Steps in the development of a *Vibrio cholerae* El Tor biofilm

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Summary

We report that, in a simple, static culture system, wild-type *Vibrio cholerae* El Tor forms a three-dimensional biofilm with characteristic water channels and pillars of bacteria. Furthermore, we have isolated and characterized transposon insertion mutants of *V. cholerae* that are defective in biofilm development. The transposons were localized to genes involved in (i) the biosynthesis and secretion of the mannose-sensitive haemagglutinin type IV pilus (MSHA); (ii) the synthesis of exopolysaccharide; and (iii) flagellar motility. The phenotypes of these three groups suggest that the type IV pilus and flagellum accelerate attachment to the abiotic surface, the flagellum mediates spread along the abiotic surface, and exopolysaccharide is involved in the formation of three-dimensional biofilm architecture.

Introduction

Bacteria in aquatic environments are rarely found in the planktonic or free-swimming phase (Costerton *et al*., 1987). Rather, they are found in association with a solid surface. Attached bacteria may take the form of a dispersed monolayer of cells on a surface, they may be clustered on the surface in microcolonies, or they may be organized into a three-dimensional biofilm (Costerton *et al*., 1995).

It has been suggested that attachment to surfaces and formation of a biofilm may provide an adaptive advantage for aquatic organisms. For example, less soluble and less easily metabolizable large organic compounds (i.e. humic acids), which are found adsorbed to aquatic surfaces, may provide nutrients for attached bacteria (Mills and Powelson, 1996). Alternatively, attachment to surfaces, such as the exoskeletons of crustaceans or insects, which are made out of chitin, may provide nutrition directly to organisms, such as *Vibrio cholerae*, which are able to metabolize chitin (Fletcher, 1996). Formation of a biofilm may also provide protection from toxic compounds, such as antibiotics, which are present in the environment (Anwar *et al*., 1992; Vess *et al*., 1993). Biofilm formation may therefore be a survival mechanism for bacteria and other microbes living in an aquatic environment.

Biofilm development is a multistep process. Bacteria approach the surface, attach and then become immobilized on the surface. In order to form microcolonies, the bacteria move along the surface and associate with one another. Finally, an ordered three-dimensional structure is formed. This structure is composed of pillars of bacteria surrounded by water channels that allow nutrients to reach biofilm-associated bacteria and allow toxic metabolites to diffuse out of the biofilm (Costerton *et al*., 1995).

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The genetic basis of colonization of abiotic surfaces has been studied in *Escherichia coli* (Pratt and Kolter, 1998) and *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998a). In *E. coli*, both type I pili and flagella are important for attachment to the surface, while flagella alone are important for bacterial movement on a surface. In contrast, in *P. aeruginosa*, flagella are important for initial attachment to the surface, while microcolony formation does not occur in the absence of type IV pili, presumably because of a lack of twitching motility. Twitching is a type of surface motility that is thought to occur through anchoring of a pilus to a fixed surface, followed by retraction of the pilus against the surface (Wall and Kaiser, 1999).

Biofilm formation has been described as a developmental process (Costerton *et al*., 1995; O'Toole and Kolter, 1998). In support of this, Davies *et al*. (1998) have shown that an acylated homoserine lactone functions as a developmental signal in the formation of the characteristic three-dimensional biofilm architecture in *P. aeruginosa*. Furthermore, the expression of alginate, an exopolysaccharide present as an extracellular matrix in *P. aeruginosa* biofilms, is induced upon contact with a surface (Davies *et al*., 1993; Hoyle *et al*., 1993).

We are interested in understanding the impact that the ability to form a biofilm has on the emergence of human pathogens that are also natural inhabitants of the aquatic environment. *V. cholerae*, a Gram-negative rod responsible for the severe diarrhoeal disease known as cholera, is a prototype of such pathogens. During recorded history, two biotypes of *V. cholerae* have devastated large regions of the world in seven pandemics (Colwell, 1996). The classical biotype is thought to have caused the first six pandemics. Currently, *V. cholerae* El Tor is the dominant biotype of *V. cholerae* worldwide and is responsible for the seventh pandemic of cholera. It has been hypothesized that the adaptation of *V. cholerae* to survival in estuarine and freshwater aquatic environments is essential to its persistence as a pandemic pathogen (Colwell and Spira, 1992). Because attachment of *V. cholerae* to aquatic surfaces is believed to be adaptive, and many studies have demonstrated attachment of *V. cholerae* to environmental surfaces such as plants, filamentous green algae, zooplankton, crustaceans and insects (Huq *et al*., 1986; Tamplin *et al*., 1990; Shukla *et al*., 1995), we hypothesize that biofilm formation may be an important factor in the survival of *V. cholerae* in the aquatic environment.

