Molecular Microbiology (1998) **30**(2), 295–304

Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development

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Summary

The formation of complex bacterial communities known as biofilms begins with the interaction of planktonic cells with a surface in response to appropriate environmental signals. We report the isolation and characterization of mutants of *Pseudomonas aeruginosa* PA14 defective in the initiation of biofilm formation on an abiotic surface, polyvinylchloride (PVC) plastic. These mutants are designated surface attachment defective (*sad*). Two classes of *sad* mutants were analysed: (i) mutants defective in flagellar-mediated motility and (ii) mutants defective in biogenesis of the polar-localized type IV pili. We followed the development of the biofilm formed by the wild type over 8 h using phase-contrast microscopy. The wild-type strain first formed a monolayer of cells on the abiotic surface, followed by the appearance of microcolonies that were dispersed throughout the monolayer of cells. Using time-lapse microscopy, we present evidence that microcolonies form by aggregation of cells present in the monolayer. As observed with the wild type, strains with mutations in genes required for the synthesis of type IV pili formed a monolayer of cells on the PVC plastic. However, in contrast to the wild-type strain, the type IV pili mutants did not develop microcolonies over the course of the experiments, suggesting that these structures play an important role in microcolony formation. Very few cells of a non-motile strain (carrying a mutation in *flgK*) attached to PVC even after 8 h of incubation, suggesting a role for flagella and/or motility in the initial cell-to-surface interactions. The phenotype of these mutants thus allows us to initiate the dissection of the developmental pathway leading to biofilm formation.

Introduction

Biofilms are sessile bacterial communities adhered to a surface. In most environments, bacteria are thought to reside predominantly in biofilms (Costerton *et al*., 1995), in contrast to planktonic or free-swimming cells typically studied in the laboratory. *Pseudomonas aeruginosa* has been shown to form biofilms on a number of surfaces, including the tissues of the cystic fibrosis lung (Govan and Deretic, 1996) and on abiotic surfaces such as contact lenses and catheter lines (Nickel *et al*., 1985; 1989; Miller and Ahearn, 1987; Fletcher *et al*., 1993). This ubiquitous organism is also the cause of nosocomial infections in immunocompromised patients and individuals with severe burns (Bodey *et al*., 1983).

Biofilms of *P. aeruginosa* (and other microorganisms) are formed from individual planktonic cells in a complex and presumably highly regulated developmental process. Planktonic cells are thought to initiate interactions with a surface in response to various signals, including the nutritional status of the environment (Wimpenny and Colasanti, 1997; O'Toole and Kolter, 1998; Pratt and Kolter, 1998). The fully developed surface-attached community can be a highly structured with distinct architectural and physical/chemical properties (Costerton *et al*., 1995). These biofilm-grown cells are thought to be markedly different from their planktonic counterparts, based on a number of lines of evidence. For example, *P. aeruginosa* growing on a surface has increased expression of *algC*, a gene required for the synthesis of extracellular polysaccharides (Davies *et al*., 1993; Davies and Geesey, 1995). Biofilm-grown *P. aeruginosa* has also been shown to acquire increased resistance to antibiotics (Hoyle and Costerton, 1991). To date, most studies of biofilms have focused on characterizing the organisms that comprise these bacterial communities, the physical/chemical properties of biofilms and the physical forces that effect the bacterium's initial interactions with a surface (Costerton *et al*., 1995; Fletcher, 1996). However, little is known about the molecular genetic mechanisms regulating the initiation of biofilm formation and the development of these complex bacterial communities.

We present the isolation of mutants of *P. aeruginosa* PA14 defective in the initiation of biofilm formation on an abiotic surface. We report the molecular characterization of two classes of mutants defective in initiation of biofilm formation: (i) mutants defective in flagellar-mediated motility; and (ii) mutants defective in type IV pili biogenesis. The analysis of the flagellar and type IV pili mutants with time-lapse phase-contrast microscopy has allowed us to begin the dissection of the early development of a *P. aeruginosa* biofilm.

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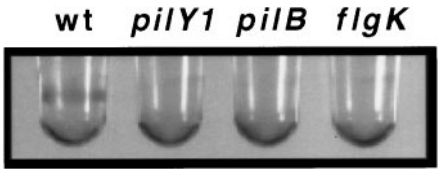


Fig. 1. Biofilm formation phenotype. Shown is the biofilm formation phenotype of the wild-type strain and three representative *sad* mutants (*pilY1*, *pilB* and *flgK*). As described previously, the biofilms form at the interface between the air and the medium (O'Toole and Kolter, 1998). Under the growth conditions used in this experiment, the only electron acceptor available is oxygen. Therefore, the biofilm forms only where oxygen levels are highest, that is at the interface between air and medium.

