispersal may also be triggered by specific responses to the presence of soluble signals derived from protozoan predators, which are produced whether in the presence or absence of bacteria (unpublished observation). An increase in nutrient availability, such as carbon (38) or iron sources (39), can also trigger dispersal and lead to mass detachment events. Possibly, dispersal in response to nutrient upshift could represent a strategy to enhance chances of successful colonization by dispersal cells by ensuring an adequate supply of nutrient sources. Interestingly, biofilms of the plant pathogen *Xylella fastidiosa* were found to use cell-cell signaling to coordinate dispersal events in response to sensing the presence of a transmission vector, e.g., leafhopper insects (40). In this case it is clear that precise regulation and rapid signal transduction are necessary for the bacteria to trigger dispersal processes upon sensing the presence of the insect before it leaves the plant surface. Transmission vectors for dispersal and spreading of bacterial populations are common in nature. In a recent study, a nonmotile *Xanthomonas* sp. was found to associate with a motile *Paenibacillus vortex*, in a hitchhiking strategy, to spread and disperse on plant surfaces (41).

Cell-cell signaling is known to play a major role in regulating dispersal in various species (42). For example, QS via *N*-acyl homoserine lactones has been implicated in dispersal in *Vibrio* species (43, 44), *Serratia marcescens* (45), and *Rhodobacter sphaeroides* (46). In *Staphylococcus aureus*, the *agr*-mediated QS system was found to control biofilm dispersal (47). In *Vibrio cholerae*, QS regulates detachment (48), and add-back of cholera QS autoinducer-1 (CAI-1) and autoinducer-2 (AI-2) resuscitate dormant cells and induce dispersal of biofilm aggregates in environmental samples (49). In *Xanthomonas campestris*, cell-cell signaling via the diffusible signal cis-11-methyl-2-dodecenoic acid triggers dispersal responses (50). This class of signal molecule has more recently been shown to be conserved across a broader range of bacteria, where it induces dispersal

(51), including cis-2-decenoic acid, a potent dispersal trigger in various bacterial species (23). Further, the addition of QS inhibitors, such as furanones, can induce detachment of biofilm bacteria and prevent biofilm formation (52, 53). The role of cell-cell signaling in regulating dispersal may allow biofilm bacteria to induce the switch at an appropriate stage of the developmental process, e.g., once a threshold density is reached, or to transmit a dispersal signal from one location to another within the biofilm population.

Several research groups have also focused their efforts on identifying signal molecules produced in mature cultures entering stationary phase that were thought to trigger dispersal events. The diffusible fatty acid cis-2-decenoic acid mentioned above was first identified after fractionation of *P. aeruginosa* spent medium that was active at inducing dispersal (23). In *Bacillus subtilis*, the readdition of a conditioned medium from late biofilm cultures was also found to induce biofilm disassembly, and D-amino acids were tentatively identified as active compounds, suggesting a role in regulating dispersal (54). However, later studies from the same team revealed that the observed effects were due to a mutation in the experimental strain which caused addition of D-amino acids to reduce metabolism in these biofilms; thus, dispersal in these studies was found to rely entirely on reduced metabolism (55), which is similar to the starvation-induced dispersal for some species as described above.

In *P. aeruginosa*, studies of cell death and dispersal events led to the identification of nitric oxide (NO) as a major signal regulating dispersal (56). NO was found to be produced in mature biofilms and trigger detachment events. NO was also found to induce dispersal in a wide range of monospecies biofilms and mixed-species biofilms (reviewed in 57). Thus, biofilm dispersal appears to be controlled by a variety of cues and signals, some of which, such as *N*-acyl homoserine lactones, play dual roles in biofilm formation as well as dispersal (7).

One important system that is involved in integrating these signals is the regulatory network centered on the secondary messenger cyclic di-GMP, because it controls both attachment and dispersal (reviewed in 58, 59). Mainly found in Gram-negative bacteria, c-di-GMP is a central element of a signaling network that integrates one or multiple signals (input) sensed by a bacterium and activates cellular effectors (output), resulting in either biofilm attachment or dispersal. Intracellular levels of c-di-GMP are controlled through the opposing activities of diguanylate cyclases (DGCs) for the synthesis of c-di-GMP, and phosphodiesterases (PDEs) for its degradation. These are encoded by conserved GGDEF-domain- and EAL- or HDGYP-domain-containing genes, respectively. Many bacterial genomes encode multiple DGCs and PDEs often associated with other putative signaling domains, suggesting that their enzymatic activities may be responsive to a range of environmental cues, including NO, O_2, and nutrients. Recently, cAMP, which was previously known to control the stringent response, was found to play a role in the regulation of biofilm formation and dispersal (25, 60). The role that nucleotide secondary messengers such as c-di-GMP and cAMP play in signal transduction in bacteria is being increasingly recognized (61, 62; cf. online census http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html), which is perhaps not surprising given the abundance of nucleotides in the intracellular milieu and their relationship to metabolic activity, a key dispersal trigger.

