Genetic analyses of bacterial biofilm formation

Leslie A Pratt and Roberto Kolter*

Bacterial biofilms are generally described as surface-associated bacterial communities comprising exopolysaccharide-surrounded microcolonies. Interspersed between these microcolonies are water-filled channels that may serve as primitive circulatory systems. Over the past few years, much progress has been made in our understanding of the development of bacterial biofilms. This progress is largely due to the recent focus on analyzing biofilms using genetic and molecular biological approaches. Specifically, researchers have begun to identify the genetic components required for the formation of single-species bacterial biofilms. These findings are leading us to an understanding of the steps involved in initiating biofilm formation and the cellular components required to accomplish these steps.

Addresses
Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
*e-mail: kolter@mbcrr.harvard.edu
Corresponding author: R Kolter

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Abbreviations

acyl-HSL	acylated homoserine lactone
EPS	exopolysaccharide
LB	Luria Bertani
MSHA	mannose-sensitive hemagglutinin pilus
PS/A	polysaccharide/adhesin
PVC	polyvinylchloride

Introduction

Traditionally, microbiologists have performed physiological experiments with bacteria grown in liquid cultures where the cells are free swimming or planktonic. These studies have been crucial in that they have led to important advances in our understanding of a wide array of basic biological processes [1]. However, studies initiated several decades ago revealed that many bacteria spend a great fraction of their existence in surface-attached sessile communities [2]. Therefore, to fully understand bacterial life, we must also focus our attention on the biofilm mode of growth.

Analyses of biofilms have revealed that they are relevant in medical, industrial, and environmental settings. Biofilm-associated bacteria generally possess increased resistance to antimicrobial agents [3–18]. For example, persistent, antibiotic-resistant biofilms can form on implanted surfaces such as catheters in the human host [7,14,19••,20–23]. Biofilms formed in industrial settings can also be recalcitrant to antimicrobial agents, thereby leading to possible infection of food products [24] and to the clogging of industrial pipes [25,26]. It is important to note, however, that biofilms can also serve beneficial functions; for example, in the environment certain bacterial species (e.g. *Pseudomonas fluorescens*) can colonize plant roots and act as biocontrol agents [27]. In addition, species of *Rhizobia* can often colonize roots, living in symbiosis with certain legumes by fixing nitrogen [28].

Extensive physical and chemical analysis of bacterial biofilms [26] began in the early 1970s, when a few investigators recognized the prevalence of bacteria in biofilms [2,19••,26]. Since biofilms form under diverse conditions, and can be composed of single or multiple species, the structures of various biofilms will necessarily have distinct features. Nonetheless, the biophysical, structural, and chemical studies performed thus far have led to a useful basic model for biofilm structure [26] (Figure 1). In this model, bacteria form microcolonies surrounded by copious amounts of exopolysaccharide. Between the microcolonies are water-filled channels [19••,26,29], and it has been proposed that these channels serve to promote the influx of nutrients and the efflux of waste products [26].

Clearly, much has been learnt about the structure and characteristics of bacterial biofilms. However, the gene products required for biofilm formation have heretofore remained elusive. Thus, the pathways leading to biofilm formation and dissolution have remained poorly understood (Figure 2). Here, we discuss the major findings revealed through genetic analyses of biofilm formation.

Genetic approaches to biofilm formation

It is important to be able to analyze bacterial attachment using a simple macroscopic assay that facilitates rapid screening of thousands of mutants for the desired phenotype. In general, microtiter plates are used to test potential biofilm defective mutants. The presence or absence of a biofilm within a well of a microtiter plate is detected by staining the wells with crystal violet. Potentially interesting mutants are then analyzed in more detail using molecular approaches and direct microscopic observation [30].