Results

Isolation of mutants defective in biofilm formation

We generated a collection of ≈2400 random transposon mutants of *P. aeruginosa* PA14 using the transposon Tn5-B30(Tc^r) (Simon *et al*., 1989). This collection of *P. aeruginosa* mutants was screened in microtitre dishes made of polyvinylchloride (PVC) to test for their ability to form a biofilm on an abiotic surface. The cells were allowed to grow in the wells of the microtitre dishes in a minimal M63 medium supplemented with glucose and casamino acids (CAA), using a technique described previously (O'Toole and Kolter, 1998), to assess their ability to form a biofilm. The biofilm was detected by staining with crystal violet (CV), a purple dye that stains the bacterial cells but does not stain the PVC plastic. After addition of CV and incubation at room temperature for ≈10 min, excess CV and unattached cells were removed by vigorous and repeated washing of the microtitre plates with water. An example of the phenotype of the wild-type strain is shown in Fig. 1. The biofilm is observed as a ring of CV-stained cells that forms at the interface between air and medium. Of the ≈2400 mutants screened, 15 mutants (0.5%) unable to form such a biofilm were isolated. These mutants were designated surface attachment defective or *sad*. The biofilm formation phenotypes of representative *sad* mutants are shown in Fig. 1.

Any strains exhibiting poor growth under these screening conditions might give the same phenotype as mutants unable to initiate formation of a biofilm. Therefore, all of the putative *sad* mutants were grown in liquid minimal M63 medium supplemented with glucose and CAA (the same medium used to screen for mutants). Of the 15 putative *sad* mutants tested, 13 grew as well as the wild-type strain but were unable to form a biofilm. The other two putative *sad* mutants had severe growth defects relative to the wild type and were not analysed further.

We performed Southern blot analysis of the 13 *sad* mutants that did not form a biofilm to determine the number of transposon insertions in each strain. A PCR-generated DNA fragment from the IS50 of Tn*5* was used to probe *Eco*RI-digested chromosomal DNA (there are no *Eco*RI sites in Tn*5*-B30). This analysis revealed a single hybridizing band for each strain, consistent with each *sad* mutant having only a single transposon insertion (data not shown). The further analyses of two classes of mutants (totalling 8 of 13) isolated in this screen is presented below. The analyses of the other five *sad* mutants will be presented elsewhere.

We reported previously that mutants of *P. fluorescens* originally identified as unable to initiate biofilm formation on PVC were also defective for biofilm formation on a variety of other abiotic surface (O'Toole and Kolter, 1998). We tested the *P. aeruginosa sad* mutants for their ability to form a biofilm on abiotic surfaces other than PVC, including polystyrene, polycarbonate and polypropylene. The wild-type strain can form a biofilm on all of these surfaces. All of the *sad* mutants originally isolated on PVC were also defective for biofilm formation on these other surfaces (data not shown).

Non-motile mutants are defective in biofilm formation

It has been reported that motility is required for both biofilm formation (on biotic and abiotic surfaces) and pathogenesis (Montie *et al*., 1982; de Weger *et al*., 1987; Grant *et al*., 1993; Korber *et al*., 1994; Simpson *et al*., 1995). However, these studies generally involved the analysis of molecularly uncharacterized non-motile mutants. Therefore, in addition to the phenotypic analyses described above, all *sad* mutants were assessed for their motility phenotype on 0.3% agar (minimal M63 medium supplemented with glucose and CAA). Of the 13 mutants tested, three strains (*sad-36*, *sad-39* and *sad-42*) were found to be non-motile (see Fig. 2). In a typical experiment after 24 h of growth at

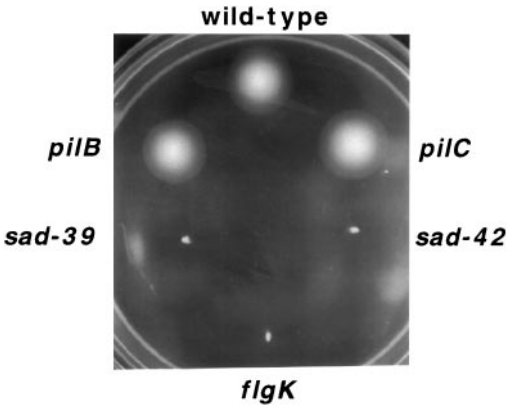


Fig. 2. Motility assays. The flagella-mediated motility of the wild-type strain, representative pili-defective mutants (*pilB* and *pilC*) and non-motile mutants (*flgK*, *sad-39*, and *sad-42*) was assessed on minimal M63 glucose/CAA medium with 0.3% agar after ≈24 h of growth at 25°C. Migration of the cells from the point of inoculation (observed as a turbid zone) indicates that the strain is proficient for flagellar-mediated motility.

room temperature, the wild type and two representative mutants defective in pili biogenesis (*pilB* and *pilC*) clearly migrated from the point of inoculation, whereas the *sad-36*, *sad-39*, and *sad-42* strains did not. One of these mutants, *sad-36*, was chosen for further analysis.

The *sad-36*::Tn*5*(Tc^r) insertion was mobilized into a wild-type genetic background by phage SN-T-mediated transduction as reported (Jensen *et al*., 1998). Eighteen out of 18 Tc^r transductants (indicating inheritance of the Tn*5* element) were non-motile and unable to make a biofilm, demonstrating that the single insertion in this strain was responsible for the observed phenotypes. The DNA sequence flanking the Tn*5* insertion in *sad-36* was determined using the arbitrary PCR method (see *Experimental procedures*) and compared with the GenBank database with BLASTX (Altschul *et al*., 1990). BLASTX translates DNA sequence in all six reading frames and compares these predicted protein sequences with GenBank. The determined DNA sequence flanking the Tn*5* element (≈375 nt), when translated, revealed a partial ORF with ≈40% identity and ≈65% similarity to HAP1 (*flgK*), the flagellar-associated hook protein 1 of *Salmonella typhimurium* and *Escherichia coli*. Mutations in the *flgK* locus in these organisms results in the synthesis of an incomplete flagellum, which renders the strains non-motile (Homma *et al*., 1990). The localization of the Tn*5* insert of the strain carrying the *sad-36* allele to a gene required for flagellar function is consistent with the non-motile phenotype of this strain.