A link between NO and c-di-GMP in the regulation of biofilm dispersal was first established in *P. aeruginosa* (29) and was then also described in *Shewanella woodyi* (63) and *Legionella pneumophila* (64). In *P. aeruginosa* several c-di-GMP–specific PDEs have been identified that appear to be involved in NO-mediated dispersal, including DipA, RbdA, and NbdA (65, 66). In some bacteria, the sensor for NO has been identified. For example, *S. woodyi* encodes a heme NO/O_2–binding (HNOX) protein, which when complexed with NO, binds to and activates a PDE enzyme, resulting in dispersal. HNOX domains are conserved hemoproteins that are highly sensitive to NO, producing responses at femtomolar levels in *Clostridium botulinum* (67). They are found in several Gram-negative and Gram-positive bacterial genomes and are often associated with a DGC or PDE (68). However, many bacterial strains known to disperse in response to NO, including *P. aeruginosa* and *Escherichia coli*, do not have HNOX-domain-encoding proteins, suggesting that other systems can sense NO signals. In *E. coli*, a redox sensor associated with a c-di-GMP PDE, *Ec*DOS was shown to increase PDE activity upon binding of NO, O_2, and carbon monoxide (69). *Ec*DOS was later shown to be part of a two-gene operon for the control c-di-GMP in response to O_2 levels where sensing of O_2 activates both DGC and PDE activity. This suggests a feedback control mechanism, similar to a thermostat, to regulate biofilm formation and dispersal precisely, depending on O_2 levels (70).

In *P. aeruginosa* the methyl-accepting protein BdlA has been found to be involved in the dispersal response to both nutrient upshift (28) and NO (29) and interacts with a PDE, possibly by stabilizing the enzyme, resulting in lower intracellular levels of c-di-GMP and enhanced

dispersal (65). Recently, an interesting mechanism of feedback regulation was uncovered when it was found that BdlA needed to be cleaved before activating dispersal. Further, cleavage of BdlA, involving the chaperone ClpD and protease ClpP, requires high levels of c-di-GMP (71). Thus, high levels of c-di-GMP, which are typical of biofilm cells, appear to be a prerequisite to induce dispersal events in mature biofilms in this strain.

EFFECTORS OF DISPERSAL

Upon sensing a dispersal cue, bacteria can activate a range of cellular effectors that allow them to break their bonds to the biofilm, including enzymes and surfactants that will degrade the biofilm EPS, resulting in dispersal (Fig. 2).

First, bacteria can produce and secrete enzymes that degrade the biofilm EPS matrix, the composition of which varies but is mainly comprised of polysaccharides, proteins, nucleic acids, and lipids. In the nonmotile species *Aggregatibacter actinomycetemcomitans*, a screen of transposon mutant biofilms deficient in the ability to release cells identified dispersinB, an endogenous β-*N*-acetylglucosaminidase capable of degrading the matrix polysaccharides, which was later found to induce biofilm dispersal in a range of bacterial species (72, 73). In *P. aeruginosa*, alginate is an essential component of the matrix, and early studies revealed that increased expression of alginate lyase leads to biofilm detachment (74). Furthermore, it was found that DNA is a major constituent of the EPS matrix, and biofilm dispersal could be induced upon treatment with DNase in *P. aeruginosa* and *S. aureus* (75, 76). DNase was also found to be endogenously produced and secreted in *Bacillus licheniformis* biofilms to induce dispersal (77). In *S. aureus*, extracellular protease activity mediated by the *agr* QS system was also found to trigger biofilm detachment (47). In *Pseudomonas fluorescens* (33), *Pseudomonas putida* (78), and *P. aeruginosa* (25), starvation-induced dispersal was linked to activation of a periplasmic cysteine protease, LapG. When activated, LapG has been shown to cleave the surface adhesion LapA to release cells from the surface (78). In *P. aeruginosa*, *in vitro* batch and continuous-flow biofilm assays revealed that a *lapG* knockout mutant strain was unable to disperse in response to nutrient starvation, O_2 depletion, or NO donor signals compared to a wild type strain (unpublished data). This suggests that dispersal events in response to these various cues all integrate via a c-di-GMP signaling cascade that relies on LapG activation to induce biofilm detachment.

