Physiological heterogeneity in biofilms

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Abstract | Biofilms contain bacterial cells that are in a wide range of physiological states. Within a biofilm population, cells with diverse genotypes and phenotypes that express distinct metabolic pathways, stress responses and other specific biological activities are juxtaposed. The mechanisms that contribute to this genetic and physiological heterogeneity include microscale chemical gradients, adaptation to local environmental conditions, stochastic gene expression and the genotypic variation that occurs through mutation and selection. Here, we discuss the processes that generate chemical gradients in biofilms, the genetic and physiological responses of the bacteria as they adapt to these gradients and the techniques that can be used to visualize and measure the microscale physiological heterogeneities of bacteria in biofilms.

Extracellular polymeric substance
A polymer, such as a polysaccharide, protein or nucleic acid, that is secreted by bacteria and forms a hydrated gel-like slime. Extracellular polymeric substances hold the biofilm together, and might serve other functions, such as nutrient trapping and protection from antimicrobial challenges.

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doi:10.1038/nrmicro1838
Published online
11 February 2008

It is well established that bacteria growing in the surface-associated communities that are known as biofilms are physiologically distinct from bacteria growing in a free-swimming planktonic state[1]. Biofilms are typically characterized by dense, highly hydrated clusters of bacterial cells. These cells secrete extracellular polymeric substances that hold the cell aggregates together. In some cases, clusters of cells are separated by channels through which fluid can move. As the bacterial cells adapt to growth in these hydrated surface-associated communities, they express phenotypic traits that are often distinct from those that are expressed during planktonic growth. These phenotypic differences are manifested in various ways, depending on the species of bacteria. For example, biofilm growth of pathogenic bacteria on tissues or indwelling devices often results in infections that have increased tolerance to antimicrobials[2,3] and the host immune response[4,5]. In an ecological context, the growth of bacteria on surfaces in the environment might influence the rate of carbon and inorganic nutrient cycling[1]. In bioreactors, biofilm growth affects reaction rates, including the biodegradation of toxic chemicals and production of chemicals during biomanufacturing processes[6,7]. Elucidating the physiologies of biofilm-associated bacteria is necessary for our understanding of infection, ecological processes and bioreactor design, as well as other processes that are mediated by microorganisms. Consequently, many studies have focused on characterizing the physiological states of bacteria that are growing in biofilms and comparing the physiologies involved with those of cells that are growing planktonically.

Early research demonstrated the physiological adaptations that certain bacteria undergo following attachment to, and growth on, surfaces and the differences in biofilm-associated bacteria compared with planktonic cells; these included the production of extracellular organelles, such as pili and flagella[8,9]. Some bacteria altered polysaccharide production[10] and even cell morphology[11]. Advances in global transcriptomic[12] and proteomic profiling of bacteria[13,14], as well as high-throughput screens to identify the genes that are required for biofilm formation[15], have revealed many other physiological differences — in addition to changes in the production of extracellular polysaccharides and organelles — that occur in bacteria after they sense growth associated with a surface and respond to the presence of the biofilm community. For example, Sauer and co-workers[13,14] suggest that biofilm formation is a process by which cells undergo a range of phenotypic switches over time. During some stages of biofilm development, as much as 50% of the proteome can be differentially produced compared with the same cells growing in planktonic culture[13,16].

It is generally assumed that the physiological activities of the bacterial cells in a well-mixed planktonic culture are uniform. To obtain enough cell material for experiments, cells are often pelleted by centrifugation and characterized *en masse*. This approach has also been used in proteomic and transcriptomic studies to characterize biofilms[12]. However, the environmental conditions and physiological responses of the bacteria to their local environment are not homogeneous throughout a biofilm. The metabolic activities of the cells, together with diffusional processes, result in concentration gradients of nutrients, signalling compounds and bacterial waste within biofilms. As the bacteria respond to these gradients, they adapt to the local chemical conditions, which can change over time as

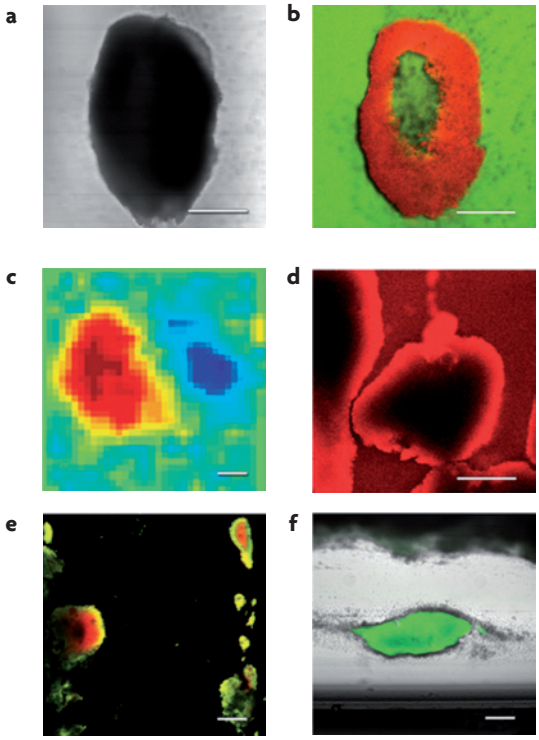

Figure 1 | **Biofilm heterogeneity.** A recurrent theme in the study of microbial biofilms is their inherent microscale heterogeneity. The physical, chemical and biological heterogeneity that can develop even in a single-species biofilm is shown. All of these patterns were imaged using the same reactor system — glass capillary flow cells containing *Staphylococcus epidermidis* biofilms[142]. In each panel, the colour or greyscale variation reflects the heterogeneity for a different property. **a** | Transmission-mode confocal scanning laser microscopy shows an isolated cell cluster (dark grey) surrounded by fluid (light grey)[143]. **b** | The same cell cluster as in **a** imaged using fluorescence-mode confocal scanning laser microscopy (biomass, red; fluid, green) shows a hollow centre[143]. **c** | Magnetic resonance microscopy image that shows measurements of water velocities and reveals complex flows; red and blue indicate velocities that are in opposite directions[144]. **d** | Confocal scanning laser microscopy image showing chemical gradients that developed during a transient diffusion study which imaged the progressive inward diffusion of the red fluorescent dye rhodamine B (a video of the entire sequence can be viewed on Montana State University's Center for Biofilm Engineering website (biofilm movies); see Further Information)[142]. **e** | An immunofluorescence image of cells that were pulse labelled with bromodeoxyuridine. DNA synthetic activity (green) was shown to be localized at the periphery of cell clusters (red)[24]. **f** | Confocal scanning laser microscope image taken during treatment with chlorine. This disinfectant permeabilizes cells at the periphery of cell clusters (dark grey), but leaves more deeply embedded cells (green) intact (W. M. Davison and P.S.S., unpublished observations). The scale bars represent 100 microns.