Type IV pili are required for biofilm formation

We analysed the DNA sequence flanking the transposon inserts of the other *sad* mutants (as described in the section above and in the *Experimental procedures*). Comparison of the translated DNA sequence flanking the Tn*5* element of a number of *sad* mutants to the GenBank database revealed that five strains carried mutations in genes required for the synthesis of type IV pili.

Type IV pili have been shown to be important for the adherence to and colonization of eukaryotic cell surfaces and are thought to play a role in pathogenesis (Woods *et al*., 1980; Ramphal *et al*., 1984; Doig *et al*., 1988; Bieber *et al*., 1998). Four of the five mutants defective in type IV pili biogenesis identified in the screen had mutations in the *pilBCD* operon, which is thought to code for accessory factors required for pili assembly and function (Nunn *et al*., 1990). The strains carrying alleles *sad-31*, *sad-33* and *sad-34* have mutations in the *pilB* gene. The DNA sequence flanking the transposon insertions in *sad-33* and *sad-34* was identical, indicating that these two strains were probably siblings. The mutations carried in *sad-31* and *sad-33*/*sad-34* map to two different locations within *pilB* (data not shown). The strain carrying allele *sad-29* has a mutation in the *pilC* gene. Because the *pilBCD* locus may form an operon, it is possible that polarity onto *pilD* is actually causing the phenotype. However, it has been shown in *P. aeruginosa* PAO1 that mutations in any of these loci result in the loss of the synthesis of pili as indicated by resistance to the pilus-specific bacteriophage PO4 and visual inspection using electron microscopy (Nunn *et al*., 1990). The fifth mutant, *sad-25*, maps to yet a third locus, a homologue of the *pilY1* gene of *P. aeruginosa* PAO1. In *P. aeruginosa*, the *pilY1* gene is in a cluster of genes (including *pilV*, *pilW*, *pilX*, *pilY2*, and *pilE*) that is required for type IV pili biogenesis (Russell and Darzins, 1994; Alm and Mattick, 1995; Alm *et al*., 1996). Mutations in the *pilY* homologues in *Neisseria* spp. (called *pilC* in these organsims) can also result in a non-piliated phenotype (Jonsson *et al*., 1991; Rudel *et al*., 1992). Consistent with the mapping of these mutations to genes required for type IV pili biogenesis was their resistance to lysis by phage F116 (Pemberton, 1973), which uses type IV pili as its receptor (data not shown).

It has been shown that type IV pili are required for a form of surface-associated movement known as twitching motility. Twitching motility is thought to be a consequence of the extension and retraction of type IV pili, which propels the bacteria across a surface by an undescribed mechanism (Bradley, 1980; Whitchurch *et al*., 1990; Darzins, 1994). We assessed the twitching motility phenotype of the mutants carrying alleles *sad-25* (*pilY1*), *sad-29* (*pilC*), *sad-31* (*pilB*), and *sad-33* (*pilB*). The wild-type, a representative flagellar mutant (*flgK*), and four type IV pili mutants are shown in Fig. 3A. In addition to forming a colony on the surface of the agar plate (1.5% agar), Twitch$^+$ strains of *P. aeruginosa* PA14 form a haze of growth that surrounds the point of inoculation (Bradley, 1980; Whitchurch *et al*., 1990). This assay differs from the test for flagella-mediated motility, which is performed by inoculating cells onto 0.3% agar plates (see Fig. 2). Furthermore, strains capable of twitching motility have a spreading colony morphology, whereas strains defective in twitching motility produce rounded colonies (Whitchurch *et al*., 1990; Darzins, 1994). This difference in colony shape can also be observed in Fig. 3A.

Twitching motility can also be assessed by phase-contrast microscopy. At the microscopic level, the edge of the colonies of strains proficient in twitching motility are highly irregular. This is thought to be a consequence of the surface movement associated with type IV pili (Whitchurch *et al*., 1990; Darzins, 1994). Mutants lacking functional type IV pili have smooth-edged colonies. To further confirm that our strains did not have functional type IV pili, we observed the edges of wild-type and pili-deficient mutants using phase-contrast microscopy. As shown in Fig. 3B, the wild-type strain has the expected irregular colony edge and the representative pili-deficient strain (*sad-31*/*pilB*) has the expected smooth colony edge