The importance of force-generating movement

In the Gram-negative bacteria subjected to genetic analysis thus far (*Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens*, and *Vibrio cholerae*), the requirement for force-generating movement has emerged as a common theme in biofilm formation. For each of these organisms, lesions in genes involved in flagellar-mediated motility hinder biofilm formation under certain conditions. In *E. coli*, flagellar-mediated motility is important in establishing cell-surface contacts during biofilm formation in Luria Bertani (LB) broth [31,32]. Similarly, non-motile mutant strains of *P. aeruginosa* and *P. fluorescens* were isolated in

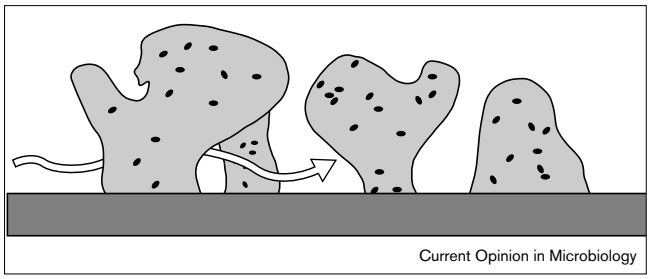
screens searching for defects in biofilm formation. These mutant strains were found to harbor lesions in genes with homology to flagellar genes of other organisms [33••].

In addition to flagellar-mediated movement, twitching motility has also been shown to be important for initial biofilm structural development by *P. aeruginosa*. Twitching motility refers to surface translocation mediated by type IV pili and appears to be widespread among Gram-negative bacteria [34]. Microscopic analysis of wild-type *P. aeruginosa*, non-motile *P. aeruginosa*, and twitch-negative *P. aeruginosa* revealed that flagellar-mediated motility is important in establishing cell-surface contacts, whereas twitching motility appears to play a role in setting up the early structure of the biofilm. Specifically, twitching motility is required for the formation microcolonies within the biofilm [33••].

It is important to note that not all studies have reported an absolute requirement for force-generating motility (i.e. under certain conditions, biofilms are formed in the apparent absence of flagellar- and pili-mediated movement). For example, a non-motile *E. coli* strain was used in one study to select for mutants that gained the ability to attach to polyvinylchloride (PVC). A gain-of-function allele in *ompR* was isolated and shown to increase production of the surface adhesin Curli. This increased production of Curli was shown to be required for biofilm formation in this non-motile strain [35•]. Similarly, motility is important for *V. cholerae* biofilm formation in LB broth, but biofilms do eventually form in such mutants at a slower rate than the wild type [36•]. Furthermore, some defects in biofilm formation in *P. fluorescens* conferred by lesions in flagellar genes can be rescued by growth in either glutamate, high iron, or citrate [37•].

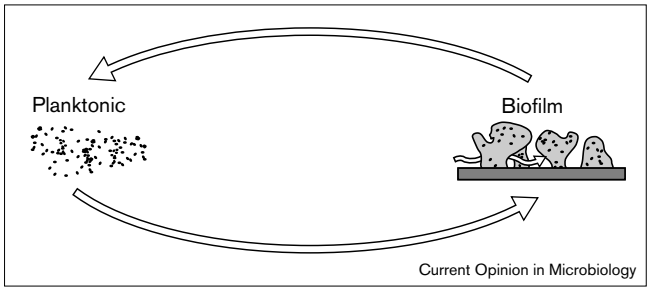
For each of these organisms, it may be that under these conditions, alternative sources of movement (that have not yet been identified) are employed during biofilm formation. Alternatively, it may be that force-generating movement helps to overcome overall repulsive forces between the bacteria and a surface, thereby increasing the chances of bacteria making initial interactions with a surface. Once these initial interactions occur, stable, productive adhesion is established by specific outer-membrane proteins. If this model is correct, then the number of initial interactions would be drastically reduced in strains lacking force-generating movement. However, the requirement for a high number of initial interactions (provided by force-generating movement) may be bypassed by the presence of cells with an increased probability of establishing stable, productive adhesion once a surface is reached. Specifically, it may be that non-motile strains of *E. coli* become 'stickier' when Curli is overproduced, and, similarly, the conditions that rescue *P. fluorescens* may cause these cells to have stronger interactions with surfaces. Such increases in stable cell–surface interactions may allow biofilms to form in the absence of force-generating movement.

Figure 1



A cartoon depiction of a bacterial biofilm, highlighting its complex three-dimensional structural characteristics. The cartoon shows matrix-enclosed bacterial populations adherent to each other and to surfaces. Note that there are microcolonies surrounded by large amounts of exopolysaccharide, and between these microcolonies are water-filled channels that may serve to promote the influx of nutrients and the efflux of waste products [26].