biofilms develop. As a result, biofilms exhibit considerable structural, chemical and biological heterogeneity (FIG. 1). Therefore, cells that are growing in biofilms are not only physiologically distinct from planktonic cells, but also vary from each other, both spatially and temporally, as biofilm development proceeds. Harvesting entire biofilm populations for experimentation is useful for identifying specific genes that are only expressed in biofilms, but does not necessarily characterize biofilm bacteria *per se,* and rather provides an average characterization of cells that are in a range of physiological states.

To fully understand the activities of bacteria that are growing in biofilms, it is important that the activities within these complex communities are resolved spatially and temporally. In this Review, we discuss several aspects of spatial heterogeneity in biofilms. First, we describe the processes that are involved in the establishment of concentration gradients of oxygen, nutrients, waste products and secreted bacterial-signalling compounds within biofilms. Second, we discuss the responses of the bacteria to chemical-concentration gradients, which can include physiological or genetic adaptation to the local chemical environment. Finally, we review the techniques that are used to characterize the physiological states of the bacteria within biofilms, and provide examples of how cells respond to biofilm chemical gradients at the local level.

Chemical heterogeneity

Much of the heterogeneity in the biological activity within a biofilm can be explained by recognizing the microscale heterogeneity in solute chemistry that is present within a biofilm. Mature biofilms contain concentration gradients of metabolic substrates and products. Oxygen is the best studied and most familiar example[17–24], and oxygen-concentration profiles in biofilms are routinely measured using microelectrodes. The microscale gradients of oxygen will vary depending on the type of biofilm and the sources and sinks for the oxygen. For example, oxygenic photosynthetic biofilms, such as those found in microbial mat communities, establish oxygen gradients in diurnal cycles[25,26]. Other biofilms can be completely anaerobic or contain predominantly anaerobic organisms, such as the biofilms that are associated with dental plaque[27].

The biofilms that have been most studied using oxygen microelectrodes are those that contain primarily aerobic or facultative anaerobic bacteria that have been exposed to aerated media. Oxygen-profile measurements in these biofilms typically reveal steadily declining oxygen concentrations as the microelectrode progresses from the fluid above the biofilm into the biofilm depths. Oxic zones that have dimensions of tens to a few hundred microns are commonly reported. The presence of oxygen-concentration gradients explains, among other phenomena, how strict anaerobes can thrive in biofilms that are continuously exposed to aerated water.

It should be emphasized that the failure of oxygen to penetrate throughout a biofilm is not a result of physical exclusion. The biofilm matrix is an aqueous solution, and solutes that are the size of oxygen diffuse in the matrix at a rate that is approximately 60% of the diffusion rate in pure water[28]. Oxygen fails to penetrate because it is actively respired by cells in the upper layers of the biofilm; this occurs in both mixed-species and single-species

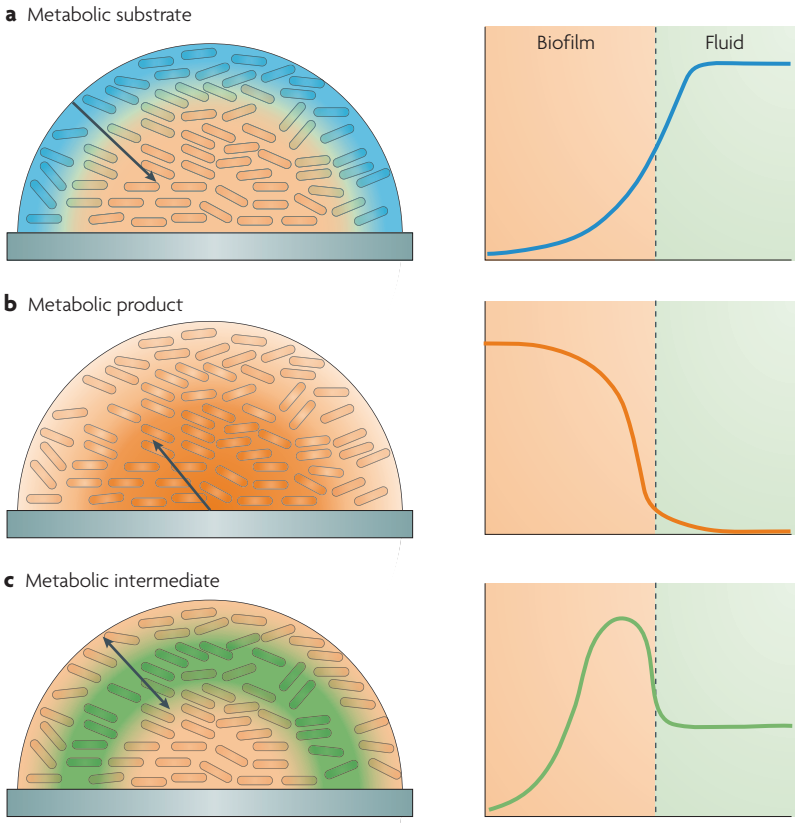

Figure 2 | **Chemical heterogeneity in biofilms.** Three qualitatively distinct patterns of chemical heterogeneity arise in biofilms owing to reaction–diffusion interactions for a metabolic substrate (blue; **a**), a metabolic product (orange; **b**) and a metabolic intermediate (green; **c**). The concentration of a substrate that is consumed in the biofilm decreases with depth into the biofilm and distance away from the source (**a**). Conversely, a metabolic product is more concentrated inside the biofilm (**b**). A metabolic intermediate that is both consumed and produced within the biofilm can exhibit concentration profiles that have local maxima (**c**).

biofilms. The dynamic balance between consumption and diffusion determines the local concentration of oxygen. In one example, measurements of oxygen concentrations within and around a mixed-species heterotrophic biofilm showed that the oxygen concentration at the biofilm–fluid interface was approximately 40% of the value in well-mixed fluid[20]. The oxygen concentration in the biofilm continued to decrease with increasing depth, and was depleted completely at a depth of approximately 175 microns into the 220-micron-thick biofilm. The measured concentration profile resembled that shown in FIG. 2a. The concentration of any nutrient that is consumed in the biofilm will also decrease with depth into the biofilm and distance from the nutrient source.

