Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal

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Abstract | In most environments, bacteria reside primarily in biofilms, which are social consortia of cells that are embedded in an extracellular matrix and undergo developmental programmes resulting in a predictable biofilm 'life cycle'. Recent research on many different bacterial species has now shown that the final stage in this life cycle includes the production and release of differentiated dispersal cells. The formation of these cells and their eventual dispersal is initiated through diverse and remarkably sophisticated mechanisms, suggesting that there are strong evolutionary pressures for dispersal from an otherwise largely sessile biofilm. The evolutionary aspect of biofilm dispersal is now being explored through the integration of molecular microbiology with eukaryotic ecological and evolutionary theory, which provides a broad conceptual framework for the diversity of specific mechanisms underlying biofilm dispersal. Here, we review recent progress in this emerging field and suggest that the merging of detailed molecular mechanisms with ecological theory will significantly advance our understanding of biofilm biology and ecology.

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Biofilms are assemblages of microorganisms, encased in a matrix, that function as a cooperative consortium. It is now well accepted that the biofilm mode of life is a feature common to most microorganisms in natural, medical and engineered systems. Consequently, recent research on biofilm communities in both natural habitats and defined laboratory model systems has revolutionized microbiology.

This basic shift in perspective for microbiology is arguably due to two key advances: the remarkable technological advances in the study of both single cells and complex microbial communities, and the incorporation of a theoretical evolutionary and ecological framework, along with more rigorous experimental designs, into biofilm research. This broader theoretical context is largely based on adopting (and adapting) theory from studies of the biology of higher organisms. The increasing use of ecological theory in biofilm studies has also now arguably reached the stage at which the information flow is reversed. The short generation times of microorganisms, the ease of replicating experiments on biofilms and an advanced technological toolbox offer the capacity for high-resolution experiments that are extremely challenging, if not impossible, in many eukaryotic systems.

Although new technologies such as metagenomics have transformed our views of complex microbial communities in a wide variety of environments, most of our current understanding of the developmental, structural and functional characteristics of biofilms derives from precise experiments on biofilms in specific experimental systems (for example, flow cells) using a limited number of model organisms. These experiments include direct visualization of biofilms at defined stages using fluorescently tagged proteins and cells together with confocal laser scanning microscopy. Using these approaches, phenomena such as the localization of different cell types and the differentiation, activity and gene expression of wild-type and mutant organisms have been determined[1–4] and have revealed striking commonalities in the structure and function of biofilms of different species (reviewed in REF. 5).

One such commonality is the biofilm 'life cycle', which consists of predictable transitions from single cells to complex microcolonies, ultimately leading to the production of differentiated, highly motile cells known as dispersal cells.

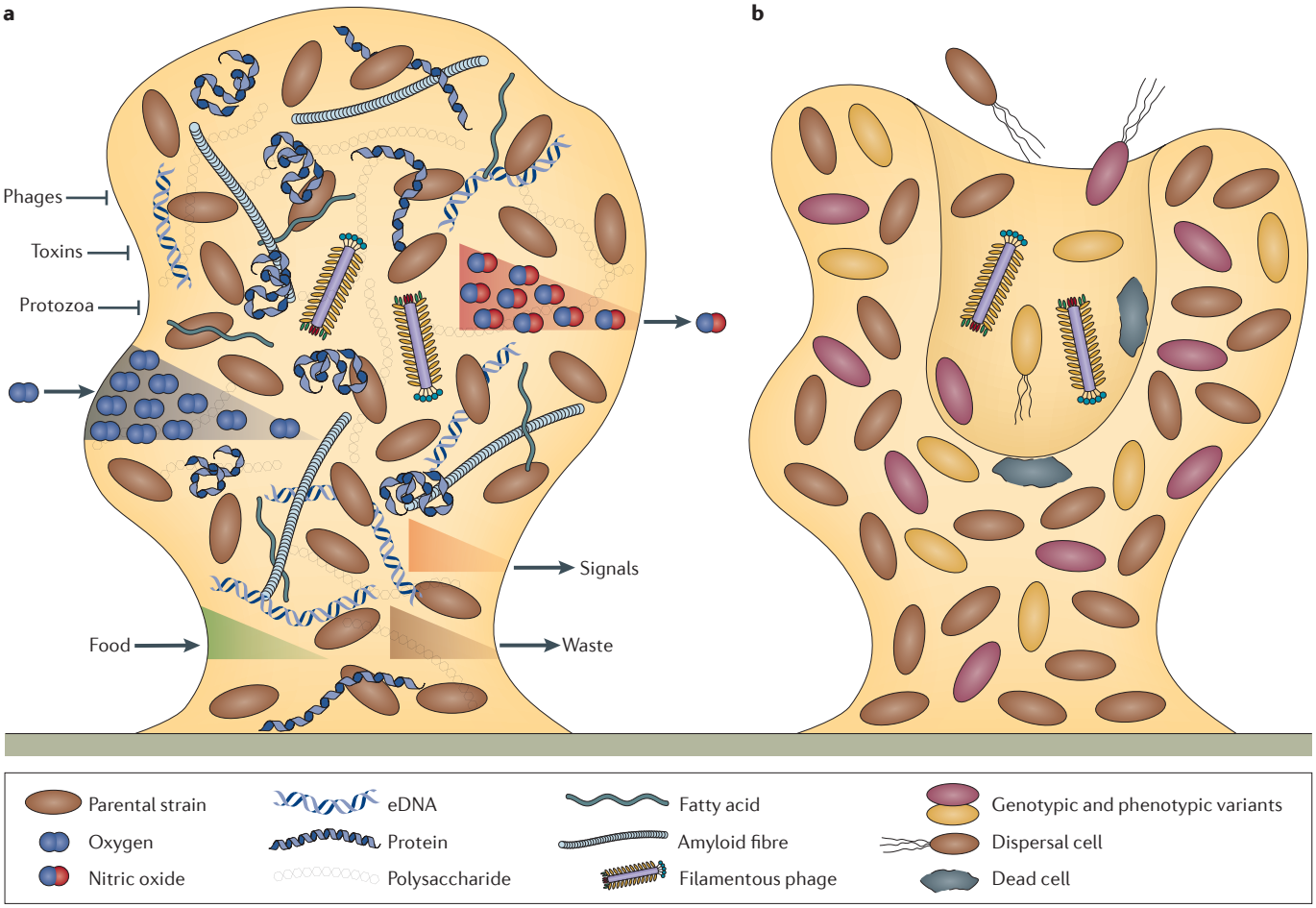


Figure 1 | **The complex structure of bacterial biofilms. a** | Microcolonies in the mature biofilm are characterized by an extracellular polymeric substances (EPS) matrix, composed of extracellular DNA (eDNA), polysaccharides, proteins, amyloid fibres and bacteriophages. The EPS matrix functions as a shield to protect the bacterial community or population from predators such as protozoa or lytic phages, as well as from chemical toxins (for example, biocides and antibiotics). The EPS matrix may help to sequester nutrients and, along with the underlying bacteria, is also responsible for the establishment of gradients (for example, oxygen and nutrients diffusing inwards, and waste products as well as signals such as nitric oxide diffusing outwards). **b** | At the time of dispersal, microcolonies undergo cell death and lysis along with active dispersal of motile bacteria to leave behind hollow colonies. This stage of development is also characterized by the appearance of superinfective bacteriophages as well as morphotypic and genetic variants in the biofilm effluent.