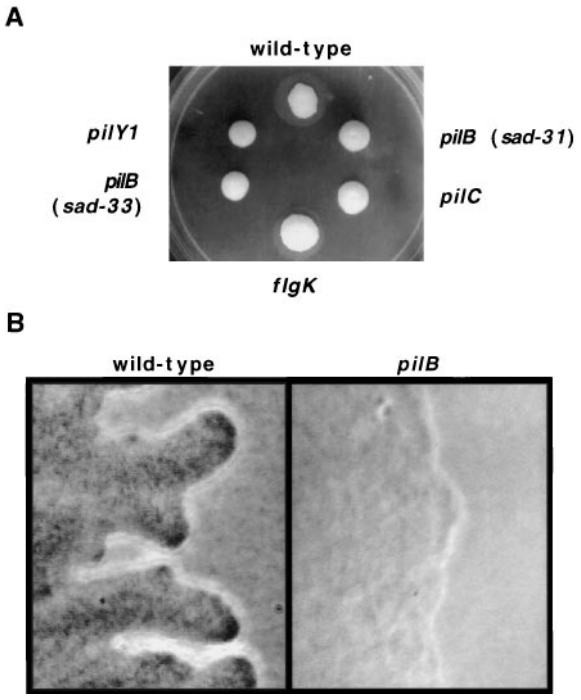


Fig. 3. Twitching motility. Twitching motility was assessed in two ways.
A. Cells were stabbed into an LB agar plate (1.5% agar) with a toothpick, incubated overnight at 37°C, then for 1–2 days at room temperature (≈25°C). Twitch$^+$ strains form a colony on the agar surface and form a hazy zone of cell growth within the agar substrate. Twitch$^-$ strains still form a colony on the agar, but lack the zone of growth within the agar. Also, the colonies of Twitch$^+$ strains are flat, spreading and irregularly shaped, whereas the colonies formed by strains defective in the synthesis of type IV pili are rounded and somewhat dome shaped (Mattick *et al.*, 1996; Darzins and Russell, 1997).
B. Direct visual inspection of the colony edges of wild type and pili mutants was performed using phase-contrast microscopy. The edge of the wild-type strain is highly irregular. In contrast, the colony edge of the representative pili mutants (*pilB*) has a smooth, regular phenotype. Micrographs were taken at 400× magnification.

phenotype. All the pili-defective mutants behaved in a fashion identical to *sad-31* (results not shown). Transmission electron-microscopic analysis of the pili mutants confirmed the lack of these structures on the surface of the mutant cells (not shown).

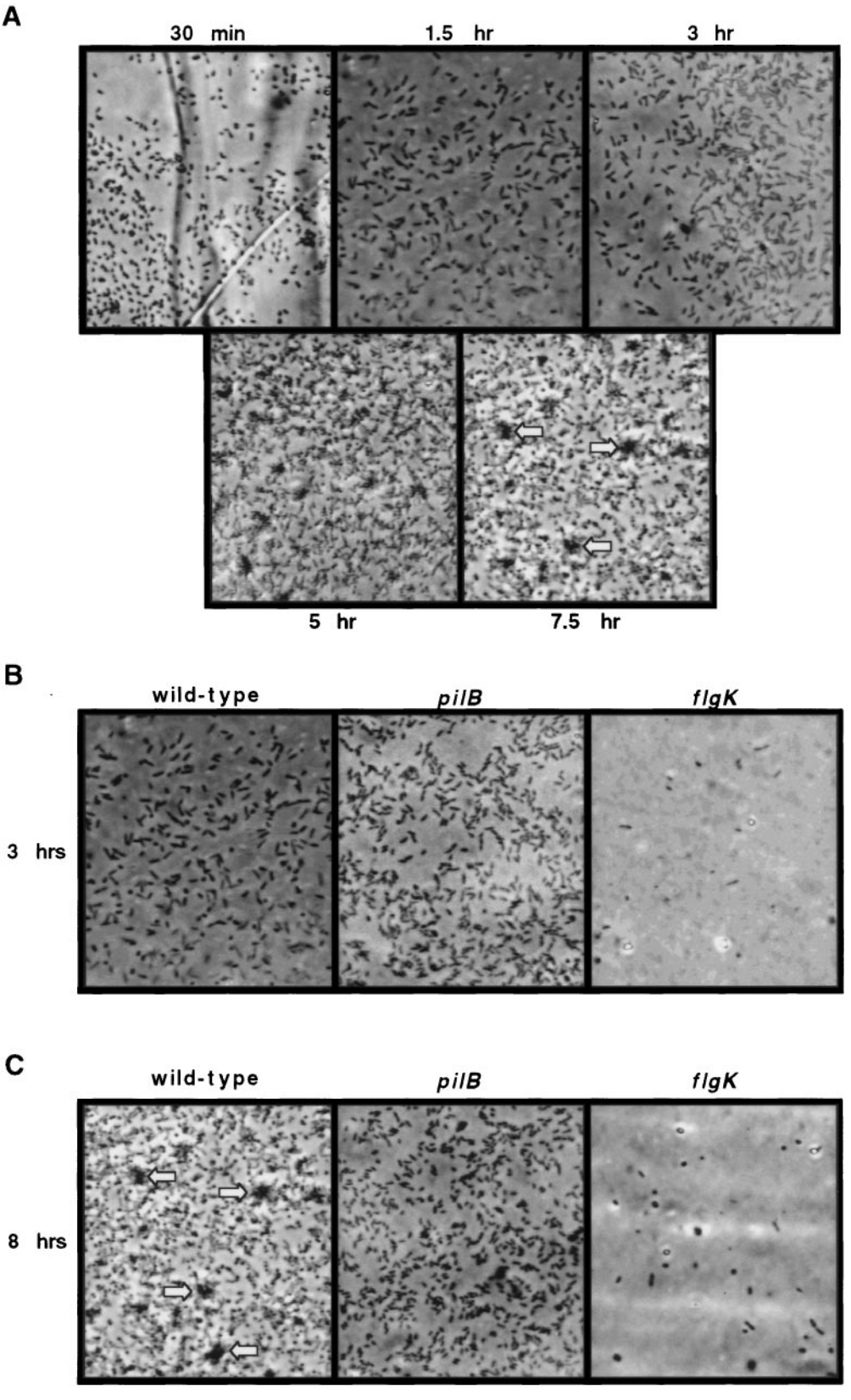
Mutants defective in flagellar-mediated motility and type IV pili biogenesis define two steps in a developmental pathway