In contrast to oxygen or nutrients, a metabolic product is characterized by higher concentration in the biofilm interior and lower concentration outside the biofilm. Net transport of the product occurs down the concentration gradient and out of the biofilm. For example, in a methanogenic biofilm that was collected from a sewage outlet and bathed in an anoxic acetate-containing medium, the methane concentration at the surface of the biofilm was approximately 10% of that measured at a depth of 2 millimetres[29]. The concentration profile resembled that shown in FIG. 2b.

Other solutes are both produced and consumed in a biofilm. This situation arises with a metabolic intermediate or when the waste product of one species can serve as the substrate for another species, in which case concentration profiles can exhibit a maximum within the biofilm. For example, in a denitrifying biofilm, nitrate is reduced to nitrogen gas through nitrite[30]. The peak nitrite concentration in the biofilm was approximately four times higher than the concentration in the bulk fluid, and in the depths of the biofilm, nitrite was nearly depleted. The concentration profile resembled that shown in FIG. 2c.

The distribution of any other solute is likewise governed by its simultaneous production, consumption and diffusion. Therefore, other electron acceptors, electron donors, cofactors, signalling molecules, metabolic products and antimicrobials will also establish concentration gradients that are dependent on their sources, sinks and diffusive mobility. As expected, the local rates of production and consumption of a solute will depend on the microscale spatial organization of the microorganisms that metabolize that solute. Microscale concentration profiles have been determined experimentally for a number of solutes, including nitrate[20,21,23], nitrite[23,30], ammonium[20,23], hydrogen sulphide[18,31,32], carbon dioxide[33,34], methane[29], hydrogen peroxide[35], chlorine[36] and chlorine dioxide[37]; microscale pH profiles have also been determined[18,38]. The measured distributions of the chemical species are generally consistent with reaction–diffusion theory[28].

Adaptation to the local microenvironment

It is reasonable to anticipate that the chemical heterogeneity which pervades biofilms will be accompanied by considerable physiological heterogeneity[22,39–41]. It is easy to imagine that there could be regions in the interior of a biofilm in which bacterial growth and activity are slowed or arrested owing to substrate limitation. In addition, the accumulation of acidic waste products in the biofilm interior could lower the local pH and affect the physiological state of the bacteria.

As an illustration of the range of microniches and physiological states that can be found in a biofilm, we consider a single-species biofilm that is growing on a single substrate, such as a carbon source. Suppose further that the bacterium is a facultative anaerobe that will grow aerobically in the presence of oxygen and fermentatively in the absence of oxygen, and the biofilm is bathed in a medium that contains both the substrate and oxygen. In a nascent biofilm, diffusive transport is rapid enough to supply all of the cells with substrate and oxygen (FIG. 3a). In a mature biofilm, at least three distinct physiological states can be anticipated (FIG. 3b–d). Cells that are located near the biofilm–bulk-fluid interface will be provided with both substrate and oxygen (FIG. 3b). These cells will presumably grow by aerobic metabolism. Because oxygen is sparingly soluble, it is usually the first metabolite to be depleted and, consequently, it would be anticipated that a region would develop below the oxic zone in which

Reaction–diffusion theory. A mathematical analysis of the distribution of a chemical solute in space and time that results from the interaction of two processes: reaction of the solute and its transport by diffusion. Reaction–diffusion interactions generate spatial gradients in the concentration of reacting solutes.

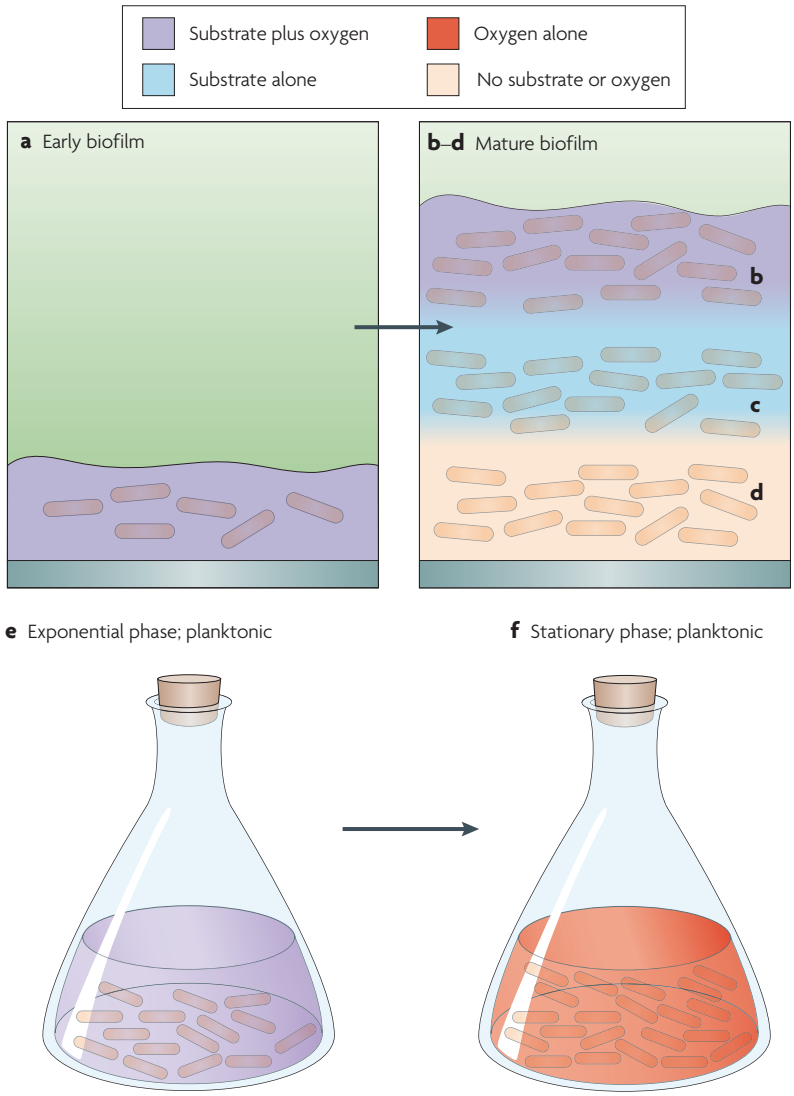

Figure 3 | **Physiological heterogeneity in a single-species biofilm.** A thin biofilm at an early stage of development (**a**) is replete with both substrate and oxygen. In the mature biofilm, environments that contain both substrate and oxygen (**b**), substrate but no oxygen (**c**) and neither substrate nor oxygen (**d**) can occur. In an exponential-phase planktonic culture, substrate and oxygen are both present (**e**), whereas in a shaken stationary-phase culture, substrate can be depleted but oxygen will still be present (**f**).

substrate is present and the cells can grow by fermentation (FIG. 3c). Deeper in the biofilm, there could be a region in which both oxygen and substrate have been depleted; in this zone, the cells might become inactive or begin to die (FIG. 3d).