Figure 2



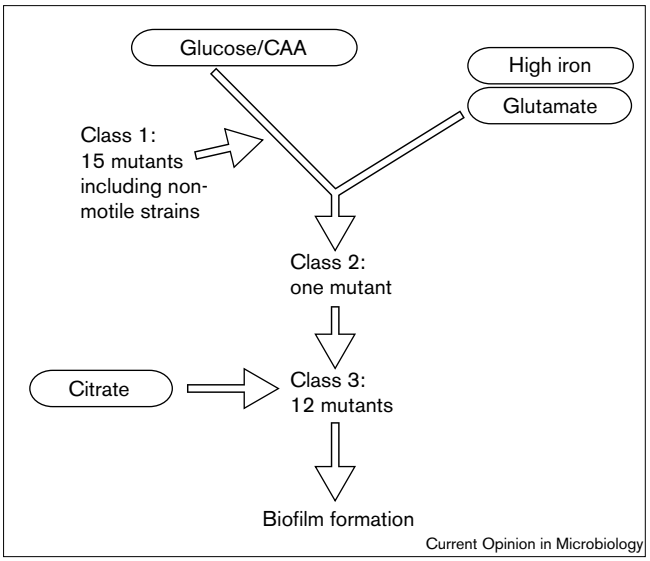
The bacterial life cycle includes both planktonic and biofilm modes of growth. Genetic approaches have focused on understanding the transition between the planktonic and biofilm mode of growth, and vice versa. The hope is to understand external cues leading to the formation or dissolution of biofilms and to identify the genetic components involved in such transitions.

It will be interesting to examine the three-dimensional architecture of biofilms that utilize distinct pathways, as the requirement for and/or the type of force-generating movement may affect the type of biofilm that is formed.

Role of specific outer-membrane adhesins

To a first approximation, interactions between bacteria and surfaces are analogous to interactions of particles with a surface. This would imply that overall repulsive and attractive forces between the bacteria (particles) and a surface dictate productive interactions. Of course such forces do exist and are likely to contribute to attachment [38,39], but genetic analyses carried out thus far argue that specific outer-membrane adhesins are required for stable attachment and biofilm formation [31,35•,36•,40•]. These results argue that essential attractive forces required for the establishment of stable interactions between bacteria and surfaces are provided by specific outer-membrane proteins (i.e. the establishment of stable interactions is not solely

Figure 3



Distinct pathways can be utilized under differing environments to initiate biofilm formation. This figure illustrates that under differing environmental conditions there are different genetic requirements for biofilm formation. Some of the mutants isolated in an initial screen for mutants defective for biofilm formation in glucose minimal medium (CAA) are able to form biofilms in the presence of either iron, glutamate, or citrate, indicating that different pathways can be utilized to form biofilms under different conditions [33••]. Class I mutants are rescued for biofilm formation by growth in high iron, glutamate, or citrate. The class 2 mutant is rescued by growth in citrate. Class 3 mutants cannot be rescued by growth under any condition tested. This class may represent genes whose products are required for all pathways to biofilm formation.

due to overall attractive and repulsive forces between bacteria and surfaces).

For example, genes encoding for the mannose-sensitive type I pilus are essential for *E. coli* biofilm formation in LB broth [31]. Indeed, type I pili are required for *E. coli* biofilm formation on all surfaces tested, including PVC, polycarbonate, polystyrene, and borosilicate glass. Since it is reasonable to assume that all of these surfaces do not resemble mannose, it appears that the pilus adhesin, FimH, has the ability to bind both specifically and nonspecifically to mannose and abiotic surfaces, respectively. Moreover, the presence of a non-metabolizable analog of mannose inhibits attachment to abiotic surfaces, indicating that binding to mannose and binding to abiotic surfaces are mutually exclusive events. This result not only argues that outer-membrane adhesins play a critical role in attachment, but also highlights possible mechanisms for preventing formation of undesired biofilms (e.g. through the addition of benign substances such as sugars or sugar analogs).