Second, the connecting biopolymers that maintain bacteria in the biofilm may be modulated by the secretion of chemicals that specifically alter adhesion appendages. Studies of *E. coli* suggested that dispersal bacteria are coated with a protein, called dispersin, that counteracts fimbriae-mediated aggregative adherence (79). Further, dispersal of *E. coli* biofilms from human epithelial cells was associated with alterations of bundle-forming type IV pili structures from thin to much longer and thicker bundles that resulted in loss of adherence and aggregation (80). To reduce surface tension and allow cells to detach, biofilm bacteria also produce amphipathic molecules, such as rhamnolipids, to induce dispersal (81). In *P. putida*, endogenous biosurfactants called putisolvins produced upon entry into stationary growth phase were able to induce biofilm detachment (82). In this scenario, the dispersal signal is amplified within an entire subpopulation of cells, where neighboring cells may degrade the matrix surrounding cells that may not have yet switched to a dispersal phenotype.

Finally, the transition from a biofilm to a planktonic mode of growth involves the activation of flagella (swimming and swarming motility) or pili (twitching motility) in motile microorganisms. Thus, flagella synthesis genes were found to be expressed in dispersed cells of *E. coli* and *P. aeruginosa* biofilms (26, 27, 38, 83), while type IV pili were found to be activated in biofilm bacteria treated with the dispersal signal NO (29). Further, the analysis of microscopy movies of *P. aeruginosa* biofilms revealed that attached bacteria use type IV pili to adopt a vertical orientation and mediate surface detachment (84).

Overall, a variety of strategies have been observed for detachment of bacteria from biofilms between different species but also in the same species. It is likely that the mechanisms used will depend on the growth conditions, including the conditional composition of the matrix, as well as the age of the biofilm.

DISPERSAL CELLS PRESENT A SPECIALIZED PHENOTYPE

Biofilm populations often display a high level of phenotypic heterogeneity (13), and not surprisingly, the dispersal subpopulation released from mature biofilms also reveals a high level of phenotypic variation. The variable phenotypes of dispersal cells are the result of a combination of changes in gene expression (transient phenotypic differences) and altered genotypes (permanent phenotypic changes). This diversity in biofilms and dispersal cells has been linked to increased fitness and

improved ability to colonize a range of habitats. Dispersal bacteria appear to be affected in key traits including attachment, metabolism, and antimicrobial resistance, as well as virulence and motility (30, 85–87).

Two studies of *P. aeruginosa* and *Streptococcus mutans* have compared stationary-phase planktonic cultures, resuspended biofilm cells, and bacteria collected from the biofilm effluent, and found differences in attachment properties and growth rates (30, 87). The entire dispersal population appeared to grow at rates similar to biofilm cells, both of which grew more slowly than planktonic cultures. While dispersal cells may have an overall slower metabolism, in *P. tunicata*, *P. aeruginosa*, and *S. mutans*, the dispersal populations have been found to utilize a wider range of nutrient sources (85–87). When variants isolated from biofilm effluents were analyzed separately, individual variants showed either decreased or increased metabolic rates compared to the parental strain (85–87). Further, dispersal cells in *P. aeruginosa* and *S. mutans* attached better and formed biofilms with greater biomass compared to both planktonic and resuspended biofilm cells (30, 87). Another study showed enhanced attachment and biofilm formation in dispersal cells of *Marinobacter hydrocarbonoclasticus* compared to planktonic cultures (88). This ability was linked to reshaping of the cell envelope and mobilization of storage reserves of cellular fatty acids and alcohol esters. Finally, increased resistance to antimicrobials and stress has been observed in biofilm-dispersed cells compared to planktonic cultures. Bacteria released from *S. mutans* biofilms had greater tolerance to chlorhexidine and acid treatments compared to stationary-phase planktonic cells (87). However, when a range of antibiotic treatments were tested by using a disc diffusion method, no difference between the dispersal and planktonic subpopulations was found in *P. aeruginosa* (30). When effluents from dual cultures of *P. aeruginosa* and *Burkholderia cepacia* biofilms were analyzed, the dispersal cells showed enhanced resistance to chlorine compared to planktonic chemostat cultures, and this resistance was proportional to the size of the detached clusters (89).