It is interesting to contrast these three physiological states with the typical outcome in a batch planktonic culture. A flask that is inoculated and placed on a shaking incubator, for example, will initially be replete with both substrate and oxygen, which enables exponential growth (FIG. 3e). As the substrate is depleted (FIG. 3f), the system is continuously aerated and so the stationary-phase culture is supplied with oxygen. This chemical and physiological state (oxygen present and substrate absent) does not match any of the three anticipated states in the biofilm. These environmental differences could help explain some of the profound differences that have been found between the transcriptomic and proteomic profiles of planktonic and biofilm cells.

Many other chemical and physiological heterogeneity scenarios can be proposed by considering different electron acceptors, electron donors and microbial metabolic capabilities. These scenarios invariably manifest multiple physiological states in a mature biofilm. This concept also extends to mixed-species biofilms. Consider a biofilm that is exposed to water which contains oxygen, sulphate and carbon sources. In this biofilm, there is the potential for aerobic heterotrophs, sulphate-reducing bacteria (SRBs) and sulphide-oxidizing bacteria to coexist. Experimental demonstrations of such systems have been published[18,32,42], and are the basis for the simplified diagram in FIG. 4. In the surface layer of the biofilm, aerobic heterotrophs consume oxygen, and in the anoxic depths, SRBs produce hydrogen sulphide from sulphate. In an intermediate zone, where both sulphide and oxygen are present, sulphide-oxidizing bacteria recycle the sulphide to sulphate. The juxtaposition of the three groups of microorganisms in a biofilm facilitates commensalistic or mutualistic interactions.

Measurements of the spatial distributions of various bacterial species in such biofilms suggest that individual species are not compartmentalized in tight strata[18,21,23,32]. Instead, the different species can be intermingled or be present in clonal pockets that are spread throughout the biofilm. For example, SRBs are found not only in the anaerobic depths of the biofilm, but also in the sulphide-oxidizing zone and even the aerobic layer[32]. Some SRBs will be located in zones that are conducive to their activity, where sulphate is present and oxygen is absent. Other SRBs can be stranded in zones where sulphate and oxygen are depleted or oxygen is present. Thus, SRBs experience a range of environments in a biofilm, ranging from conditions that allow metabolism and growth to starvation and oxygen toxicity. The same is true of other species — they are sufficiently scattered in a biofilm, such that a spectrum of physiological states must exist within the population of each species.

Cell motility and the hydrodynamic-driven deformation and flow of biofilm material could increase the possibility for cells of a particular species to distribute across a spectrum of chemical niches. Although this discussion has been framed in terms of the chemical environment, it is also possible that microbial cells respond to their local mechanical environment. For example, bacteria might be able to detect local viscosity[8], sense surfaces[43] or transduce contact with specific ligands on the surface of neighbouring cells[44].

Bacteria are exquisitely adept at sensing, and adapting to, their environmental conditions. In some bacterial species, as much as 10% of the genome is composed of regulatory genes[45]. The genetic circuits for responding to conditions such as anaerobiosis, starvation, oxidative stress, osmotic stress, antimicrobial challenges and pH stress are well known and

differentially regulated in bacteria. Therefore, as the cells within biofilms respond to chemical gradients, which can intersect and/or overlap in biofilms (FIG. 2), the gene expression and physiological activities of the bacteria will vary, possibly at the micrometre scale, as cells adapt to the local and unique environmental conditions within a biofilm (FIG. 5a).

Genetic heterogeneity and adaptation

Although chemical heterogeneity and physiological adaptation to the local environment explain much of the biological heterogeneity in biofilms, it is likely that other mechanisms also contribute to the phenotypic heterogeneity. Recent studies have demonstrated that genetic variants arise in biofilms through mutation or recombination. These variants have been detected primarily as changes in the colony morphology of subpopulations of cells following biofilm growth. In addition, stochastic gene expression might also lead to heterogeneity. In contrast to changes in DNA sequence, subpopulations of cells express genes differently from the larger population in a stochastic manner and not in response to a particular environmental cue. In this section, we discuss two mechanisms of cell diversification: genetic variation and stochastic gene expression.

Genetic variation. Several recent studies have demonstrated that during the growth of bacteria in biofilms, variant subpopulations often emerge[46–53]. This phenomenon has been observed in a wide range of bacterial species, including both Gram-negative and Gram-positive bacteria. Following the growth of the parent strain in biofilms, small percentages of isolated strains exhibit colony morphologies that are distinct from the parent. Examples include the small rough or wrinkly variants of *Pseudomonas aeruginosa*[52,54], the small non-mucoid variants of *Streptococcus pneumoniae*[46] and the rugose colony type of *Vibrio cholerae*[49]. In some cases, the cause of the phenotypic variation has been mapped to changes at specific genetic loci. For example, in *S. pneumoniae*, the small non-mucoid colony variant often results from deletions or single-nucleotide polymorphisms in the *cps3* gene cluster[46,50], which encodes genes that are involved in capsular polysaccharide production. Variants of *Pseudomonas putida* that were isolated from mixed-culture biofilms with an *Acinetobacter* sp. were found to have mutations in the *wapH* homologue, which is involved in core lipopolysaccharide biosynthesis[55]. In addition, small rough variants of *P. aeruginosa* that were isolated from aged biofilms were found to hyperexpress *psl* and *pel*, two of the three *P. aeruginosa* polysaccharide gene clusters[52]. Therefore, as described above, the diversification of these phenotypic variants in biofilms is not necessarily a result of the physiological regulation of gene expression in response to the local environmental conditions, but rather results from DNA sequence changes. Because variants can constitute 10% or more of a biofilm population within a few days of being formed from a homogenous inoculum[52,56], it is unlikely that mutation processes alone are responsible for the accumulation of variants. Mutation and genetic rearrangements probably generate variants that are then enriched through natural selection in the local biofilm environment. In a mixed-species biofilm, genetic changes during biofilm development can lead to the evolution of species interactions[47].