We used the *sad* mutants isolated in this study as tools to initiate the dissection of the early steps in biofilm formation. To follow the initiation of biofilm formation by the wild-type and *sad* mutants, we directly visualized the formation of the biofilm on PVC using phase-contrast microscopy. A small tab of PVC plastic (≈3 mm × ≈6 mm) was incubated in the well of a microtitre dish that has been inoculated with $\approx 10^6$ cfu ml^{-1} of the appropriate strain in minimal M63 medium supplemented with glucose and CAA. After incubation for various times at 37°C, the plastic tab was removed from the microtitre dish with ethanol-sterilized forceps, rinsed with 1 ml of sterile minimal M63 medium and placed on a slide. The slide was examined using phase-contrast microscopy (400× magnification) as described in *Experimental procedures*.

Figure 4A shows a time-course of the development of a biofilm on PVC by the wild-type strain over 7.5 h at 37°C as observed using phase-contrast microscopy. As early as 30 min after inoculation, the wild type formed a dispersed monolayer of bacterial cells attached to the surface of the PVC plastic. A progressively more dense monolayer of cells formed on the surface over the next 3–4 h. By 5 h, and continuing until at least 7.5 h, this monolayer almost completely covered the PVC surface and became punctuated by microcolonies that are distributed across the surface of the PVC plastic and comprise multiple layers of cells. Typically, the wild-type microcolonies were ≈3–5 layers of cells thick.

We directly visualized the ability of the type IV pili-deficient and non-motile strains to form a biofilm on PVC using phase-contrast microscopy and compared their phenotypes with the wild-type strain. For the representative non-motile strain (carrying a mutation in *flgK*), few to no cells were observed attached the PVC plastic even after 8 h of incubation in the presence of the PVC surface. All other non-motile strains analysed had a phenotype identical to the *flgK* mutant. This observation is consistent with previous results showing the importance of motility in biofilm formation (Montie *et al.*, 1982; de Weger *et al.*,

Fig. 4. Direct observation of biofilm formation on PVC using phase-contrast microscopy.
A. Direct visualization of the initiation of biofilm formation over 7.5 h in our model system. Incubation of *P. aeruginosa* for up to 3 h in the presence of PVC results in the attachment of cells in a monolayer on this surface. At later time points (5 and 7.5 h), microcolonies can be observed dispersed throughout the monolayer (microcolonies are indicated by arrows).
B. Shown are phase-contrast micrographs of the wild-type strain, a representative pili-defective mutant (*pilB*) and a representative non-motile mutant (*flgK*) after incubation for 3 h at 37°C in the presence of PVC plastic. The wild-type and *pilB* strains attach to the PVC surface in a monolayer. Few or no cells are seen on the PVC plastic when the biofilm formation phenotype of the non-motile mutants is assessed.
C. The biofilm formation phenotype of the wild type, *pilB* (*sad-31*) and *flgK* at 8 h is shown. The wild-type strain forms a biofilm on the surface of the PVC plastic, which comprises a monolayer of cells punctuated by dispersed microcolonies. The *pilB* mutant has a slightly more dense monolayer than observed at 3 h but does not form the microcolonies evident with the wild-type strain. The *flgK* mutant has very few cells attached to the PVC plastic. Micrographs were taken at 400× magnification and ≈50 fields were searched for each strain tested and representative fields are shown.


A
30 min
1.5 hr
3 hr
5 hr
7.5 hr
B
wild-type
pilB
flgK
3 hrs
C
wild-type
pilB
flgK
8 hrs

1987; Grant *et al*., 1993; Korber *et al*., 1994; Simpson *et al*., 1995).

We also directly visualized the biofilm formation phenotype of a representative mutant defective in pili biogenesis (*pilB*). At the early time points (≤ 3 h), there was little difference in the biofilm formation phenotype of the wild type and the type IV pili mutants; both the wild-type and the pili-defective strain form a dispersed monolayer of cells on the surface of the PVC plastic. By 8 h, in contrast to the aggregates of cells formed by the wild-type strain, the pili-defective mutants did not develop these characteristic microcolonies. Furthermore, the wild-type strain almost completely covered the PVC surface with a dense, tightly packed layer of cells. The phenotype of the type IV pili mutants at this 8 h time point was unchanged from that observed at 3 h, that is a dispersed monolayer of cells. The other mutants defective in pili biogenesis (*pilC* and *pilY1*) had similar phenotypes (data not shown).

A role for twitching motility in biofilm formation

To define better the events that lead to microcolony formation by the wild type and to determine whether surface-based twitching motility plays a role in biofilm formation, we used phase-contrast time-lapse microscopy to follow a developing biofilm. Using time-lapse microscopy, we watched individual microcolonies formed by the wild-type strain over a period of 56 min (with images acquired at 15 s intervals). Shown in Fig. 5 is a montage of nine phase-contrast micrographs taken during biofilm formation by the wild-type strain every 7 min between 360 and 416 min after inoculation. Several microcolonies were followed through the course of this experiment to illustrate the movement of cells across the PVC plastic surface.