The physiological plasticity of dispersal bacteria is in part due to a high level of heterogeneity, resulting in the formation of phenotypic variants. Individual variants have been isolated from biofilm effluents with a frequency ranging from 5 to 60% of the dispersal subpopulation, which increased as the biofilm matured, in various strains including *P. aeruginosa*, especially from undomesticated clinical isolate biofilms (86, 90) and *S. marcescens* (91). In *P. tunicata*, a mutant unable to undergo cell lysis and dispersal released fewer variants in their effluent, with an overall reduced ability to form biofilms, reduced motility, and homogeneous growth rate. In contrast, wild type biofilms showed extensive variation and overall increased values in all three aspects (85). Interestingly, when all variants collected from the dispersal effluent were grown in mixed cultures and tested for resistance to predation, the assemblage of variants showed increased resistance compared to monocultures grown from individual variants or the parental strain (92). The effect of self-generated genetic variation on increasing resistance of the mono-species biofilm population is also observed when genetic diversity is increased at the species level in mixed-community biofilms (93).

Dispersal cells appear to have a specialized phenotype, different from biofilm bacteria and also clearly distinct from cells grown in planktonic cultures (Fig. 3). The dispersal phenotype appears to show various degrees of stability resulting from phase variation to more permanent genetic alterations. Variants that were isolated from biofilm effluents, e.g., in *P. aeruginosa* (86) or *S. marcescens* (91), were found to be stable and maintain a distinct phenotype after subculturing. The physiological characterizations of entire dispersal populations have been performed immediately after detachment events, within hours following induction or seeding dispersal, and it is not entirely clear whether the observed specialized traits, such as antibiotic resistance (e.g., persisters) or metabolic activity may be stable or transient and whether reculturing of these cells would lead to a phenotype closer to planktonic cells.

Genotypic diversity has been observed in dispersal cells; for example, in *S. marcescens* biofilms, variants isolated from the biofilm effluent were found to result from a single mutation in a regulatory gene (92), which likely represents a favorable genetic switch as opposed to *de novo* mutation. Recently, whole-genome deep sequencing of biofilm dispersal populations revealed single nucleotide mutations in several hot spots, indicative of parallel evolution, but no extensive genetic variation could be observed (94). The latter observation is surprising given the high frequency of phenotypic variation observed.

Thus, it appears that the dispersal population harbors a high level of heterogeneity. However, there has been no investigation of whether this diversity may be directly linked to dispersal or its regulation or whether it was already found within the biofilm, although we can detect such variants within the biofilm biomass prior to dispersal (S.A. Rice et al., unpublished). The generation