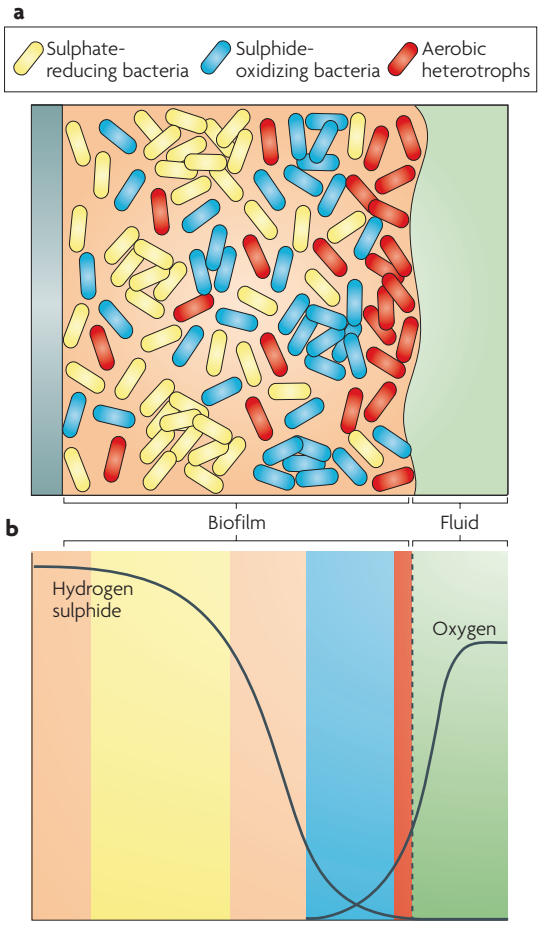

Figure 4 | **Physiological heterogeneity in a mixed-species biofilm.** Three groups of microorganisms are distributed within a biofilm (**a**), and each experiences a range of chemical microenvironments (**b**). In panel **b**, shaded zones denote the regions of oxygen respiration (red), sulphide oxidation (blue) and sulphate reduction (yellow), and the black curves describe the concentration profiles for hydrogen sulphide and oxygen.

Genetic variation and natural selection provide a slow, but long-lasting, mechanism to generate physiological heterogeneity in biofilms (FIG. 5b). Although still a subject of experimentation[57], it has been proposed that genetic change and diversification of bacteria within a biofilm community provide an insurance policy against future antimicrobial challenges or environmental changes, with the diversified population predicted to be more robust than a single parent strain[2,56]. Other investigators have suggested that phenotypic variation generates cells that are more adept at surface colonization[49,58]. Genetic variation might be both a mechanism for enhanced initiation of biofilm formation by planktonic cells and an adaptive mechanism in mature biofilms.

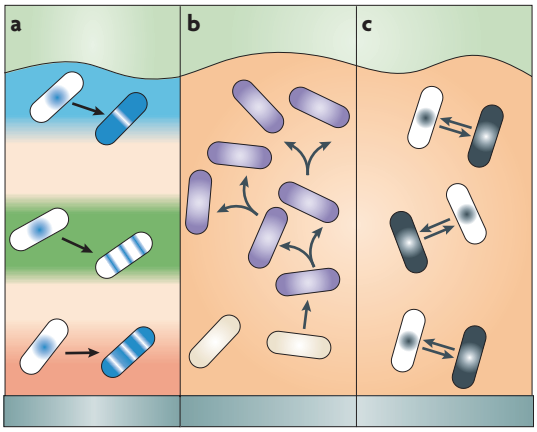

Figure 5 | **Multiplicity of phenotypic states in biofilms.** The three hypothesized mechanisms of phenotypic diversification in a biofilm. **a** | Physiological adaptation. Cells respond adaptively to local environmental conditions by turning on or off certain genes; the responses depend on the local chemical microenvironment and, therefore, allow for a range of distinct localized adaptations. **b** | Genotypic variation and natural selection. A mutation or chromosomal rearrangement results in a variant (purple) that multiplies according to its fitness in the biofilm. **c** | Stochastic gene switching. Cells toggle between discrete physiological states by gene switching, which is random in nature.

Stochastic gene expression. Researchers have used reporter-gene constructs to enable the microscopic visualization of the activity of single bacterial cells on surfaces and have noted striking variation in the activities of neighbouring cells[10,59,60]. This variation could lead to a broad distribution of expression levels, or it could enable a bistable switch, in which genes are either 'on' or 'off' (REFS 61–63). Therefore, stochastic gene expression provides a mechanism to generate phenotypic diversity in a population that is independent of the prevailing environmental conditions (FIG. 5c). An example of stochastic processes with respect to biofilms is extracellular matrix production by *Bacillus subtilis*[64]. The extracellular matrix of this organism is composed of exopolysaccharide that is encoded by the *epsA–O* operon and the TasA protein[65,66]. The genes that encode the matrix material are regulated by the repressor protein SinR and its anti-repressor SinI. By using different coloured fluorescent reporter genes that were fused to the promoters for *sinR*, *sinI* and the matrix operon, Chai and colleagues[64] found that, although the repressor *sinR* was expressed in almost all cells, the *sinI* anti-repressor was expressed in only a subset of cells. The induction of *sinI* generally correlated with cells in which matrix production was induced. Chai *et al.*[64] proposed that matrix production in *B. subtilis* is under the control of a bistable switch, and that only a subset of cells is responsible for this matrix production. This stochastic switching mechanism might be independent of the differential sensing of the local environmental condition.

In another example, Baty and colleagues[59,60] studied the expression of a chitinase gene (*chiA*), in a marine *Pseudoalteromonas* species, in young biofilms that were attached to chitin and silicone films. They observed that individual cells which strongly expressed *chiA* could be found adjacent to cells that were not expressing *chiA*, and suggested that this differential expression reflects a 'division of labour' strategy by bacterial subpopulations. Because these biofilms were thin, and cells that exhibited differential activity were so close to each other, it is likely that they experienced nearly identical chemical environments, which again suggests a bistable mechanism for gene expression in an originally clonal population.