Dispersal
The movement of an individual organism away from the parent organism or population to a new niche.

Cell communication signals
Molecules that are produced and perceived by an organism. Signals are produced at a particular stage of growth under specific conditions or in response to changes in the environment. They accumulate extracellularly and are recognized by a dedicated receptor to induce a concerted response when a critical threshold has been reached. To be classed as cell communication, this response must extend beyond that which is required for metabolism or detoxification of the substance.

These specialized cells can colonize new surfaces to initiate the surface-associated phases of the life cycle. In contrast to passive dispersal, resulting from sloughing of cells and erosion from the biofilm, the production of specific dispersal cells at the final stage of the biofilm life cycle is an active and highly regulated response. The sequence of life cycle stages and the production of motile dispersal cells by otherwise sessile organisms bear striking similarities to certain developmental processes of many higher organisms, for which the ecology and evolution of life cycles and dispersal have long been central research themes.

In this Review, we focus on the biology of dispersal from bacterial biofilms. We describe the mechanisms that underlie the biofilm life cycle and dispersal, and conclude by exploring the broader evolutionary and ecological context for dispersal from microbial biofilms, including the comparative biology of life cycles and dispersal in bacterial biofilms and eukaryotes.

The life cycle of a biofilm

Biofilms develop through several stages. Here, we consider the start of the life cycle as dispersal from the mature biofilm, when planktonic cells are released, migrate to new surfaces and subsequently attach and mature into three-dimensional communities, including microcolonies. In the mature biofilm, cells are encased in an extracellular matrix composed of proteins, exopolysaccharides and extracellular DNA (eDNA)[6] (FIG. 1a). In addition to immobilizing the bacteria, the matrix is a scaffold that traps nutrients and various biologically active molecules, such as cell communication signals. The matrix may resemble an external digestion system, as it also accumulates enzymes that can degrade various matrix components as well as any nutrients or other substrates[6]; once degraded, the products are then in close proximity to the cells, facilitating uptake. Moreover, the matrix acts as a shield against toxins, antimicrobials and predators.

Gene expression, morphology, phenotype, and the stage of differentiation and development vary between the cells in a mature biofilm[7]. Indeed, the architecture of biofilms, such as mushroom- or tower-like structures, often derives from a division of labour between cells in the biofilm[8]. The heterogeneous nature of the mature biofilm may be a consequence of differential gene expression by cells across different regions in the biofilm structure, as a result of gradients of nutrients, electron acceptors and waste products, but may also be caused by mutations that lead to a diversity of genotypic and phenotypic variants[9] (FIG. 1b). Moreover, the complex interactions among the various species in multispecies biofilms also affect the structure and function of these communities, with antagonism (competition) and synergism (metabolic cooperation) being common themes[10]. The interactions between microorganisms associated with oral biofilms are relatively well understood[11], but for the majority of systems the specifics of these interspecies interactions are mostly unknown.

Genetic control of biofilm development. The observation that biofilms of many different bacterial species develop through similar stages suggests that biofilm formation is a genetically regulated process. For example, *Pseudomonas aeruginosa* displays stage-specific expression of particular genes, and mutants lacking these genes stall biofilm formation at specific stages[3]. Similarly, mutations in the *Serratia marcescens* genes *bsmA* and *bsmB*, which encode a putative two-component regulatory protein pair, stall filamentous biofilm formation at the cell cluster stage[1]. However, transcriptomic analyses of different stages of biofilm development across different bacterial species have generally failed to show a consistent correlation between these stages and the expression of specific sets of genes[5]. This may be due to the differential expression of genes with similar functions in diverse species, the physiological heterogeneity within the biofilm or differences in experimental systems.

A consistent theme in the regulation of biofilm formation and dispersal is the central role of the intracellular second messenger cyclic di-GMP (c-di-GMP), which was first identified in *Gluconacetobacter xylinus* as a regulator of cellulose synthesis[12]. The intracellular level of c-di-GMP regulates the transition from biofilm to planktonic phenotypes in response to various environmental cues and cell–cell signals in bacteria[13]. Although the genes encoding the diguanylyl cyclases (DGCs) that synthesize c-di-GMP and the phosphodiesterases (PDEs) that degrade it are redundant and their expression is not well understood in all cases, the outcome is the same: a decrease in the intracellular c-di-GMP level leads to dispersal (see below). Moreover, the correct timing of gene expression is essential for the development of the typical three-dimensional biofilm architecture in each of these systems[1,7].

Dispersal. Active dispersal from biofilms is typically (but not in all species) preceded by localized death and lysis of cells in the centre of mature biofilm structures[4,14]. Because of the heterogeneous nature of the cells in the mature biofilm, only a subpopulation of cells will undergo lysis. For some well-studied biofilm systems, the genetic regulators, the effectors and their modes of action for killing a subpopulation of cells are known[14–16]. These killed cells provide nutrients for the bacteria that will become the dispersal cells. The dispersal cells 'escape' by coordinated evacuation from break-up points[17], leading to the characteristic hollowing of biofilm microcolonies that is observed during the dispersal stage for many biofilms (FIG. 1b). In the dispersal cells, genes that regulate features of the sessile biofilm phenotype, such as exopolysaccharides and fimbriae, are downregulated, whereas genes that encode factors which are important for a motile lifestyle, including flagella and proteins involved in chemotaxis, are upregulated[18,19].

Cues, signals and regulators of dispersal

As many of the ecological and evolutionary drivers of dispersal are thought to be substantial changes or even slight variations in habitat or environmental quality, it is not surprising that there is a range of environmental cues that triggers dispersal from biofilms, including alterations in the availability of nutrients (such as carbon sources[20–23]), oxygen depletion[24,25], low levels of nitric oxide (NO)[18,26,27], changes in temperature[28] and high[29] or low[30,31] levels of iron (TABLE 1). In addition, there are several bacterially derived signals that induce dispersal (reviewed in REF. 32), including acyl-homoserine lactones[33,34], cell–cell autoinducing peptides[35], diffusible fatty acids[36,37] and D-amino acids[38] (TABLE 1). The fact that dispersal is induced by many different cues or signals potentially allows precise regulation of the attached and planktonic phenotypes in response to changes in environmental conditions.

Nutrients. Both increases and decreases in nutrient concentration have been correlated with biofilm dispersal. Biofilms of *Aeromonas hydrophila* and *P. aeruginosa* increase their detachment rates in response to nutrient depletion[39]; *P. aeruginosa* biofilms formed on a solid substratum[21] and those formed in suspension as aggregates[23] disperse in response to starvation. Furthermore, *Pseudomonas fluorescens* biofilms detach when dissolved organic carbon, glucose or nitrogen concentrations decrease[40,41], and biofilms of *Pseudomonas putida* rapidly disperse in response to carbon starvation[20].