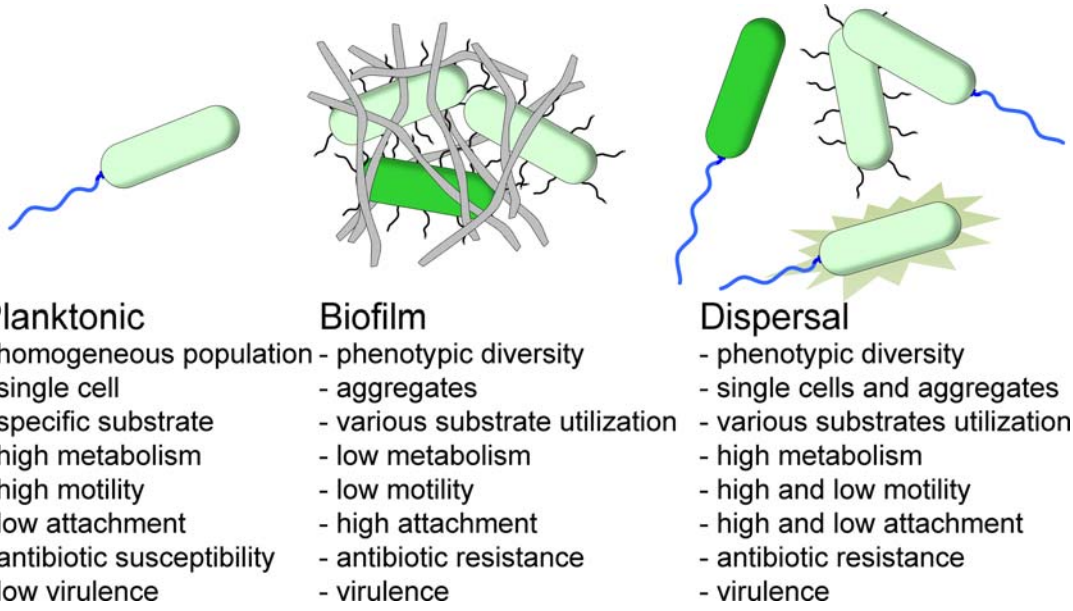


FIGURE 3 Physiological traits of planktonic, biofilm, and dispersal cells. Symbols are defined in Figure 2. doi:10.1128/microbiolspec.MB-0015-2014.f3

of phenotypic diversity in dispersal bacteria has been linked to a range of triggers including oxidative stress (22, 95), activation of a superinfective filamentous phage that is also linked to reactive oxygen and nitrogen species activity (96), as well as anaerobic conditions (97). In *P. aeruginosa*, the mismatch repair system was found to generate genetic mutants after repair of oxidative stress–induced DNA damage (95). The emergence of stable phenotypic variants was also associated with the activation of a superinfective prophage by nitrosative stress (56, 96). In *P. tunicata*, an autolysis protein, AlpP, was shown to generate production of hydrogen peroxide, which led to phenotypic diversification and increased fitness (85). Indeed, deletion of the filamentous phage, the AlpP protein, and RecA-mediated recombination resulted in the loss of variant formation and an increase in sensitivity of the biofilms formed by the mutants when exposed to stressors.

ECOLOGICAL AND EVOLUTIONARY ASPECTS OF BIOFILM DISPERSAL

From an evolutionary perspective, biofilm dispersal represents an important adaptive strategy with profound impact on the survival and fitness of bacteria. The nature of dispersal, whether it benefits the biofilm population as a whole or only individual dispersal cells, is still not entirely clear. Because biofilms display a high level of cell differentiation and show close similarities to multicellular organisms (98), biofilm development may share some similarities with the evolution and transition to multicellularity of higher organisms. In this context, dispersal has been considered an important step for the transition to multicellularity (99) and has been explained within the framework of each of the two main theories of multilevel (group) (100) and kin (individual) (101) selection. Group selection implies dispersal is an altruistic event that conserves the genetic inheritance of the population. In this scenario, diversity in the dispersal subpopulation should be restricted to reversible phenotypic switches and not derive from genetic mutations. In contrast, dispersal as an event selected at the individual level, kin selection, correlates with a high level of genetic variation within dispersal cells and thus a gradual change in genetic content or function relative to the parental population.

These questions of genetic evolution and dispersal in biofilms have important implications, for instance, in the context of the emergence of antibiotic resistance, a significant concern of our modern society. Biofilms play a major role in the resistance of bacteria toward antimicrobials and antibiotics, because bacteria in biofilms often display a high level of tolerance, via the generation of persister cells (102) or by accumulating resistant mutants via spontaneous mutations (103) as well as horizontal transfer of resistance genes (104). A direct link between increased genetic diversity specifically associated with a dispersal program rather than as a result of diversity that occurred within the biofilm has not been firmly established yet. In contrast, specific diversity in

the dispersal population appears to result from stable cell differentiation or phase variation and thus derive from a program encoded within bacterial genomes to maximize chances of colonization and survival.

The lungs of cystic fibrosis (CF) patients chronically infected with bacterial pathogens, which are mostly clonal in nature due to the apparently infrequent transmission between patients, offer a rare opportunity to observe evolutionary adaptation of bacterial populations. Genomic analyses of *P. aeruginosa* infections in CF patients over 8 years showed strong positive selection signals (ratio of nonsynonymous to synonymous changes per site, dN/dS >1) (105). Later analyses of bacterial infections over 35 years, representing 200,000 generations, revealed that after a period of adaptation *P. aeruginosa* stabilizes its phenotype in CF lungs and then applies a negative selection for mutations (106, 107). Thus, it is clear that clonal populations in a restricted environment undergo genetic evolution, which is either positively or negatively selected. While bacterial infections in CF patients' lungs are often associated with the biofilm mode of growth, and thus chronic infections are likely to go through dispersal events, the role that biofilm dispersal plays in generating genetic mutations over time remains unclear. Another study specifically analyzed genetic changes in *B. cepacia* biofilms through successive dispersal events *in vitro*. Beneficial changes occurred in the population, leading to mixed communities of stable variants that were more productive (colony-forming units number) than any monoculture (108).