A study of the genetic basis of biofilm development by *V. cholerae* El Tor is necessary to begin to unravel the role played by biofilm formation in the emergence of this pathogen. Recently, the type IV pilus, MSHA, which is responsible for mannose-sensitive haemagglutination by *V. cholerae* El Tor, has been implicated in biofilm formation on non-nutritive, abiotic surfaces (Watnick *et al*., 1999). In addition, studies have clearly demonstrated that the colony morphology of the rugose form of *V. cholerae* El Tor, which forms thicker biofilms than non-rugose El Tor, results from an exopolysaccharide (EPS) encoded by the *vps* locus (Yildiz and Schoolnik, 1999). The steps in the development of large, three-dimensional structures of *V. cholerae* on abiotic surfaces, however, have not been defined. In this work, we undertake a genetic and microscopic analysis of the development of a *V. cholerae* El Tor biofilm.

Results

A simple experimental system for the development of three-dimensional biofilms

Flow cells have generally been used for the growth and study of three-dimensional biofilms (Lawrence *et al*., 1991). Such systems, however, require a specialized apparatus, maintenance of sterility over weeks and a large amount of liquid medium. In the experiments reported here, we placed a glass coverslip in a conical tube partially filled with

medium, inoculated the medium with the strain of interest and incubated the bacteria with the coverslip surface without agitation. The coverslip was then removed from the medium for observation. For *V. cholerae*, such an experimental design results in the development of a three-dimensional biofilm with pillars of bacteria and water channels (Fig. 1). Such biofilms reach a thickness of ≈ 25 μM after 5 days of incubation at room temperature. Although the flow cell is an excellent means of assessing biofilm formation, the systems described here greatly simplify the processing of a large number of strains or mutants.

Genetic screen for biofilm-deficient mutants

A screen for biofilm-deficient mutants of *V. cholerae* El Tor was undertaken. Briefly, transposon mutagenesis was used to create a library of mutants that was then screened for the ability to form biofilms in the wells of a polyvinylchloride (PVC) microtitre dish as described in *Experimental procedures*. Of the 10 000 mutants screened, 80 were operationally defined as defective in biofilm formation because of their inability to produce a crystal violet ring when stained in the microtitre dish assay. All of these mutants were screened for defects in flagellar-mediated motility. Nine of the 80 mutants were non-motile using the motility agar assay. The sequence of the transposon junctions of five of these mutants was determined. All five were shown to have insertions in known flagellar structural or motility genes. Sequence analysis of arbitrary polymerase chain reaction (PCR) products showed that transposon insertions in 45 mutants mapped to various genes involved in the production of a functional MSHA pilus. These results were confirmed by haemagglutination assays. Twenty-six additional mutants were both motile and able to agglutinate red blood cells. Sequence analysis of arbitrary PCR products demonstrated that five of these mutants had transposon insertions in the *vps* locus, which is responsible for exopolysaccharide production (Yildiz and Schoolnik, 1999). Six mutants had transposon insertions in genes of unknown function, and we were unable to obtain transposon insertion sequences from 15 additional mutants.

Description of mutant groups

Table 1 shows representative mutants of the three groups isolated, namely, mutants in the MSHA biogenesis and secretion pathways (MSHA), mutants in synthesis of exopolysaccharide (EPS) and flagellar mutants (MOT).

The first group of mutants included transposon insertions in many open reading frames (ORFs) of the operons controlling both biosynthesis and secretion of the MSHA pilus (Marsh and Taylor, 1999). All these mutants failed to agglutinate red blood cells. The MSHA group also included two mutants with independent transposon insertions in a gene with 66% identity in protein sequence to the *pilT* gene of *P. aeruginosa*. PilT, which has also been identified in *Myxococcus xanthus* and *Neisseria gonorrhoeae*, is believed to be responsible for the retraction of type IV pili (Whitchurch *et al*., 1991; Wu *et al*., 1997; Wolfgang *et al*., 1998). In these three bacteria, although *pilT* mutants are hyperpiliated and produce pili that are indistinguishable from wild-type pili when visualized by electron microscopy, they do not exhibit twitching motility. Using electron microscopy of negatively stained *V. cholerae* to study multiple specimens, we were able to detect single pili on occasional wild-type cells and multiple pili on occasional *pilT* mutant cells (data not shown). The frequency of piliated MSHA mutant cells was ≈ 20-fold less, possibly suggesting that another pilus may be present infrequently under these conditions. In *V. cholerae* El Tor, however, we have thus far been unable to demonstrate MSHA-based twitching motility by standard experimental techniques. We hypothesize, however, by analogy with *P. aeruginosa*, *N. gonorrhoeae* and *M. xanthus*, that MSHA pili do impart twitching motility to *V. cholerae* and that *pilT* mutants of *V. cholerae* possess MSHA pili that are non-functional.

MSHA pili, however, may not be able to generate sufficient force to effect twitching under the conditions we have tested.