In some instances the molecular mechanisms triggered by nutrient depletion have been described. Carbon starvation of *P. putida* results in a decrease in levels of the adhesin LapA, a major component of the extracellular matrix of the biofilm[42]; LapA is degraded by the protease LapG, which is derepressed following a decrease in levels of c-di-GMP in response to starvation.

By contrast, biofilms of *Acinetobacter* sp. str. GJ12 become more tightly packed when starved[43]. When nutrients are plentiful, cells within the biofilm spread across the surface, with some completely dispersing and reattaching to the surface when nutrient levels decrease. Similarly, *P. aeruginosa* biofilms disperse when carbon availability rapidly increases[22]; compared with the other biofilm cells, dispersal cells have higher expression of

Second messenger
An intracellular molecule (usually small and rapidly diffusible) that transmits information from a receptor to a target molecule; for example, cyclic AMP and cyclic di-GMP.

Chemotaxis
The movement of cells or organisms according to chemical concentration gradients in the environment, either towards or away from the stimulus.

Nitric oxide
(NO). A small, reactive gas and a universal signalling molecule in biological systems (as initially discovered in the 1970s, for its role in regulating vasodilation in mammals). In bacteria, NO is generated as a by-product of anaerobic metabolism or by NO synthases (NOSs).

Autoinducing peptides
Extracellular peptides, ranging from 5 to 34 amino acids in length, that are generated by cleavage from precursor peptides and then further post-transcriptionally modified. These peptides are used by Gram-positive bacteria as cell communication signals.

Table 1 | **Dispersal factors**

Inducers	Organism	Mechanisms	Refs
Signals			
AHLs	*Pseudomonas aeruginosa*	Quorum sensing-mediated induction of rhamnolipid synthesis	82
	Serratia marcescens	Unknown	34
	Rhodobacter sphaeroides	Dispersal of planktonic aggregates, possibly through regulation of EPS production	62
AIP and *agr*	*Staphylococcus aureus*	Regulation of serine and metalloproteinase production	35
DSF	*Xanthomonas campestris*	Induction of endo-β-(1,4)-mannosidase, and modulation of c-di-GMP levels	36, 68
	P. aeruginosa	Unknown	37
	Escherichia coli		
	Klebsiella pneumoniae		
	Proteus mirabilis		
	Streptococcus pyogenes		
	Bacillus subtilis		
	S. aureus		
	Candida albicans		
AI-2	*Vibrio cholerae*	Quorum sensing-mediated inhibition of EPS production	61
Physiological cues			
D-amino acids	*B. subtilis*	Release of amyloid fibres	38
	P. aeruginosa	Unknown	38
	S. aureus		
NO	*P. aeruginosa*	Modulation of c-di-GMP levels via BdlA and aerotaxis genes	18
	Staphylococcus epidermidis	Unknown	26
	E. coli		
	S. marcescens		
	Bacillus licheniformis		
	C. albicans		
	V. cholerae		
	*Legionella pneumophila**	Modulation of c-di-GMP levels via HNOX family binding of NO	48
	Nitrosomonas europaea	Regulation of motility and/or chemotaxis responses	49
Nutrients			
Increased nutrient levels	*P. aeruginosa*	Induction of motility genes and denitrification genes	22
	Acinetobacter sp. str. GJ12	Unknown	43
Carbon and nitrogen limitation	*P. aeruginosa*	Modulation of c-di-GMP levels	23
	Pseudomonas fluorescens	Possible modulation of cell hydrophobicity	40, 41
Carbon limitation	*Pseudomonas putida*	Modulation of c-di-GMP levels, and induction of LapG-mediated cleavage of the surface adhesin LapA	20, 42
Oxygen limitation	*Shewanella oneidensis*	Modulation of c-di-GMP levels by MxdB and the phosphodiesterase YhjH, and regulation of anaerobic metabolism by ArcA and Crp	25
	P. aeruginosa	Reduction in c-di-GMP levels via RdbA, a phosphodiesterase with a putative oxygen-sensing domain, leading to the induction of rhamnolipid production and motility, and to a decrease in EPS synthesis	24
Iron	*P. aeruginosa*	Reduction in biofilm formation (for high iron levels, via the *pqs* system, and for low iron levels, via the induction of rhamnolipid production)	29, 30
Effectors			
EPS-degrading enzymes	*P. fluorescens*	Cleavage and degradation of exopolysaccharide by exopolysaccharide lyase	73
	P. aeruginosa	Cleavage and degradation of alginate by alginate lyase	81
Chitinase	*Pseudoalteromonas* sp. S91	Degradation of chitin (by ChiA and ChiB)	74
Nuclease	*S. aureus*	Degradation of extracellular DNA (by a thermonuclease)	75
	P. aeruginosa	Degradation of extracellular DNA (by exogenously added DNase)	80
Dispersin	*Aggregatibacter actinomycetemcomitans*	Degradation of *N*-acetylglucosamine polymers (by DspB)	76

AHLs, acyl-homoserine lactones; *agr*, accessory gene regulator; AIP, autoinducing peptide; AI2, autoinducer 2; ArcA, aerobic respiration control protein A; c-di-GMP, cyclic di-GMP; Chi, chitinase; DSF, diffusible signal factor; DspB, dispersin B; EPS, extracellular polymeric substances; HNOX, haem nitric oxide- and/or oxygen-binding protein; NO, nitric oxide; *pqs*, *Pseudomonas* quinolone signal. *NO is presumed to be the signal.

fliC, the gene that encodes flagellin, and lower expression of *pilA*, the gene that encodes pilin (also known as fimbrial protein). The sensor regulator for this carbon-dependent dispersion in *P. aeruginosa* is BdlA[2], a chemotaxis regulator that is affected by c-di-GMP levels.

Oxygen. Dispersal can be induced by hypoxia, an environmental stress that is commonly experienced by cells in a biofilm. For example, *Shewanella oneidensis* rapidly disperses on oxygen depletion[25]: 80% of the *S. oneidensis* biofilm biomass disperses when the cells are deprived of a flow of oxygen for 15 minutes. The molecular mechanism underlying the dispersal response to oxygen deficiency is known in some cases. A mutation in the *P. aeruginosa* gene *rbdA* leads to a dispersal-deficient phenotype when the cells are deprived of oxygen[24]. RbdA is a phosphodiesterase that breaks down c-di-GMP, and thereby positively regulates motility and the production of rhamnolipids, both of which are connected to dispersal and negatively regulate the production of the exopolysaccharides that are needed for biofilm formation. During the early stages of biofilm formation, the activity of RbdA is suppressed by oxygen bound to its sensing domain[24]. This suppression is relieved when oxygen becomes depleted, resulting in the upregulation of genes involved in swarming, swimming and rhamnolipid synthesis, and the downregulation of genes required for the production of extracellular polymeric substances (EPS).