Further, the mixed-variant biofilms obtained in these experiments resembled that of CF infections, suggesting that parallel evolution occurs *in vitro* and in CF lungs (109). In particular, evolved *B. cepacia* populations harbored mutations in the *wsp* operon regulating c-di-GMP signaling, suggesting that the mutants were affected in their ability to form biofilms and disperse (109). This supports a close association between the evolution of strains in the CF lungs and biofilm dispersal events. Overall, it is likely that dispersal plays a critical role in evolutionary processes, because it allows spreading of any new phenotypic trait to colonize new habitats where competition with the parental population is limited. Interestingly, the importance of biofilm dispersal in evolutionary processes may be observed in the apparent evolutionary conservation of the regulatory pathways that mediate dispersal. For example, multiple components of the NO-mediated dispersal signaling pathways from biofilms, NO/c-di-GMP, are also conserved in higher multicellular organisms, where the NO/cyclic GMP system regulates diverse physiological functions in mammals, regulating vasodilation, platelet aggregation, and sensory systems, as well as in plants, regulating development and pathogen defense responses (110). These similarities suggest an intriguing relationship between biofilm dispersal signaling and the evolution of eukaryotic regulatory pathways (29).

Intriguingly, bacteriophages have been associated with phenotypic diversity in the dispersal population. In particular, in *P. aeruginosa* a filamentous phage, Pf4, which is present as a prophage, is highly expressed during biofilm development compared to planktonic cells (8). The activity of the Pf4 phage was linked to killing and lysis of a subpopulation of cells within biofilms (16) and the emergence of phenotypic variants (96). Addition of the superinfective phage to planktonic cultures resulted in the formation of morphotypic variants, which were not observed in uninfected control cultures (111). Further, the Pf4 phage was found to increase fitness and virulence in a murine infection environment (111). However, it is not yet understood whether the Pf4 phage superinfective switch is coregulated with dispersal pathways. In *P. aeruginosa*, modulating c-di-GMP levels had no effect on phage superinfection, and both the wild type and a prophage mutant dispersed equally upon NO addition, suggesting that the NO dispersal pathway is not dependent on the Pf4 phage (unpublished data). A lytic RNA-containing bacteriophage, PP7, has also been implicated in regulating phenotypic variation in *P. aeruginosa*, leading to diversification and the evolution of a small-rough colony phenotype (112).

Dispersal is thought to be beneficial to biofilms for several reasons and, in particular, allows microbial populations to spread and survive. Arguably, dispersal may be considered the most important phase of biofilm development. Mathematical models have suggested that dispersal mostly benefits a population in an open space, while it can lead to its collapse in restricted environments (5). Dispersal is beneficial when a population faces catastrophic events that may eradicate a colony at a particular location, while leaving the location habitable for new organisms.

The lack of detailed studies of biofilm dispersal in natural systems leaves a number of questions unanswered. Can biofilm dispersal be detrimental as the result of utilizing crucial resources while increasing competition in a local environment and thus ultimately damaging the parental biofilm population? Do biofilms adopt a strategy of mass dispersal in harsh environments, risking extinction by misallocating energy resources to dispersal cells that lose the inherent protection of the biofilm? In the context of infection, a