In Fig. 5, the white arrow indicates the position of a microcolony that is first clearly visible in B, becomes larger (C) but has dispersed by D. This microcolony does not reform during the course of this experiment (D–I). A series of time-lapse micrographs taken at 15 s intervals between 374 min (C) and 381 min (D) show that this microcolony disperses because the cells comprising the colony move apart while still remaining associated with the plastic surface (data not shown but can be viewed as a time-lapse movie at http://gasp.med.harvard.edu/GO5.html).

The black arrow points to a large microcolony evident in Fig. 5A. This large microcolony becomes progressively smaller (B–F) and eventually splits into two small, adjacent microcolonies (G). In H, these two adjacent microcolonies form a larger single colony that has grown slightly in size when visualized 7 min later (I).

The formation of microcolonies in this system is due to, in a large part, the aggregation of cells found dispersed in the monolayer of cells on the surface and not solely to the growth of the bacterial cells. This point is illustrated further by data presented in H and I. The dark circle in I indicates a dense, well-formed microcolony. However, this colony is

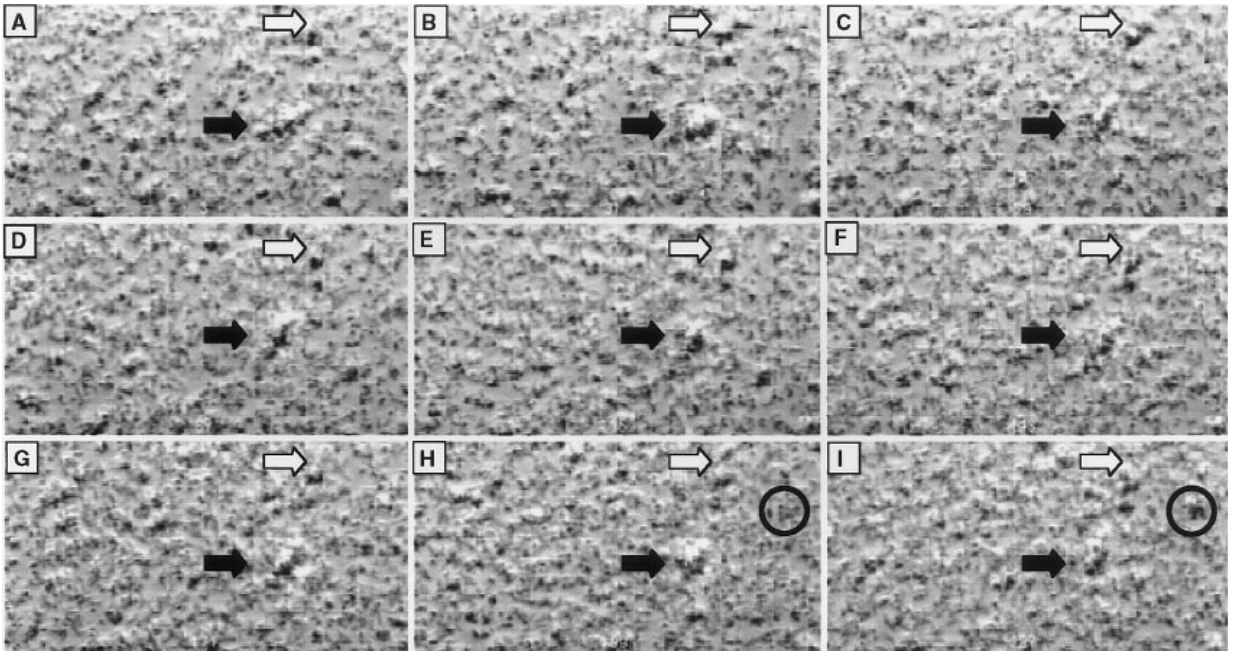


Fig. 5. A role for twitching motility in biofilm formation. Shown are a series of phase-contrast micrographs of a biofilm prepared as described in *Experimental procedures*. The first image (A) was taken at 6 h after inoculation and the subsequent images (B–I) are at 7 min intervals. Arrows indicate microcolonies that form and/or disperse over the course of the experiment. The black circles indicate the identical spot on the field in H and I. See the text for additional details.

not evident 7 min previously in H. The elapsed 7 min between the micrograph shown in H and the micrograph shown in I represents less than the time needed for a single population doubling under these growth conditions. Furthermore, analysis of the time-lapse film shows that this microcolony forms by recruiting adjacent cells from the monolayer (data not shown here, but can be viewed as a time-lapse movie at http://gasp.med.harvard.edu/GO5.html). The data described above and shown in Fig. 5 demonstrate the dynamic nature of microcolony formation and dispersal during the course of biofilm development.

As discussed above, type IV pili are required for surface-based twitching motility and mutants defective in type IV pili biogenesis do not make the microcolonies characteristic of the wild-type strain. It is important to note that none of the behaviours described above for the wild-type were observed in the representative type IV pili mutant, *pilB*. As shown above in Fig. 4, this strain does not form microcolonies when observed either after 8 h of growth or when monitored by time-lapse microscopy (data not shown).