Cyclic di-GMP signalling. Recently, the ubiquitous intracellular nucleotide c-di-GMP has emerged as the central element of a signal transduction network that regulates the transition from biofilm to planktonic phenotypes in response to various environmental cues and cell–cell signals in bacteria[13]. In general, an increase in c-di-GMP levels facilitates the biofilm mode, whereas a decrease results in a switch to dispersal and the planktonic mode of existence (BOX 1). The cellular concentration of c-di-GMP is dynamically controlled by the opposing activities of multiple DGCs (which catalyze the formation of c-di-GMP from two molecules of GTP) and PDEs (which degrade it to 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or two GMPs). A wide variety of DGC and PDE sensor domains have been identified, and these domains integrate the environmental signals into the production or breakdown of c-di-GMP. In turn, c-di-GMP binds to specific domains of regulatory molecules that control the expression of genes relevant to the biofilm or planktonic mode of life (BOX 1). This signal transduction system is strongly dependent on differentiation events that occur during biofilm development and that may induce or repress the expression of DGC- and PDE-encoding genes, as well as that of the effectors targeted by c-di-GMP.

One of the interesting paradoxes of signal transduction by c-di-GMP is the redundancy of DGCs and PDEs. This overlap in function allows for the integration of multiple cues through a global intracellular pool of the second messenger, but it also appears that distinct phenotypes can be controlled by specific DGCs or PDEs. One plausible explanation for the specificity of the c-di-GMP generated or hydrolysed by any single enzyme is the possible existence of subcellular pools of c-di-GMP in close proximity to target receptors[44,45]. Furthermore, several studies suggest that, in some instances, c-di-GMP functions within local complexes formed between the DGC or PDE and a cognate adaptor that can bind c-di-GMP and direct a specific response before the signalling molecule can diffuse throughout the cell[13,46].

Nitric oxide signalling. Low, non-toxic concentrations of NO, derived endogenously or from external sources, also induce biofilm dispersal[18]. Low NO concentrations regulate a widely conserved genetic pathway that controls the conversion between the planktonic and surface-attached lifestyles through the action of c-di-GMP[47]. NO induces the dispersal of monospecies biofilms that contain *P. aeruginosa, Escherichia coli*, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus licheniformis*, *S. marcescens*[26], *Bacillus subtilis*, *Legionella pneumophila*[48], *Nitrosomonas europaea*[49] or *Neisseria gonorrhoeae*[50] and also causes the dispersal of multispecies microbial biofilms that are formed in drinking water and recycled-water systems[26]. One consequence of NO-mediated dispersal is that, owing to the reversal of the genetic programme that drives biofilm development, both the biofilm and the dispersal cells lose their high level of resistance to antimicrobials[18]. This effect may also be linked to a general increase in metabolic activity of the biofilm as a consequence of NO exposure, a response that may prepare the bacteria for a more active, motile lifestyle as free-living cells[47].

In *P. aeruginosa*, the chemotaxis transducer protein BdlA forms part of a direct link between NO levels and the activity of c-di-GMP-targeting PDEs[47]. NO can reversibly bind to the haem of the PER–ARNT–SIM (PAS) domain present in some DCGs and PDEs, stimulating PDE activity and therefore leading to dispersal[51]. Interestingly, a BdlA orthologue, AerC, acts as an energy sensor via the FAD cofactor associated with its PAS domain, suggesting that BdlA mediates NO-dependent biofilm dispersal by acting as an energy sensor; this hypothesis is consistent with the observation that biofilm dispersal is a metabolism-dependent process[52].

From an evolutionary perspective, the role of NO in broadly controlling biofilm dispersal has striking similarities with the role of NO in the regulation of other bacterial lifestyles and of eukaryotic systems. For example, NO also regulates seed dormancy in plants[53] and dormancy in bacteria[54]. Indeed, it has been suggested that NO is a conserved messenger of life history transitions across multiple domains of life[55].

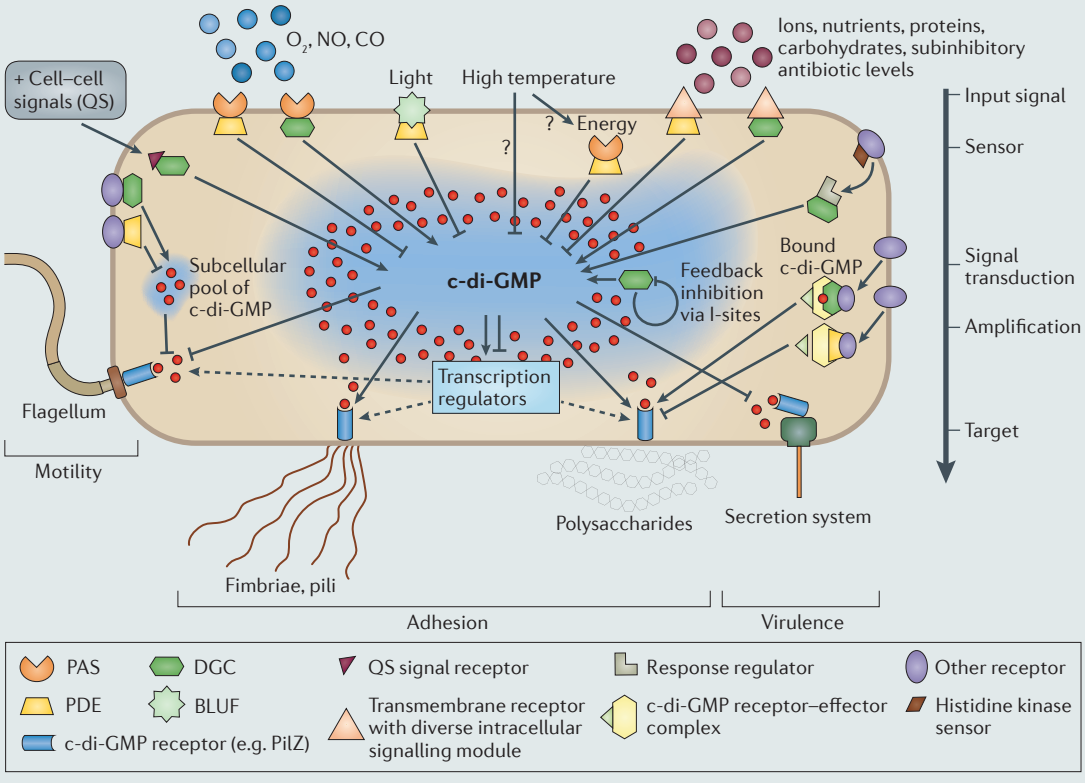
Quorum sensing. Although quorum sensing-based extracellular cell–cell signalling systems have been primarily linked to biofilm formation[56], quorum sensing also has an important role in dispersal from biofilms. In *S. aureus*, the accessory gene regulator (*agr*) quorum sensing system has variable effects on biofilm development, depending on the medium and the flow conditions (reviewed in REF. 57). This system is repressed in biofilms but is actively expressed in the dispersal population[58]. Artificially inducing *agr* in

Sensor regulator
A protein that receives and responds to information about changes in the environment, either by binding second messengers or through phosphorylation, to induce transcriptional changes.