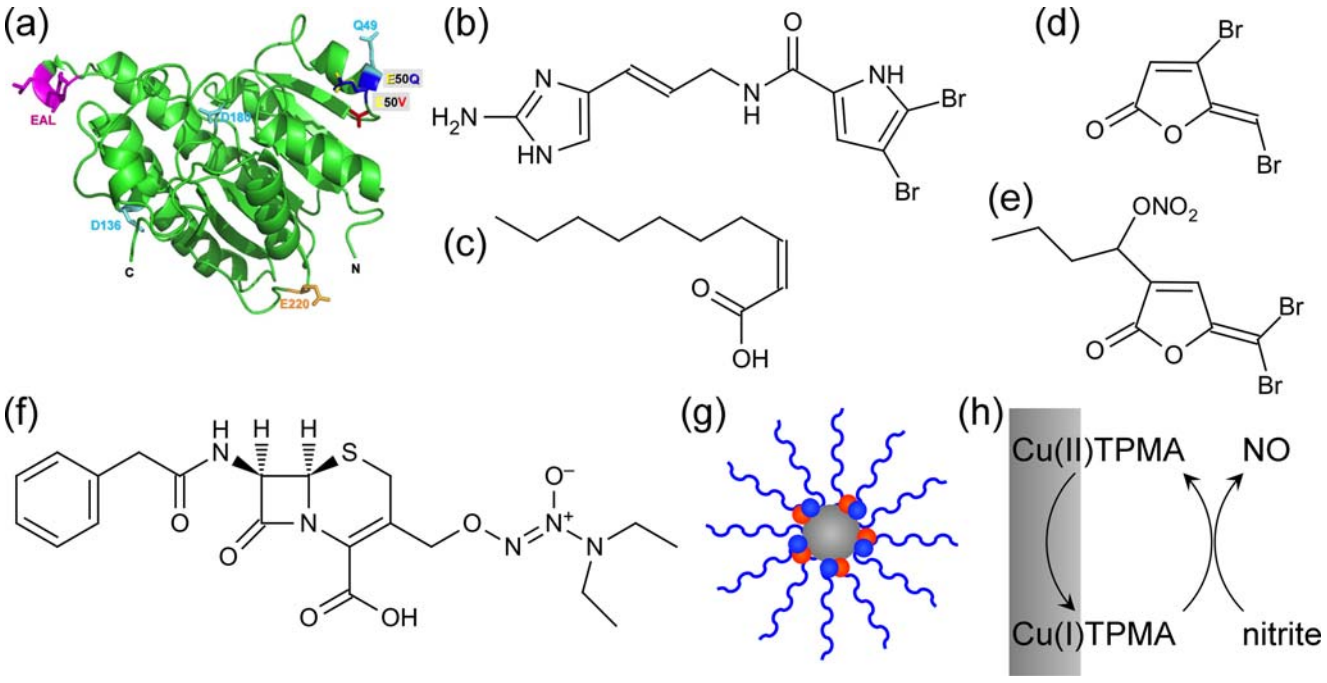
number of reports revealed that dispersal is a critical strategy for pathogens to successfully colonize a host. A functioning dispersal response appears to be crucial to ensure transmission of disease in both animals (113, 114) and plants, for instance, transmission of the plant pathogen *X. campestris* on the Chinese radish (50). A recent study found that *P. aeruginosa* mutant strains impaired in their ability to disperse from biofilms had reduced virulence and reduced ability to cause persistent infections in both murine and plant *in vivo* infection models (115). These beneficial effects of dispersal in a context of infection may be due to the association of dispersal signaling pathways with activation of virulence in some pathogens, e.g., via a decrease in c-di-GMP levels leading to expression of an endo-β-1,4-mannanase in *X. campestris* (50) and activation of type III secretion in *P. aeruginosa* (116).

Further studies will be required to fully grasp the role of dispersal in complex ecological systems involving multispecies biofilms in highly heterogeneous habitats. Metagenomics and molecular analysis–based studies appear highly suitable to reveal complex patterns of diversity in microbial communities but require precise temporally and geographically linked sampling points to track and analyze biofilm dispersal processes in natural environments (117). While not biofilm specific, some elegant model studies have been performed to determine the impact of dispersal events on microbial community composition and changes, which showed that environmental filtering, functional plasticity, and competition are all important mechanisms influencing the fate of dispersed communities (118).

OPPORTUNITIES FOR BIOFILM CONTROL

Manipulation of the biofilm development program by inducing dispersal signals has emerged as a strategy for developing novel biofilm control measures in recent years (Fig. 4). One of the first strategies was to treat biofilms with EPS-degrading agents to disperse attached

FIGURE 4 A range of strategies targeting dispersal have been developed to control biofilms and biofilm-related infections. (a) BdcA protein with enhanced c-di-GMP binding (124). (b) Oroidin and synthetic derivatives that were identified as potent dispersal inducers after screening chemical libraries (125). (c) Diffusible fatty acid signal cis-2-decenoic acid (23). (d) Furanone 30, a synthetic QS inhibitor derived from natural furanone compounds isolated from the red alga *Delisea pulchra* (131). (e) Fimbrolide-nitroester with dual action QS inhibition and NO release (134). (f) β-lactam-NO prodrugs for the targeted delivery of NO to infectious biofilms (132). (g) Controlled delivery of NO using nanoparticles (135). (h) Catalytic generation of NO from endogenous nitrite sources to disperse and prevent biofilm for long-term applications (138). doi:10.1128/microbiolspec.MB-0015-2014.f4



cells. These treatments were later combined with antibiotics to eradicate the dispersed cells, for instance dispersinB in combination with the broad-spectrum biocide triclosan (119), or alginate lyase together with the aminoglycoside tobramycin (120). Given the diversity in the components of biofilm EPS matrices (121), enzyme-based treatments are likely to be biofilm specific, which can be beneficial when designing narrow-spectrum treatments.