Similar results have been obtained with genetic analysis of biofilm formation by *V. cholerae* El Tor [36•,40•]. Specifically, the type IV mannose-sensitive hemagglutinin pilus (MSHA), which does not play a role in host colonization [41], is required for biofilm formation on borosilicate glass in LB broth. Similar to the results obtained with *E. coli*, the addition of a non-metabolizable analog of mannose inhibits *V. cholerae* El Tor biofilm formation on borosilicate glass [40•]. In contrast, MSHA is not required for *V. cholerae* attachment to the nutritive surface, chitin [40•]. This suggests that a different, yet unidentified, adhesin may be required for attachment to chitin.

As discussed above, in non-motile strains of *E. coli* an alternative adhesin, Curli, plays a role in biofilm formation. Therefore, it is possible that under these conditions, a Curli-dependent pathway is utilized that bypasses the requirement for flagellar-mediated motility, and this distinct pathway utilizes the distinct adhesin Curli [35•]. Whether or not these strains also require type I pili remains to be determined.

Distinct pathways

As alluded to above, genetic analyses of biofilm formation have led to the important realization that under distinct environmental conditions, distinct pathways are utilized to form biofilms. Evidence supporting this view was reported in a paper describing a genetic analysis of the initiation of biofilm formation in *P. fluorescens* [33••]. In this work, the authors isolated transposon insertion mutations that rendered the cells defective in attachment to PVC when the cells were grown in M63 glucose minimal medium supplemented with casamino acids. Some of the biofilm-defective mutants could be rescued by the addition of high concentrations of iron or glutamate, and some could be rescued by the addition of citrate (Figure 3). These results define distinct genetic requirements for biofilm formation by *P. fluorescens* in differing environments.

Further evidence supporting this view was reported for *V. cholerae* El Tor biofilm formation. As noted above, MSHA was shown to be critical for biofilm formation on borosilicate glass when the cells were grown in LB broth. In contrast, MSHA was not required for attachment to the nutritive surface chitin [40•]. Moreover, attachment of *V. cholerae* El Tor to chitin occurred whether the cells were grown in LB or in M63 minimal medium. This was in contrast to the MSHA-dependent attachment to glass as attachment to glass was greatly reduced when the cells were grown in minimal medium. Together, these results illustrate that both the genetic requirements (MSHA) and environmental factors (LB versus minimal) for biofilm formation on nutritive and nonnutritive surfaces are distinct [40•].

Signaling

Several reports have addressed the potential role of extracellular factors in biofilm formation. Acylated homoserine lactones (acyl-HSLs), which are quorum-sensing signal molecules, have been demonstrated to be present both in aquatic biofilms grown on submerged stones [42], and in

biofilms formed on urethral catheters [43•]. Since these acyl-HSLs are synthesized and secreted at high levels in dense cultures, one would predict a role for these compounds late in biofilm formation.

An elegant study illustrated the importance of an acyl-HSL synthesized by *P. aeruginosa* in the formation of the characteristic three-dimensional architecture within the biofilm [44••]. In this study, *lasI* mutant cells unable to synthesize N-(3-oxododecanoyl)-L-homoserine lactone were shown to form biofilms with abnormal structure. The mutant biofilms contained cells that were tightly packed, and the biofilms were approximately 20% the thickness of wild-type biofilms. This is in stark contrast to the microcolonies interspersed with water-filled channels characteristic of wild-type biofilms.

Very recently, a molecular link between the environment and the decision between planktonic and biofilm modes of growth has been identified. Specifically, the *P. aeruginosa crc* gene has been shown to be required for biofilm formation [45•]. Crc plays a role in sensing carbon source availability [46,47] and has been shown to affect expression of the type IV *pilA* structural gene [45•]. Since type IV pili-mediated twitching motility is required for *P. aeruginosa* biofilm formation [37•], Crc links carbon availability to the decision whether or not to enter a biofilm mode of growth.