Bistability might help to explain the subsets of bacterial cells, called persister cells, that are found in biofilms[67–69]. Persister cells are proposed to be a subpopulation of cells that have enhanced resistance to antibiotics. These cells might be in a dormant state, against which the activities of antibiotics are less effective. Following the treatment of biofilms with antibiotics, and killing of the rapidly growing cells, these dormant cells can repopulate the biofilm. Using a microfluidics approach, the concept of persister cells was demonstrated for a clonal population of *Escherichia coli* at the single-cell level[70]. Although all cells experienced nearly identical environmental conditions, individual cells divided at different rates[70,71]; the non-dividing cells survived antibiotic treatment and switched to rapid growth following the removal of the antibiotic. Researchers are currently pursuing the genetic basis of persistence[72–75].

The above discussion describes processes that are involved in the generation of physiological heterogeneity in biofilms, which include (but are probably not limited to) adaptation to the spatially localized environmental conditions; the generation of variants that have phenotypically distinct traits; and stochastic gene-expression processes that can be independent of the prevailing environmental conditions. As more information is generated on genetic heterogeneity in biofilms, additional mechanisms might be proposed.

Measuring physiological heterogeneity

In recent years, the experimental techniques that are used to visualize and measure microscale physiological heterogeneity in biofilms have advanced greatly. Many of these techniques are performed using fluorescent reporter genes or fluorescent physiological indicator stains, coupled with epifluorescence or confocal scanning laser microscopy. The advantages and limitations of each technique are summarized in TABLE 1.

Respiratory activity. One of the oldest staining approaches for visualizing respiratory activity in biofilms involves the use of tetrazolium dyes, such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). This soluble, non-fluorescent substrate generates a red fluorescent precipitate if it is reduced by respiring bacteria. Because the fluorescent product is insoluble, this technique can be used to locate respiratory activity within a biofilm[39,76–78]. For example, Huang and co-workers[39] used CTC staining, followed by frozen sectioning, to compare the patterns of respiratory activity in biofilms before and after treatment with a disinfectant. They found greater loss of respiratory activity near the biofilm–bulk-fluid interface compared

Persister cell
A metabolically quiescent cell that neither grows nor dies when exposed to cidal concentrations of antimicrobial compounds.

Table 1 | **Advantages and limitations of experimental techniques for mapping physiological activities in biofilms**

Technique	Advantages	Limitations
Analysis of respiratory activity by CTC staining	A strong, stable fluorescence signal allows the localization of respiratory activity. Minimal sample processing required.	Only detects general respiratory activity. Not specific for other cellular activities. Not applicable to some anaerobic biofilms. Destructive and, therefore, only allows end-point analyses.
Differential membrane permeability staining	Allows the localization of cell-membrane integrity at the single-cell level. Useful for characterizing the effectiveness of antimicrobials. Simple commercial kit available.	Prone to artefacts, including incomplete stain penetration, matrix staining and false staining of live cells as dead.
DNA synthetic activity	Strong, stable fluorescence signal. Minimal sample processing required. Has a record of successful application to eukaryotes.	Useful for general DNA-synthesis activity. Not specific for other cellular activities. Requires uptake of BrdU substrate. Destructive and, therefore, only allows end-point analyses.
Reporter genes	No exogenous inducer or substrate required. Non-destructive and, therefore, allows continuous monitoring of gene expression over time. Allows the expression of specific genes to be monitored, rather than general metabolic activity. Different coloured fluorescent proteins allow several genes to be monitored simultaneously.	Requires genetically engineered strains and the reintroduction of engineered strains into the environment. The energy that is required for expression of the reporter genes might affect cell physiology. GFP requires oxygen for the activation of fluorescence.
FISH	Effective for localizing species diversity in natural biofilms. Engineered strains and exogenous substrates are not required and, therefore, any biofilm can be analysed.	Effective at quantifying cell numbers, but questionable as a quantitative method for gene expression. Not effective for low abundance RNAs without amplification.
MAR	Can be used in combination with FISH to characterize the activities of diverse microorganisms in natural biofilm assemblages at the single-cell level.	Only biosynthetic (substrate uptake) activity can be detected. Less effective for catabolic processes and, therefore, only semi-quantitative. Requires radioactive material and long exposure times. FISH and MAR are destructive and, therefore, only allow end-point analyses.
Fluorescent lectin staining of extracellular products	Lectin specificity and differential labelling allows the detection of multiple types of polysaccharides simultaneously.	Lectin must be specific for the polysaccharides that are produced by the particular microorganisms which are being tested. Lectins might not fully penetrate into the biofilm.
Laser capture microdissection microscopy and qRT-PCR	Allows the isolation of small groups of cells from defined locations in biofilms. Sensitive quantification of low-abundance mRNA transcripts, without prior genetic engineering of the bacteria. Multiplex qRT-PCR allows the quantification of several genes simultaneously.	Destructive and, therefore, only allows end-point analyses. Monitoring is not continuous.

CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; FISH, fluorescent *in situ* hybridization; GFP, green fluorescent protein; MAR, microautoradiography; qRT-PCR, quantitative reverse transcriptase real-time PCR.

with deeper regions. Although useful, investigators have reported that CTC staining sometimes fails to illuminate all of the active cells in a population[79], perhaps, in part, because of toxicity of the dye[80] or inhibition by solutes, such as phosphate[81].

Differential cell-membrane permeability. One of the most popular approaches for assessing the antimicrobial activity of an agent against biofilms is the use of stains that depend on cellular membrane integrity. The widely used commercial kit LIVE/DEAD *Bac*Light contains a pair of nucleic acid stains: a green fluorescent stain, Syto 9, which is membrane permeable and expected to stain all cells, and a red fluorescent stain, propidium iodide, which is membrane impermeant and should only stain cells that have compromised membranes. This staining approach has been used to visualize the spatial patterns of antimicrobial action in biofilms[3,82–87]. For example, Hope and Wilson[86] used this technique to study the effect of chlorhexidine on mixed-species biofilms of oral bacteria; they found greater loss of viability in the surface layers of the biofilm compared with deeper layers. Use of the *Bac*Light kit can produce potential artefacts, such as the non-specific binding of propidium iodide and the scoring of cells as both live and dead simultaneously that can occur if cells are stained with both dyes[88–90]. This staining approach should, therefore, be tested in each system first to ensure accurate scoring of live and dead cells.