Discussion

Flagellar-mediated motility, type IV pili and the initiation of biofilm formation

Among the non-biofilm forming mutants isolated in this screen were those defective in flagellar-mediated motility (Fig. 2). Motility has also been suggested to be involved in biofilm formation in other model systems (Montie *et al*., 1982; de Weger *et al*., 1987; Smit *et al*., 1989; Korber *et al*., 1994; O'Toole and Kolter, 1998; Pratt and Kolter, 1998). Therefore, the isolation of non-motile strains helps to validate our experimental approach. Furthermore, for one of the mutants (*sad-36*), we have shown that the insertion element in this strain is in a structural gene required for the synthesis of a functional flagellum. For a more complete discussion of the role of flagella-mediated motility in biofilm development in *Pseudomas* and *E. coli* see Pratt and Kolter (1998).

Another class of mutants isolated in this screen are those defective in the synthesis of type IV pili, including strains with mutations in *pilB*, *pilC* and *pilY1*. These polar-localized pili are virulence factors and have been implicated in both the adherence of bacteria to eukaryotic cell surfaces (Woods *et al*., 1980; Ramphal *et al*., 1984; Bieber *et al*., 1998; Doig *et al*., 1988; Tang *et al*., 1995) and in a form of surface-associated movement termed twitching motility (Bradley, 1980; Mattick *et al*., 1996; Darzins and Russell, 1997). The *pilBCD* operon is thought to encode accessory factors required for type IV pili biogenesis (Nunn *et al*., 1990). The *pilY1* gene has sequence similarity to the *pilC* genes of *Neisseria meningitidis* and *Neisseria gonorrhoeae*, which have been shown to be required for type IV pili biogenesis and attachment to eukaryotic cells in these organisms (Jonsson *et al*., 1991; Rudel *et al*., 1995; Rahman *et al*., 1997).

Using the sad *mutants to initiate the dissection of the biofilm developmental pathway*

We have used the *sad* mutants, in conjunction with phase-contrast microscopy, to begin to elucidate the early steps in biofilm formation on an abiotic surface. The goal of these studies is to correlate specific bacterial structures with defined steps in the biofilm developmental pathway. Our current model for biofilm formation is shown in Fig. 6. The direct visual inspection of the biofilm formation phenotype of non-motile strains on PVC plastic revealed that, compared with the wild-type strain, only very few cells could make a stable interaction with this abiotic surface (Fig. 4B). Furthermore, our studies were performed with a molecularly characterized non-motile strain with a defined defect in flagellar synthesis. Earlier studies used uncharacterized strains that may have had pleiotrophic defects (Lawrence *et al*., 1987; Mills and Powelson, 1996). Our observations, which concur with previous studies (Lawrence *et al*., 1987), suggest that motility is important for the cells to make initial contacts with an abiotic surface. From these experiments it is not clear whether the flagellum plays a direct role as an adhesin as previously suggested for *P. fluorescens* (Lawrence *et al*., 1987), or, as also proposed, that flagellar-mediated motility is required to bring the cell within close proximity of the surface to overcome repulsive forces between the bacterium and the surface to which it will eventually attach (Mills and Powelson, 1996). It is possible that flagellar-mediated motility is required for both of these processes.

The direct visual analysis of mutants defective in type IV pili biogenesis revealed that early biofilm formation by these mutants ($\leq$ 3 h) was very similar to the wild-type strain, in

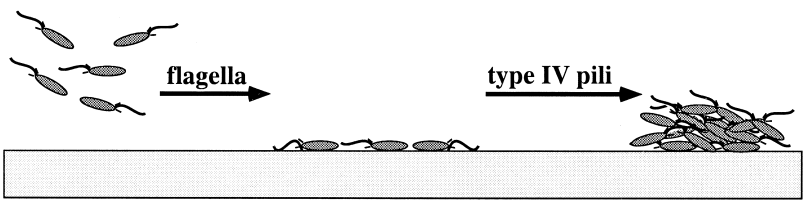


Fig. 6. A model for biofilm formation. Shown is our current model for the role of flagella and type IV pili in biofilm formation. Flagella or flagella-mediated motility appears to be important for the formation of a bacterial monolayer of the abiotic surface. Type IV pili appear to play a role in downstream events such as microcolony formation. See the text for additional details.

that all of these strains form a dispersed monolayer of cells on the PVC plastic. However, the wild-type strain eventually forms a dense, tightly packed monolayer of cells punctuated by microcolonies on the plastic surface; the pili-defective mutants remained as a dispersed monolayer of cells (Fig. 4B and C). Based on our observations, it appears that type IV pili are required downstream of flagella but still early in this developmental pathway.

The data above suggest possible roles for type IV pili and type IV pili-mediated twitching motility in *P. aeruginosa* biofilm development. It is possible that type IV pili play a direct role in stabilizing interactions with the abiotic surface (that may have been initiated via flagella or flagella-mediated motility) and/or in the cell-to-cell interactions required to form a microcolony. Type IV pili-mediated twitching motility may also be necessary for cells to migrate along the surface to form the multicell aggregates characteristic of the wild-type strain. In support of such a role for twitching motility in biofilm formation, we present evidence that the wild-type strain does move across the surface and form cell aggregates by recruiting cells from the adjacent monolayer (Fig. 5). It is important to note that strains defective in pili biogenesis (like the *pilB* mutant) express neither twitching motility nor microcolony formation phenotypes.