Box 1 | **Cyclic di-GMP and the regulation of dispersal**

Various cyclic di-GMP (c-di-GMP) receptors have been linked to specific physiological processes, ranging from polysaccharide biosynthesis, to direct regulation of gene expression, to motility. These processes indicate that c-di-GMP plays an important part in the regulation of dispersal. The first cellular c-di-GMP receptors identified, PilZ domains, are found in regulatory proteins in a wide variety of microorganisms[13,110]. These regulatory proteins include Alg44, which positively controls alginate synthesis in *Pseudomonas aeruginosa*; PilZ, which downregulates twitching motility in *P. aeruginosa*; diguanylate receptor protein (DgrA), which controls flagellum synthesis in *Caulobacter crescentus*[111]; and BcsA, which regulates cellulose synthesis in *Gluconacetobacter xylinus* and other Gram-negative bacteria[110]. Furthermore, c-di-GMP inhibits *P. aeruginosa* FleQ, a transcription factor that activates the synthesis of flagella and represses the production of the extracellular polymeric substances (EPS) component Pel[112]. Pel is also regulated by pectate lyase E (PelE), which contains a PelD domain receptor of c-di-GMP[113]. Interestingly, the *Escherichia coli* c-di-GMP-binding protein BdcA (also known as YjgI) was recently engineered to increase its affinity for c-di-GMP and was then used to scavenge intracellular c-di-GMP to trigger biofilm dispersal[114], further indicating that c-di-GMP is important for this process.

The nucleotide c-di-GMP therefore has a central role in the regulation of biofilm dispersal (see the figure). Intracellular c-di-GMP levels are dynamically controlled by the antagonistic activities of diguanylyl cyclases (DGCs) and phosphodiesterases (PDEs), which are often found in multiple copies associated with amino-terminal sensor domains, such as PER–ARNT–SIM (PAS) for sensing gaseous ligands, and blue light using flavin (BLUF) for sensing light. On sensing cell–cell signals and environmental cues, such as oxygen (O_2), nitric oxide (NO), nutrient levels, temperature or light, DGCs or PDEs are stimulated. In turn, c-di-GMP interacts with, or is degraded and so prevented from interacting with, specific receptors that coordinately activate a cascade of proteins at the transcriptional, translational or post-translational levels to induce changes in the cell surface that promote either biofilm formation (high levels of c-di-GMP) or virulence and dispersal (low levels of c-di-GMP). The regulatory pathways may operate either via a global intracellular pool of c-di-GMP (or localized subcellular pools of c-di-GMP that have been hypothesized to exist) or via direct interactions of the DGCs or PDEs with potential cognate adaptors based on adaptor binding to c-di-GMP.



an *S. aureus* biofilm or adding the cognate autoinducing peptide causes dispersal of cells from the biofilm[35]. This *agr*-mediated dispersal requires the induction of a protease that is probably involved in degradation of the EPS matrix. Further, RNA III-inhibiting peptide (RIP), which negatively regulates the quorum sensing response, can reduce *S. aureus* biofilms *in vivo*[59]. Such compounds are now undergoing clinical trials to control biofilm infections associated with chronic wounds[60] (BOX 2).

In Gram-negative bacteria, acyl-homoserine lactones regulate attachment, biofilm development and biofilm dispersal. For example, *S. marcescens* quorum sensing mutants are impaired in dispersal[34], and genes involved in EPS synthesis are repressed by quorum sensing in *V. cholerae*, facilitating the release of dispersal cells at high cell densities[61]. In suspended biofilms (flocs) of *Rhodobacter sphaeroides*, the induction of quorum sensing leads to the dispersal of aggregates. This

Box 2 | **Applications of dispersal research**

One of the clear motivations for studying the biofilm 'life cycle' is to identify and develop novel targets to control biofilm development, as this control has applications for industry and medicine (reviewed in REF. 115). The manipulation of biofilm development does not require killing of the biofilm, but rather modification of the genetic switches that interfere with the life cycle. In this regard, the mechanisms that drive dispersal from biofilms would be suited for such applications, because dispersal is associated not only with removal of the biofilm but also with reversal of the gene expression programme, whereby the dispersal population once again becomes sensitive to biocides and antibiotics.

The broad spectrum of dispersal cues, signals, regulatory systems and effectors provides multiple opportunities to induce biofilm dispersal and ameliorate biofilm-associated problems. For example, the enzyme dispersin B can be used to disrupt biofilms of *Staphylococcus* spp.[116]. However, the use of dispersin B or other enzymes that degrade extracellular polymeric substances (EPS) may be limited to industrial applications owing to the potential immunogenic properties of such biomolecules.

Another approach has been to interfere with the extracellular signalling systems that drive biofilm development and stability. This can be achieved by either degrading the signal (called quorum quenching) or using signal analogues that outcompete the native signal for its cognate receptor. Interestingly, both approaches have solutions found in nature. For example, there are many reports of aquatic and terrestrial plants that produce secondary metabolites which can bind to the signal receptor and hence block the signal-mediated regulatory cascade that drives biofilm development.

In addition, it has recently been shown that reactive oxygen and nitrogen species, specifically NO, can induce biofilm dispersal via regulation of the intracellular concentrations of cyclic di-GMP[18]. Based on its ability to control biofilm stability and phenotypes in a range of bacteria, NO may represent a promising therapeutic for the control of biofilm-related infections. Because NO is an important intracellular signal in humans, a number of compounds, called NO donors, have been developed to release NO in the body in order to modify gene expression. Such compounds are active in dispersing single-species biofilms as well multispecies biofilms from engineered systems.

The use of other dispersal signals in an applied context may also be possible. For example, exogenous addition of the unsaturated fatty acid cis-2-decenoic acid induces dispersion of biofilms for a number of Gram-negative and Gram-positive bacterial species, as well as for *Candida albicans*[37].

presumably ensures that cells are not masked from their primary energy source (light) when growing in suspension[62]. However, although the quorum sensing regulon has been characterized in several bacterial species and represents as much as 10% of the genome in some species, its role in dispersal is still little studied, and only a few quorum sensing-regulated dispersal effectors, such as rhamnolipids[63] and detachases[64], have been identified.

Other cell–cell signals. Many other signals can affect biofilm properties and processes such as dispersal. In the plant pathogen *Xanthomonas campestris*, a signalling system based on diffusible signal factor (DSF; unsaturated fatty acid *cis*-11-methyl-2-dodecenoic acid) regulates biofilm formation and dispersal in addition to playing a part in virulence[36]. Mutants defective in DSF production have an aggregative phenotype, and addition of exogenous DSF induces dispersal of aggregates[65]. Interestingly, the response regulator of this signalling system is a PDE[66], so the presence of the DSF signal results in a decrease in c-di-GMP and leads to the induction of planktonic phenotypes. Moreover, this quorum sensing system is also linked to cyclic AMP signalling and, thus, to the nutritional status of the cell[67]. DSF appears to be one member of an emerging family of fatty acid-derived signals that modulate dispersal[68]. For example, *P. aeruginosa* produces *cis*-2-decenoic acid, an unsaturated fatty acid that is structurally related to DSF[37]; *cis*-2-decenoic acid inhibits biofilm formation and induces the dispersion of established biofilms[37].

Many studies have demonstrated that nutrient availability affects biofilm development. For example, glucose starvation induces dispersal in *P. aeruginosa* biofilms[21,23]; this dispersal is energy dependent and also requires the signal metabolite cAMP, as a *P. aeruginosa* mutant deficient in the adenylyl cyclase CyaA does not disperse[69]. Furthermore, inhibition of cAMP production in biofilms by the addition of an adenyl cyclase inhibitor, atropine, prevents dispersal. It is interesting to note that starvation-induced dispersal does not require NO-mediated signalling, suggesting that there are multiple, independent pathways that induce dispersal[69].