The recent discoveries of the central role of the secondary messenger c-di-GMP in regulating the transition between biofilm and free-swimming cells across many bacterial species attracted strong interest. Enzymes involved in c-di-GMP turnover appear very potent for controlling the switch, and the global signaling network has become a primary objective for developing novel antibiofilm measures. The first proof of concept studies of targeting c-di-GMP to treat infections showed that *in vivo* manipulation of c-di-GMP levels can effectively clear (by decreasing c-di-GMP) or prolong (by increasing c-di-GMP) *P. aeruginosa* infections in murine models (122, 123). Further, in an effort to develop a potential therapeutic drug, a BdcA protein was modified to enhance its c-di-GMP binding, thus reducing the intracellular c-di-GMP concentration. When added to biofilms *in vitro*, the modified BdcA protein caused nearly complete dispersal (124).

Improved high-throughput assays for biofilm dispersal allowed for the screening of large numbers of synthetic compounds and are particularly amenable to screening chemical libraries. Using this approach, 2-aminoimidazole derivatives targeting QS were designed and found to disperse established biofilms (125, 126). Studies of aerobic granules, a type of suspended biofilms used for wastewater treatment, revealed that the fatty acid 2-decenoic acid, which was previously identified to regulate dispersal in *P. aeruginosa* and a range of single-species biofilms, can also trigger dispersal in these mixed-species biofilms (127). In this context, strategies to inhibit 2-decenoic acid signaling may be useful to maintain granule integrity and sludge performance (127). QS signals and inhibitors have also been examined for their potential to enhance performance outputs in wastewater treatment biological reactors (128, 129), as well as for the treatment of biofilm-related infections and bacterial virulence (130, 131).

Of particular interest is NO, a simple and versatile dispersal signal that is highly conserved across biofilm species and activates c-di-GMP signaling to induce dispersal. Much progress has been made in recent years to design efficient NO delivery strategies, making it an outstanding candidate for novel therapeutic strategies. A range of NO-based technologies have been developed that offer a versatile range of solutions to control biofilms (reviewed in 57), which include a new class of β-lactam-NO prodrugs for the targeted delivery of NO to infectious biofilms (132, 133), dual quorum-sensing antagonists–NO releasing compounds (134), and novel polymers for sustained delivery of NO to prevent biofilm formation in a nontoxic fashion (135). NO-based treatments have been applied to the treatment of fouling on water filtration membranes (136, 137). A promising approach for long-term prevention and dispersal of biofilms in industrial and clinical settings is the use of copper-based catalytic technology to reduce endogenous nitrite ions to continuously produce NO at the surface (138). Recently, the first clinical trial was conducted to evaluate the use of low-dose inhaled NO gas combined with standard antibiotic therapy for the disruption of *P. aeruginosa* biofilms in patients with CF (139). The results demonstrated that patients who received NO gas at 5–10 ppm (~200 nM NO) showed significant reductions in the number of *Pseudomonas* biofilm aggregates compared to patients who received a placebo. These data suggest that using NO as an adjunctive therapy may be highly beneficial for the treatment of CF-related biofilm infections.

CONCLUDING REMARKS

Advances in biofilm and molecular biology studies have led to the elucidation of key signaling molecules and genes that govern biofilm development and dispersal processes. The existence of a developmental program appears clearer, but the challenge remains to address how these molecules and genes work together as control systems to generate patterns in space and time in biofilms. The biofilm mode of growth represents the predominant lifestyle for bacteria in environmental, industrial, and medical settings. A precise understanding of biofilm development and dispersal is crucial to better control microbial communities, with profound implications for global health, and to overcome the threat of antibiotic resistance as well as to develop innovative solutions for improved management of environmental microbes.

ACKNOWLEDGMENTS

Conflicts of interest: We disclose no conflicts.

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