The microscopic colonies formed by *P. aeruginosa* PA14 on PVC plastic during biofilm development are reminiscent of the macroscopic aggregates formed by *Myxococcus* during the development of fruiting bodies (Kaiser, 1984). Furthermore, the development of these fruiting bodies by *Myxococcus* has also been shown to require type IV pili (Wu and Kaiser, 1995). Interestingly, recent studies suggest a requirement for homoserine lactones (HSLs) to express type IV pili-mediated twitching motility (Glessner *et al*., 1998). These data suggest a role for cell-to-cell signalling early in microcolony formation in addition to the established role of HSLs in the later stages of biofilm development (Davies *et al*., 1998). However, it is also important to note that type IV pili mutants can still interact with the abiotic surface, suggesting the existence of additional, unidentified adhesions that promote cell-to-surface interactions.

The requirement for type IV pili in biofilm formation on an abiotic surface has an additional important implication. As mentioned above, type IV pili have been shown to be important for bacterial adhesion to eukaryotic cell surfaces and pathogenesis (Woods *et al*., 1980; Ramphal *et al*., 1984; Sato *et al*., 1988; Ramphal *et al*., 1991; Tang *et al*., 1995; Bieber *et al*., 1998). These data suggest that there may be an overlap in factors required for the initiation of biofilm formation on an abiotic surface and the factors necessary for bacterial attachment and pathogenesis *in vivo*. If this is the case, the biofilm formation assay presented here may serve as a simple primary screen for identifying novel virulence factors. The analysis of additional mutants isolated in this screen and their testing in models of pathogenesis is in progress.

Experimental procedures

Bacterial strains, media and chemicals

P. aeruginosa PA14 was grown on rich medium (Luria Bertani; LB) or minimal medium (as indicated in each experiment) at 37°C, unless otherwise noted. The minimal medium used was minimal M63 salts (Pardee *et al*., 1959) supplemented with glucose (0.2%), $MgSO_4$ (1 mM) and, where indicated, casamino acids (CAA, 0.5%). Antibiotics were added at the following concentrations: for *E. coli*, ampicillin (Ap), 150 μg ml^{-1}; naladixic acid (Nal), 20 μg ml^{-1}; for *P. aeruginosa*, Tc, 150 μg ml^{-1}. All enzymes for DNA manipulation were purchased from New England Biolabs. All plasmids were constructed in *E. coli* JM109 using standard protocols (Ausubel *et al*., 1990). Plasmids were transferred to *P. aeruginosa* by electroporation (Bloemberg *et al*., 1997). Transductions were performed as reported (Jensen *et al*., 1998). Assays for assessing flagellar-mediated motility were performed as reported (O'Toole and Kolter, 1998). Twitching motility was assessed as described (Whitchurch *et al*., 1990).

Genetic techniques

Transposon mutants were generated with Tn*5*-B30(Tc^r) using a modification of published protocols (Simon *et al*., 1989) as described (O'Toole and Kolter, 1998). The resulting transposon mutants were screened as described below.

Biofilm formation assay

Screen for mutants defective in biofilm formation

This assay is based on the ability of bacteria to initiate biofilm formation on polyvinylchloride plastic (PVC). The initiation of biofilm formation was assayed as described (O'Toole and Kolter, 1998).

Quantification of biofilm formation

Biofilm formation was quantified as described (O'Toole and Kolter, 1998). Briefly, the crystal violet was solubilized in 95% ethanol and the absorbance was determined at 600 nm.

Microscopy

Visualization of bacterial cells attached to PVC was performed by phase-contrast microscopy (400× magnification) using a Nikon Diaphot 200 inverted microscope (Nikon). The images were captured with a black and white CCD72 camera integrated with a Power Macintosh 8600/300 computer with video capability (Cupertino). The images were processed using SCION IMAGE software, a modification of NIH IMAGE (NIH) by the Scion Corporation.

Molecular techniques

DNA sequence flanking transposon mutants were determined

using arbitrary PCR (Caetano-Annoles, 1993) as described (O'Toole and Kolter, 1998). Southern blots were performed as follows: chromosomal DNA of the *sad* mutants was prepared (Pitcher *et al*., 1989), digested with *Eco*RI (Tn*5*-B30 does not have a *Eco*RI site) and transferred to GeneScreen Plus (NEN Research Products) as reported (Ausubel *et al*., 1990). The hybridization was performed with the ECL direct nucleic acid labelling and detection system (Amersham Life Science) according to the manufacturer's instructions without modification. The DNA probe used was derived from the insertion sequence element (IS50) of Tn*5* and generated using PCR with the Tn*5* element as a template. The PCR primers used to generate the probe were IS50R.1 (5′-GCTTCCTTTAGCAGCCCTTGCGC-3′) and IS50R.2 (5′-CTTCCATGTGACCTCCTAACATGG-3′).

Acknowledgement

We thank L. G. Rahme for providing us with strain *P. aeruginosa* PA14. This work was supported by grant number GM58213 from the NIH to R.K. and Fellowship DRG of the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation to G.A.O. We also thank the Micro Core Facility at the Harvard Medical School for DNA sequencing and L. A. Pratt and S. E. Finkel for a critical review of the manuscript.

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