Amino acid 'signals' also modulate biofilm dispersal[38], including the D-amino acids (D-leucine, D-methionine, D-tyrosine and D-tryptophan) in *B. subtilis*. In *V. cholerae*, D-amino acid concentrations are low in exponential phase but begin to accumulate in early stationary phase[70]. Thus, the production of these amino acid isomers may be induced in stationary phase as an adaptation for remodelling the cell envelope in non-growing cells. These D-amino acids seem to be incorporated into the bacterial cell wall, causing the release of amyloid fibres that are anchored to the cell wall and are a major protein component of the biofilm matrix[71]. Exogenous addition of a mixture of D-amino acids prevents biofilm formation in *S. aureus* and *P. aeruginosa*, and these amino acids have been proposed to be a widespread signal for biofilm dispersal[38].

Effectors of dispersal

To disperse, bacteria encased in a biofilm must be able to 'break their biofilm bonds' (REF. 72). To do so, bacteria have developed a number of effectors, such as enzymes, surfactants and bacteriophages, that will degrade the biofilm matrix, eDNA, polysaccharides and proteins, leading to dispersal (TABLE 1). These effectors mediate the extensive structural and metabolic changes that a biofilm undergoes during the production and release of dispersal cells, including the killing of bacteria in the microcolony centre and the formation of a hollow microcolony as bacteria evacuate the biofilm to disperse for new habitats[14]. This process may also be associated with the substantial loss of biofilm biomass more generally.

Enzymes and surfactants. A key aspect of dispersal-related changes in the biofilm is the partial breakdown of the matrix, allowing release of the dispersal cells. The production of dispersal cells and the release mechanisms should be closely linked within the biofilm, both spatially and temporally, and initial evidence suggests that directed dissolution of the matrix occurs at specific sites from which dispersal cells are released[17]. The effectors of matrix dissolution and cell release often have enzymatic activity and include polysaccharide-degrading enzymes[73], chitinases[74], proteases[35,42] and nucleases[75].

Response regulator
The phosphorylation-dependent modulator of a two-component phosphorelay system. The partner sensor protein responds to environmental stimuli to modulate the phosphorylation status of the regulator, and the resultant phosphorylation cascade drives the response through differential expression of target genes.

One such dispersal cell-releasing enzyme is dispersin B, an endogenous poly-*N*-acetylglucosaminidase capable of degrading matrix polysaccharides[76]. This enzyme was identified in a transposon screen of the non-motile species *Aggregatibacter actinomycetemcomitans* for genes that are essential for dispersal[77]. Dispersin B was later found to induce biofilm dispersal in a range of bacterial species, including *Staphylococcus epidermidis*, suggesting a conserved mechanism for dispersal[77,78] from biofilms that contain poly-*N*-acetylglucosamine[79].

Several additional enzymes have been linked to biofilm dispersal. The *agr* system of *S. aureus* controls the expression of an extracellular protease that degrades proteins such as adhesins and EPS and thus allows dispersal[35]. The development and stability of *S. aureus* biofilms also relies on eDNA secretion. *S. aureus* not only controls the release of eDNA into the biofilm matrix, but also produces enzymes that degrade the eDNA and may therefore play an important part in biofilm dispersal[75]. Similarly, biofilms of *P. aeruginosa* have considerable amounts of eDNA in the biofilm matrix, and exogenously added DNase disperses biofilms in this species[80]. Alginate also has an important role in *P. aeruginosa* biofilm persistence and, consequently, in diseases such as lung infections. Increased expression of alginate lyase leads to biofilm detachment[81].

In addition to enzymatic activity, amphipathic molecules reduce surface tension to facilitate detachment and dispersal. For example, addition of exogenous rhamnolipids triggers dispersal in *P. aeruginosa*[82], and rhamnolipid production has been linked to cell death and microcolony hollowing[63]. Moreover, putisolvins (biosurfactants) produced by *P. putida* during entry into stationary growth phase induce biofilm detachment[83]. By contrast, rhamnolipids produced by *S. marcescens* str. MG1, which are regulated by quorum sensing and are important for swarming motility, do not appear to play a part in dispersal of filamentous biofilms[34].

Bacteriophages and dispersal. Bacteriophages are another major class of dispersal effectors. Approximately 60–70% of all bacterial genomes surveyed contain prophage sequences[84]. The traditional view of host–phage interaction is that phages primarily cause lysis or mediate horizontal gene transfer (HGT). However, recent results suggest that bacteriophages have important roles in biofilm development, particularly in the dispersal phases, by inducing cell death and providing enzymes that can help break down the biofilm matrix. For example, cell death and dispersal in *E. coli* biofilms are linked to the activity of bacteriophages D1P12 and CP4-57 (REF. 85). Bacteriophages also affect multispecies biofilms that contain *P. fluorescens* and *Staphylococcus lentus*, in which they can induce dispersal of the non-target *S. lentus* cells[86].

P. aeruginosa provides one of the best studied links between phages and cell death during dispersal. Prophage genes are strongly induced during the later stages of *P. aeruginosa* biofilm formation[87], and bacteriophages are readily isolated in the biofilm effluent produced by the laboratory strain *P. aeruginosa* str. PAO1 (REF. 14) and from biofilms of clinical isolates[88] (FIG. 1b). Indeed, in the absence of the bacteriophage Pf4, cell death of *P. aeruginosa* str. PAO1 is not induced during biofilm development, and the biofilms are less stable than those formed by the wild-type strain[16]. Pf4-driven dispersal is associated with a switch from the lysogenic to the superinfective phage form; cell death is induced when superinfective phages are added to biofilms of mutant cells that lack Pf4 (REF. 16). Further evidence for the role of superinfection comes from the role of bacteriophages in dispersal of *P. aeruginosa* str. PA14. In this strain, lysogeny inhibits biofilm formation and swarming, but deletion of the CRISPR (clustered regularly interspaced short palindromic repeats) elements, which are host defence systems against phages, restores biofilm formation and swarming[89].

In some cases, the specific mechanisms by which bacteriophages mediate biofilm dispersal are known. Some bacteriophages incorporate active EPS-degrading enzymes into the phage particle, presumably to allow it to access cell surface receptors[90,91]. The combined effects of EPS degradation and lytic activity of the bacteriophage thus facilitate cell dispersal from the biofilm. In an interesting adaptation of this natural activity, when bacteriophage T7 was engineered to produce dispersin B, biofilm biomass was reduced by 4.5 orders of magnitude compared with the mass of biofilms containing non-modified T7 (REF. 92).

In addition to carrying enzymes, bacteriophages may drive biofilm cell death and dispersal in other ways. In *Treponema denticola* biofilms, an increase in the expression of genes with homology to a toxin–antitoxin (TA) system correlates with dissolution of the biofilm and release of dispersal cells[93]. In *E. coli*, the induction of genes with homology to certain TA genes involved in bacteriophage-mediated lysis also leads to biofilm cell death and dispersal[85]. In this case, the response regulator Hha negatively regulates the expression of genes that encode rare tRNAs, and this activates the toxin proteins in the TA pairs through the activity of proteases. Hha induction may occur in response to nutrient limitation or the presence of uncharged tRNAs, and cellular physiology and growth status would therefore be intimately linked to cell death via this pathway.