Propidium iodide has also been used to detect permeabilized cells either on its own[91] or against bacteria that have been intrinsically tagged with green fluorescent protein (GFP)[4,92–94]. Because propidium iodide stains dead cells but does not affect live cells, this approach allows time-lapse imaging during antimicrobial treatment. Haagensen *et al.*[93] used this technique to show that the antimicrobial peptide colistin acts preferentially on bacteria in the interior of a *P. aeruginosa* biofilm.

Another method for assessing cell permeability uses staining by fluorogenic esterase substrates, such as fluorescein diacetate. The application of this approach to biofilms is largely unexplored[95,96] and merits further investigation.

DNA synthetic activity. An immunohistochemical technique for identifying replicating cells has recently been applied to biofilms[24]. The method involves pulse labelling with the thymidine analogue 5-bromo-2-deoxyuridine, followed by probing with a fluorescently tagged antibody that specifically recognizes brominated DNA. Rani and co-workers[24] used this technique to demonstrate that a *Staphylococcus epidermidis* biofilm contains replicating cells, as well as many cells that are not actively synthesizing DNA. A recent innovation in the application of this method to bacteria couples the immunocapture of labelled DNA with sequencing approaches to identify the community structure of the actively replicating population in a particular environment[97,98]. This modification has yet to be applied to biofilms.

Fluorescent staining of extracellular matrix material. Extracellular polysaccharides, as well as embedded proteins and DNA, often provide the structural integrity for the three-dimensional biofilm matrix[99–104]. The distribution of extracellular polysaccharides is usually studied using confocal scanning laser microscopy coupled with biofilm staining by fluorescently labelled lectins that bind specific oligosaccharide subunits. Studies of this type have demonstrated that the distribution of polysaccharides in biofilms is heterogeneous[105–108]. For example, Laue and colleagues[107] characterized the localization of three polysaccharides in biofilms that were produced by the plant pathogen *Pseudomonas syringae*. They found that one polysaccharide, levan, localized primarily to the interstitial voids in the biofilm interior. However, an unidentified polysaccharide that binds the *Naja naja* lectin was distributed in a fibrous structure in regions that were not occupied by levan. Lawrence and co-workers[106] used fluorescently labelled lectins to characterize the three-dimensional architecture of natural biofilm assemblages. They identified biofilms that had three general, but distinct, staining zones: cell-associated polymer, intercellular polymer and a differently staining polymer that encases the biofilm exterior surface. Taken together, these studies suggest that specific extracellular-matrix materials are not homogenous throughout biofilms. The mechanism for this heterogeneous distribution is not yet clear, but might be a result of subsets of cells that express different polymers or the accumulation of polysaccharides in specific regions of the biofilm. Nevertheless, the results suggest that, in addition to catabolic processes, which are heterogeneously distributed in biofilms, anabolic processes, such as polysaccharide biosynthesis, can also be differentially regulated in biofilms.

Reporter genes. Perhaps the most widely used approach for characterizing localized levels of gene expression is the use of reporter genes fused to promoter regions of interest. Gene-expression activity within biofilms can then be analysed by epifluorescence or confocal scanning laser microscopy. β-galactosidase and alkaline phosphatase have both been used as reporter genes[10,22,41,66], but, for each, the biofilm must be incubated with an appropriate fluorogenic substrate. Fluorescent proteins, such as GFP, obviate the need for incubation with a substrate, and allow gene expression to be monitored, mainly non-invasively, over time. GFP is stable in most bacteria, and by using unstable versions of GFP that have a short half-life[109], an indication of recent gene activity can be gained, thereby allowing temporal, as well as spatial, aspects of local gene expression to be determined.

Several research groups have used the growth-rate-dependent *rrnBP1* promoter fused to an unstable GFP to image overall patterns of growth activity within a biofilm[109–111]. These investigations generally indicate greater growth activity near the biofilm–nutrient interface, at least in mature biofilms. Another strategy for visualizing patterns of protein synthetic activity is to use an inducible GFP construct[24,110,112–114]; a few hours after the inducer is added to a mature biofilm, the pattern of fluorescence reveals the spatial distribution of the capacity for *de novo* protein synthesis. This approach often leads to sharply stratified patterns of activity, in which the interior regions of the biofilm lack protein synthetic activity.

In addition to growth and protein synthetic activities, reporter genes have allowed researchers to discern localized gene activities that are related to quorum sensing[115–118], drug efflux pumps[119], β-lactamase induction in response to antibiotic exposure[114], stationary phase[120] and anaerobiosis[111]. In one example, the anaerobically expressed *mtrB* gene of *Shewanella oneidensis* was localized in the interior of mature clusters, but was not expressed in immature biofilms[111]. This pattern is consistent with the development of an oxygen concentration gradient in mature biofilms.

Reporter genes have also been used to investigate interactions between different bacteria in mixed-species biofilms[121]. Klausen *et al.*[122] used dual-colour fluorescent-protein labelling of wild-type *P. aeruginosa* and type IV pili mutants to demonstrate the spatial localization of motile and non-motile mutants that form spatially distinct subpopulations in mushroom-shaped biofilm cell clusters.

Although useful, reporter-gene technology requires prior genetic manipulation of the bacteria and reintroduction of the constructed strains back into the environment. This approach, therefore, precludes the study of gene expression in wild-type strains in their native states.

Fluorescence* in situ *hybridization. Fluorescence *in situ* hybridization (FISH) has been used to characterize bacterial distribution and, in some cases, expression activity *in situ*[123–127]. Using FISH, single cells are probed with fluorescently labelled nucleic acids that are specific for RNA molecules. Generally, ribosomal RNAs (rRNAs) are probed, because they tend to be in high abundances in cells and, therefore, provide bright signal intensities. As some regions of the rRNA sequence can be highly conserved between species, and other regions are divergent, probes can be designed at differing levels of cell specificity. For example, probes can be designed that encompass entire domains (Bacteria or Archaea) or can be specific for individual species. By using different coloured fluorescently labelled dyes, it is possible to detect the distribution of many species of bacteria in individual biofilm samples. Advances in FISH technology, using new probe designs, have increased cell detection and helped overcome some of the difficulties that are

Peptide nucleic acid probe–FISH
Sequence-specific identification of nucleic acids using fluorescently labelled probes that contain peptide backbones. Useful for identifying individual cells in biofilms, generally by probing specific ribosomal RNA sequences.