Exopolysaccharide

Initial molecular/genetic analysis of alginate production in *P. aeruginosa* indicated that alginate expression was induced upon contact with a surface [48,49]. More recent genetic evidence supporting the importance of exopolysaccharide (EPS) in biofilm formation has come from both the Gram-positive bacterium *Staphylococcus epidermidis* and the Gram-negative bacterium *V. cholerae* El Tor. The production of EPS in *V. cholerae* El Tor has been shown to be dependent on the *vps* locus [50••]. In a study that looked at biofilm formation over a 72 hour time period, wild-type *V. cholerae* El Tor formed biofilms 10–15 μm in depth with characteristic pillars of bacteria. In contrast, an EPS mutant remained a monolayer of isolated, attached cells [36•].

It is important to recognize that Gram-positive bacteria are non-motile. Yet, similar to the Gram-negative *V. cholerae* El Tor, transposon mutations have been isolated in *S. epidermidis* that render the strains deficient in capsular polysaccharide/adhesin (PS/A) [51–53]. These PS/A deficient cells were shown to be defective in biofilm formation. More recently, expression of PS/A has been shown to be regulated in a phase-variable fashion [54•]. It is interesting to note that type I pili genes in *E. coli* are also phase variable, and, as noted above, are required for biofilm formation. It may be that phase-variable expression of adhesins important in biofilm formation is an emerging theme, and future study may lead to the identification of additional factors that are regulated in a phase-variable fashion in both Gram-positive and Gram-negative organisms.

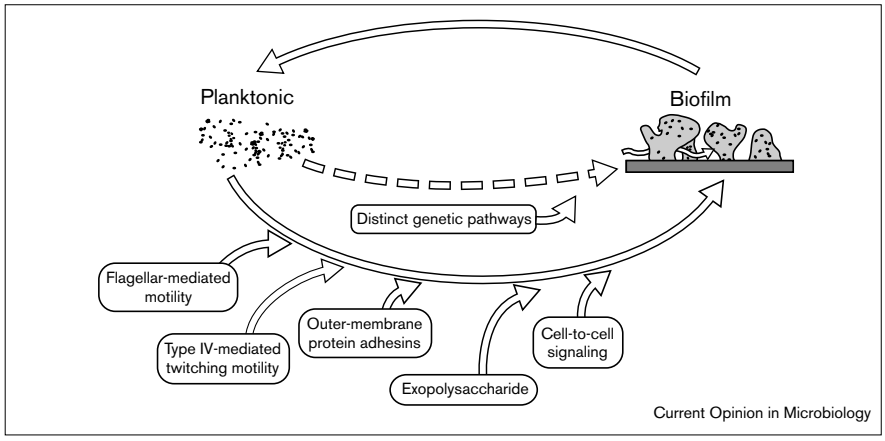
Conclusions

It is now clear that flagellar-mediated motility and/or pilus-mediated motility provide mechanisms enabling Gram-negative bacteria to reach surfaces and to move along surfaces, contributing to the initial stages of biofilm structure development. Cell surface protein adhesins and EPSs have been shown to play roles in establishing constructive interactions between cells and surfaces. Finally, both external environmental cues and bacterial signaling molecules have been shown to be important in triggering the initiation of biofilm formation and establishing the complex three-dimensional structures characteristic of biofilms (Figure 4).

It appears that bacteria are not simply unicellular organisms, but rather, they often persist in a biofilm state of existence that can be compared to a multicellular organism. We, as microbial geneticists, have been slow to

Figure 4

Summary of genetic components important in biofilm formation. Note that not all bacteria require all of these factors, and that distinct genetic pathways can be utilized by a single species to form a biofilm.



realize this, and consequently have steadfastly examined bacteria grown in batch culture, thereby treating and studying them as unicellular species. Only recently have we begun to grasp the significance of the multicellular nature of microbes. Yet, within this relatively short period, genetic approaches to studying biofilms have revealed much about both the cellular factors and the steps during which these factors function during biofilm formation. But we have only begun, and we should note that we have not yet learned about the genetic components involved in the dissolution of biofilms (Figure 4). Through additional genetic analysis of single-species biofilms, through microscopic observation of mutants, through examining gene expression within biofilms composed of wild-type or mutant cells, and through studying the genetic requirements for the dissolution of biofilms, we should further our understanding of the steps in forming and dispersing single-species biofilms. Only then will we be prepared to embark on studying the molecular complexities necessarily involved in the formation and dissolution of multi-species biofilms [55].

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