Ecological and evolutionary aspects

The study of biofilm life histories and dispersal is in its infancy, but there is a long history of such studies for eukaryotes (BOX 3). Analogies between many eukaryotic life histories and those of biofilms appear to be strong, and key features — the alternation of relatively sessile phases with mobile dispersal phases, the generation of variation associated with dispersal, and programmed production of the dispersal phase — are common to biofilms and the life cycle of many eukaryotes. Here, drawing further on the literature about the ecology and evolution of eukaryote dispersal (for recent reviews, see REFS 94–96), we highlight several possible explanations for the benefits of dispersal from biofilms, including

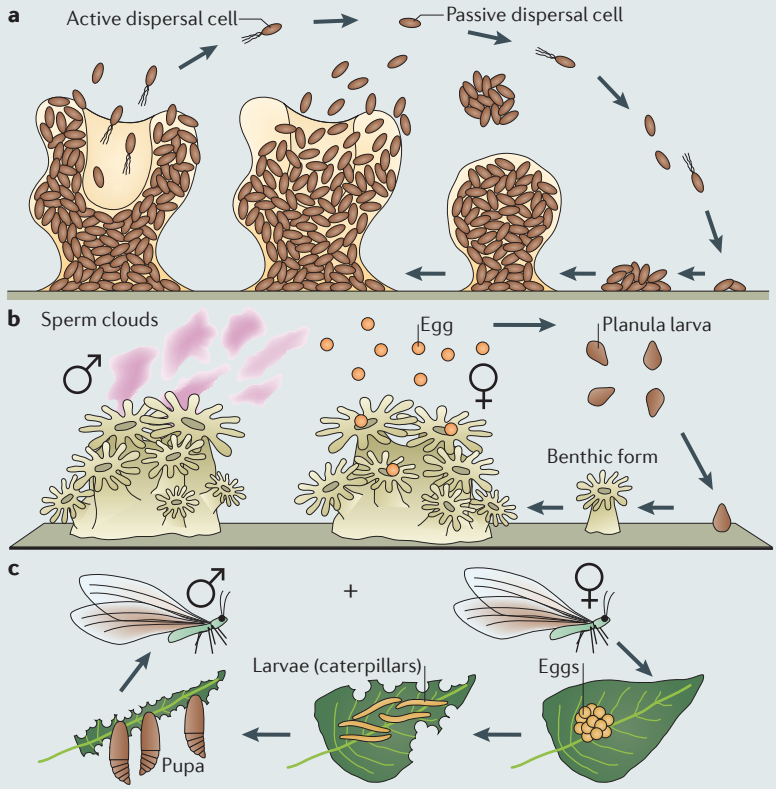
Lysogenic
Pertaining to a bacteriophage genome: being incorporated into the chromosome of the host bacterium, resulting in transmission to daughter bacterial cells on cell division. Lysogenic phages are referred to as prophages.

Box 3 | **Life cycles and dispersal in biofilms and eukaryotes**

The 'life cycle' of biofilms (see the figure, part **a**) bears striking similarities to the life cycles of many higher organisms for which the ecology and evolution of life histories and dispersal have long been central research themes[105,117,118]. Perhaps the closest analogies to biofilms are colonial marine invertebrates such as corals and bryozoans[119,120] (see the figure, part **b**). The life cycle of such organisms starts when a planktonic larva settles onto the sea bed (or epibiotically onto another benthic organism) and metamorphoses into the benthic form. Through asexual replication, this single coral polyp becomes a colony, which becomes reproductively mature and produces gametes (sperm and eggs), often in response to environmental signals[121]. These gametes are fertilized either in the plankton or within the maternal colony, and the resultant embryos develop into larvae, which restart the life cycle. Importantly, for most such organisms larvae are the result of sexual reproduction, and thus the production of dispersive larvae results in offspring that have a greater range of genetic variation than parental colonies, analogous to the enhanced variability observed in bacterial dispersal cells.

Such 'biphasic' life cycles, in which there are two distinct and separate life history stages, are extremely common in marine animals[105,118,122,123], and alternating between a dispersive phase and a sessile or at least less motile phase is widespread among eukaryotes. For example, holometabolous insects have less motile larvae (such as caterpillars) that alternate with dispersive adults (such as butterflies) (see the figure, part **c**). One consequence of such biphasic life histories is that larvae and adults use different resources and are subject to different habitat cues, as are planktonic dispersal cells and benthic biofilms.

In eukaryotes, as in bacterial biofilms, the production of dispersive propagules and any sequential life history stages are programmed, with predictable timing. In eukaryotes, this programming arises as a consequence of reproductive and developmental timing and periodicity, including breeding and maturation cycles. The biofilm life cycle is also characterized by an orderly and predictable transition through a series of stages, each with a set of genes that is uniquely expressed during that stage, leading to particular phenotypes and culminating in a dispersal event. The biofilm life cycle consists of active dispersal (triggered by signals such as starvation, rhamnolipids, phages and cell death) and passive dispersal (as occurs through streamers, rolling dispersal and sloughing), both of which result in the release of planktonic cells that undergo attachment and biofilm maturation to form microcolonies. As for eukaryotic life cycles, environmental cues (for example, low nutrient conditions[39]) can induce transitions between, or initiation of, these different stages in the biofilm life cycle.



avoidance or minimization of both competition and the resultant depletion of resources from local environments, exploitation of particular characteristics of the habitat (including its quality and variation), the generation or maintenance of genetic variation, and bet hedging in variable environments.

Depletion of resources and competition among cells in the local environment, with a subsequent decrease in habitat quality, is the most frequently cited explanation for the evolution of the dispersal phase in biofilms[22,23,25]. It is an intuitively attractive explanation, as presumably without the ability to disperse, the biofilm becomes a dead-end for the bacteria. Indeed, the regulated detachment of cells from a surface after the depletion of easily utilizable carbon substrates is part of the life cycle of *Acinetobacter calcoaceticus*[97]. More generally, microarray studies have shown that cells in biofilms appear to be in stationary phase[98,99], indicating that there is a resource limitation, although it is worth noting that such studies are typically laboratory based and carried out in static and undisturbed conditions.

Stress as a result of habitat decay can be a strong initiator of dispersal in biofilms (although in some instances it can also result in increased biofilm formation)[24,25,28,74,100,101]. Stress can occur as a result of shifts in conditions, such as the accumulation of reactive oxygen and nitrogen species, or attack by protozoan predators and bacteriophages (the main causes of mortality for bacteria). Endogenous oxygen and nitrogen molecules derived from cellular metabolism can also act as initiators of biofilm dispersal, and bacteria have developed regulatory systems to drive differential gene expression in response to these changing conditions[18,25]. An example of such a response is the coordination of cellular regulation through intracellular levels of c-di-GMP. Similarly, defence against natural predators such as protozoa and bacteriophages appears to be highly regulated, as different responses occur at different stages of the biofilm life cycle[102]. Dispersal from mature biofilms may also be triggered by specific responses to signals derived from protozoan predators (D.M., unpublished observations).