Catalyzed reporter deposition–FISH
A method for increasing the sensitivity of FISH by using horseradish peroxidase-labelled oligonucleotide probes. The enzyme catalyzes the deposition of tyramine molecules, which results in fluorescent signal amplification at the site of probe hybridization.

In situ reverse-transcription polymerase chain reaction
A method to convert mRNA to cDNA and amplify the cDNA from cells that are fixed on microscope slides. Gene expression from individual cells is then assayed by using sequence-specific fluorescent probes that hybridize to the amplified product.

Microautoradiography
A method in which the activity of individual cells is determined by assaying incorporation of a radiolabelled substrate into cell material. Cell activity is determined by exposing labelled cells to photographic emulsion and quantifying exposed silver grains adjacent to the cells.

Laser capture microdissection microscopy
A microscopic method in which a laser is used to excise subsets of cells from the surrounding population. The excised cells can then be isolated for further analysis by laser catapulting.

associated with probe penetration through the matrix. For example, Lehtola and co-workers[128] used FISH that incorporated peptide nucleic acid (PNA) probes (Peptide nucleic acid probe–FISH) to detect *Mycobacterium* spp. in potable-water biofilms. PNA probes can have greater penetration ability in biofilms, but still maintain the ability to hybridize to target nucleic acids. Catalyzed reporter deposition–FISH (CARD–FISH) has also provided an advance in cell detection, and has been used to characterize biofilms in soil and marine environments[129–131].

To characterize gene expression, Magnuson and colleagues[132] used the *in situ* reverse-transcription polymerase chain reaction to amplify low-copy-number mRNA from intact cells. The amplified product was then detected using fluorescently labelled probes. Although non-quantitative owing to the amplification step, this FISH modification should be useful for identifying the presence or absence of mRNA transcripts within individual cells from a biofilm community.

Microautoradiography. FISH provides information on the distribution of cells within complex communities such as biofilms. To detect the physiological activities of individual cells, FISH is often combined with other methods, such as microautoradiography (MAR)[133] and, more recently, Raman spectroscopy[134]. Using MAR, radiolabelled substrates are taken up by active cells and incorporated into cellular material. The degree of uptake can then be determined by exposing the cells to liquid film emulsion. This approach has been combined with FISH to detect the activities of genetically distinct bacterial cells[133,135,136]. For example, this approach has been used to study an autotrophic nitrifying biofilm that used CO_2 as the sole carbon source and NH_4^+ as the sole source of electron donors[137]. Using FISH, the investigators were able to localize and quantify the members of the biofilm community, including the ammonia- and nitrite-oxidizing bacteria, as well as the heterotrophs in different Proteobacteria subclasses. The activities of the individual members were determined by analysing the uptake of different ^{14}C-labelled carbon substrates, including bicarbonate, acetate, amino acids and *N*-acetylglucosamine. Although only semi-quantitative, the combination of FISH and MAR allowed the investigators to characterize which members of the biofilm community carried out specific carbon-uptake activities. For example, ammonia- and nitrite-oxidizing bacteria used ^{14}C-bicarbonate, whereas the primary utilizers of *N*-acetylglucosamine were cells from the Cytophaga–Flavobacterium–Bacteroides group. Similar studies have been performed using this approach in biofilms in which sulphate reduction is a primary process[138].

FISH was also recently combined with Raman spectroscopy[134], which is used to quantify the incorporation of ^{13}C-carbon into cellular material. This approach was used to characterize the involvement of *Pseudomonas* spp. in naphthalene degradation, and allowed ^{13}C uptake to be resolved at the single-cell level. Therefore, Raman–FISH should be particularly useful in future studies on the localized activities of individual bacteria in biofilm communities.

Laser capture microdissection microscopy. Laser capture microdissection microscopy was developed to isolate subsets of cells from surrounding tissues[139,140] and can, therefore, be used to characterize the spatial distribution patterns of bacteria within biofilms. Lenz *et al.*[141] recently used this approach, in combination with quantitative reverse-transcriptase real-time PCR, to dissect and capture subsets of cells from vertical strata within *P. aeruginosa* biofilms and quantify mRNA transcripts, as well as 16S rRNA. This approach allowed low-abundance mRNA transcripts from different regions of the biofilms to be quantified, without prior genetic manipulation of the cells. The results indicated that the abundance of specific transcripts, including a housekeeping gene, differs by orders of magnitude depending on the location of the bacteria within the biofilms.

Concluding remarks

In addition to the environmental conditions to which biofilms are exposed, the metabolic activities of bacteria within biofilms result in microscale heterogeneities in the chemical and physical parameters of the biofilm interstitial fluid. Chemical gradients of nutrients, waste products and signalling compounds develop, and can overlap or intersect, resulting in unique environmental niches. Microorganisms within the biofilms can respond to these local environmental conditions in various ways, including: altering gene-expression patterns or physiological activities to adapt to a particular location within the biofilm; enriching for populations of a particular species that are best adapted to a particular condition; and selecting for fitter mutant strains that can better survive under a given condition. Natural variants within biofilm subpopulations can also increase fitness by providing tolerance to antimicrobial challenges and resilience to changing environmental conditions. Technological advances for identifying and characterizing the physiological activities of bacteria at the cellular level will provide an increased understanding of the behaviour of bacteria within the complex biofilm environment.

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Acknowledgements
Work in the laboratory of P.S.S. has been supported by National Institutes of Health grant 5R01GM67245 and an award from the W. M. Keck Foundation. M.J.F.'s work on this topic is supported by Public Health Service grant AI-065906 from the National Institute of Allergy and Infectious Diseases.

DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Bacillus subtilis | *Escherichia coli* | *Pseudomonas aeruginosa* | *Pseudomonas putida* | *Pseudomonas syringae* | *Shewanella oneidensis* | *Staphylococcus epidermidis* | *Streptococcus pneumoniae* | *Vibrio cholerae*

FURTHER INFORMATION

Philip S. Stewart's homepage: http://www.biofilm.montana.edu
Montana State University's Center for Biofilm Engineering Resource Library (biofilm movies): http://www.erc.montana.edu/Res-Lib99-SW/Movies/Database/MD_DisplayScript.asp

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