Many studies of the advantages of dispersal in biofilms do not focus on the benefits of dispersal per se, but rather argue for the advantages of the associated generation of variation. This link between dispersal and the generation or maintenance of genetic variation is also commonly mentioned in the literature about eukaryote dispersal, as dispersal events are typically associated with the generation of variation through sexual reproduction in eukaryotes. Moreover, one frequently proposed advantage of dispersal in eukaryotes is as a means to avoid inbreeding[94] and the consequent decrease in variation and, usually, fitness. Inbreeding has no easy direct analogy in asexually reproducing biofilm bacteria; although the frequency of HGT among biofilm bacteria is often high[103], the consequences of this genetic exchange are not entirely clear. Depending on the rate of exchange, such gene transfer could increase or decrease genetic variation among cells in the biofilm. The situation is further complicated by the fact that HGT can

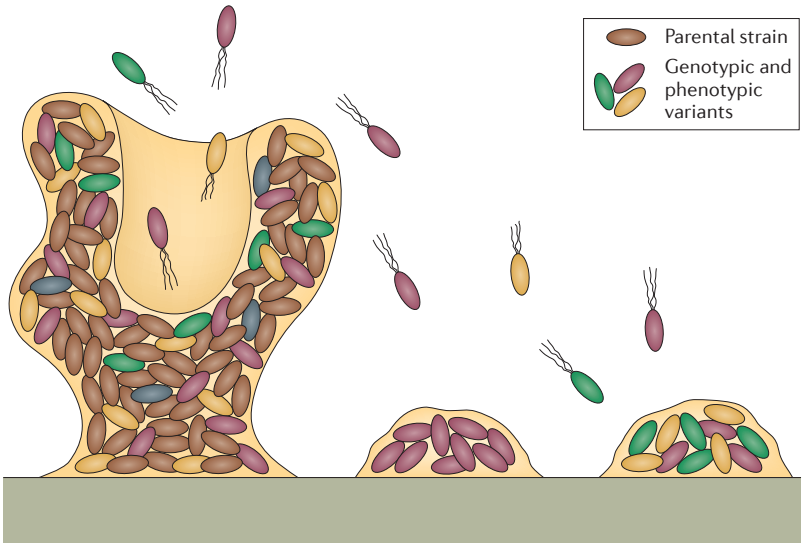


Figure 2 | **Active biofilm dispersal and variant formation.** Active dispersal of the parental strain and genetic variants, represented by different cell types, leads to subsequent attachment and colonization that can be initiated by either single variants, to generate clonal biofilms, or multiple variants, to form mixed-variant biofilms.

occur both within and between bacterial species, and interspecies exchange is difficult to align with standard models for the selective advantage of intraspecies genetic variation.

Models of bet hedging presume that a major advantage of dispersal is the ability to colonize a greater range of habitat types than just the parental habitat[95]. This greater breadth of colonization should be facilitated by the production of a diversity of dispersal cell types that can then access a wider range of habitat types or resources, an argument that features strongly in the literature about variable larval types in marine invertebrates[104,105]. Dispersal variants from at least some species of bacterial biofilms can colonize different and/or more environments than the bacteria that initiated the biofilm, with significant outcomes for the success of those bacteria in the environment (FIG. 2). For example, morphotypic variants from *P. fluorescens* colonize different parts of the root to wild-type bacteria[106], supporting the notion that differentiation of dispersal cells is important for niche expansion. The generation of variants during dispersal is common to other bacterial species as well. For example, biofilm dispersal variants have been identified in *P. aeruginosa*[88,107], *Pseudoalteromonas tunicata*[15] and *S. marcescens*[108], and these variants often differ in traits that may facilitate colonization in general or colonization of a greater range of habitats. For example, dispersal variants of *P. tunicata* vary in their attachment and biofilm formation profiles[15].

The variation generated by the production of dispersal cells may also convey benefits to the mature biofilm. *S. marcescens* str. MG1 generates different colony variants during biofilm development and dispersal, and these variants differ in colonization traits such as motility, expression of virulence factors and biofilm development[108]. Biofilms formed from a mixture of wild-type cells and several variants are also more resistant to predation by protozoa than are monovariant or wild-type biofilms[109]. By contrast, these mixed-variant populations are not protected in the planktonic phase, suggesting that biofilms facilitate resource partitioning or cooperation within the population. Thus, the formation of biofilms consisting of mixed variants through the generation of dispersal cells may benefit the biofilm as well as increase the habitats that can be colonized by these cells.

Two final aspects of the literature on dispersal of eukaryotes suggest that biofilms will be particularly useful for understanding dispersal more generally. The first aspect is the increasing recognition that the interplay between environmental conditions and intrinsic characteristics of organisms is an important driver of dispersal, as conceptualized in the 'condition-dependent' versus 'phenotype-dependent' model[96]. The roles of habitat and intrinsic characteristics in dispersal are readily experimentally amenable for bacteria and biofilms owing to our ability to experimentally vary specific bacterial characteristics using molecular tools and to readily vary habitat quality, persistence, and so on. The second aspect is that much of the experimental and empirical literature on dispersal by eukaryotes necessarily focuses on proximate (that is, ecological) causes and consequences of dispersal. Because of the short generation times and the accessible genetics of bacteria, experimental studies of the ultimate (or evolutionary) factors driving dispersal are also likely to be more achievable for biofilms than for most eukaryotes.

Conclusions and future directions

It is now increasingly clear that the programmed generation of dispersal cells is a crucial component of the life cycle of biofilms for many species of bacteria. The mechanisms by which these dispersal cells are produced are diverse and are affected by numerous factors, both intrinsic and extrinsic to the biofilm. As is the case with many other biofilm-related phenomena, both intercellular and intracellular signalling appear to play crucial parts in the production and regulation of dispersal cells. Our increasing understanding of the regulation of dispersal cell production, including the role of signals, offers promise for novel approaches to the control of bacteria in industrial, environmental and medical applications.

Looking to the future, an increased understanding of the molecular details behind dispersal cell production will probably be further enhanced by advances in genomic and imaging tools. However, given the seemingly close analogies between the life cycles of biofilms and of eukaryotes with 'biphasic' life histories, we suggest that the greatest conceptual advances will result from the integration of ecological and evolutionary theory with a mechanistic understanding of biofilm dispersal. We suggest that such a framework will guide both the design and interpretation of experiments in ways that are not typically considered within the more restricted disciplinary context of traditional biofilm biology.

Bet hedging
An evolutionary response to variable environments. In the context of dispersal, it is predicted to manifest in a number of ways, including the production of different types of dispersal cells to maximize colonization of different habitats, and spreading dispersal in time to accommodate temporally varying habitats.

Colonial
Of an organism: able to form replicate, more or less identical units ('modules') via asexual means; these units then often connect physically and physiologically to form a colony. Monospecies biofilms are colonial (or modular, or clonal) in this sense.

Holometabolous
Pertaining to an insect: with a life cycle in which there is a larval phase that is morphologically and ecologically distinct from the adult phase and which must undergo 'complete metamorphosis' via a pupal phase before becoming an adult. Examples include butterflies and true flies.

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