The second group of mutants was defective in flagellar motility (MOT). Based on similarity to previously studied flagellar genes, this group included both mutants lacking flagella and mutants with paralyzed flagella. The predicted presence or absence of flagella was confirmed by electron microscopy. These mutants were also unable to agglutinate red blood cells. The inability of non-motile mutants of classical *V. cholerae* to haemagglutinate has been reported previously (Gardel and Mekalanos, 1996). Using electron microscopy of negatively stained *V. cholerae*, no pili were observed on non-motile cells, suggesting that motility affects the production of MSHA pili.

The third group of biofilm-deficient mutants had transposon insertions in genes involved in exopolysaccharide biosynthesis (EPS). Two of these genes have been identified previously as essential for exopolysaccharide production in the rugose form of *V. cholerae* El Tor (Yildiz and Schoolnik, 1999). All these mutants were able to agglutinate red blood cells in a mannose-sensitive fashion, indicating the presence of functional MSHA pili.

A crystal violet quantification of the biofilms formed by representative mutants on borosilicate glass after a 24 h incubation is shown in Fig. 2. This demonstrates (i) that biofilm formation defects of the mutants from all these classes are virtually indistinguishable by crystal violet staining at 24 h; and (ii) that mutants defective in biofilm formation on PVC are also defective on glass.

Rates of biofilm formation by biofilm-deficient mutants

We hypothesized that some of the biofilm mutants might have a lower rate of biofilm formation, and that, if given sufficient time, they would be able to form biofilms indistinguishable from the wild-type strain of *V. cholerae* El Tor. To begin to test this hypothesis, biofilm development by both wild-type and mutant *V. cholerae* was studied over the course of 72 h. Figure 3 quantifies biofilm formation over 72 h for the *V. cholerae* wild-type strain and representative biofilm-deficient mutants. All mutants in each group were tested and found to follow the pattern of the representative mutant illustrated in Fig. 3. In addition, time course assays were repeated on borosilicate with similar results.

As illustrated in Fig. 3, the wild-type strain reached a plateau in crystal violet staining after 29 h of incubation, while the MSHA mutant reached an equivalent plateau in crystal violet staining after ≈ 48 h. Furthermore, the rate of biofilm formation by the *pilT* mutant was indistinguishable from that of other MSHA mutants. This suggests that the presence of a non-functional MSHA pilus does not measurably enhance biofilm-forming ability beyond that of an MSHA structural mutant.

EPS mutants did not form a biofilm detectable by crystal violet staining during the course of the experiment. This suggests that EPS mutants are not simply slower but unable to form a biofilm under the conditions of this experiment.

Flagellar mutants were able to form a biofilm measurable by crystal violet staining after ≈ 72 h. No difference in the rate of biofilm formation was observed between mutants with a paralyzed flagellum and mutants with no flagellum at all. As was found for MSHA, therefore, the presence of a non-motile flagellum does not improve biofilm-forming ability beyond that of a flagellar structural mutant.

Microscopic analysis of wild-type and mutant biofilm structure

Although measurement of the crystal violet incorporated into a biofilm allows easy estimation of the bacterial biomass adherent to a surface, microscopy is required to determine the spatial distribution of the adherent bacteria. To study the development of the wild-type and mutant biofilm architectures further, we observed the biofilms microscopically at 15 min, 28 h and 72 h after inoculation. These experiments were repeated multiple times with similar results. Furthermore, the total surface area examined in each of these studies was at least 0.06 cm^2, and images shown are representative of what was observed in multiple fields. Figure 4 shows attachment of the wild-type and mutant cells to the bottom of a microtitre well at 15 min. This demonstrates clearly that, while wild-type and EPS mutants were able to attach readily in 15 min, no surface-associated MSHA and flagellar mutants were observed. This pattern was also observed on borosilicate (results not shown).

After 24 h of incubation with a borosilicate glass coverslip, the pattern of attachment we observed for wild-type and representative *V. cholerae* mutants was quite different (Fig. 5). The wild-type *V. cholerae* formed a confluent biofilm on the coverslip, the MSHA mutant was just beginning to form the microcolonies that are characteristic of incipient biofilms, the EPS mutant remained a monolayer of isolated, attached cells, and the flagellar mutant was not associated with the surface.

After 72 h of incubation on the borosilicate surface, the wild-type biofilm reached a thickness of 10–15 μm, and confocal microscopy was necessary to visualize the biofilm structure. In Fig. 6, representative vertical cross-sections are shown for 72 h biofilms formed by the various mutants. In this simple system, wild-type *V. cholerae* forms a biofilm with the characteristic architecture of pillars of bacteria and water channels that is observed in continuous flow systems (Costerton *et al*., 1995). Furthermore, the MSHA mutant has formed a biofilm that is indistinguishable from the wild type. In contrast, the flagellar mutant has a few localized areas of attachment. Within these areas, however, the biofilm is approximately as thick as the wild type and contains water channels that characterize the wild-type biofilm. Finally, even after 3 days, the EPS mutant remains attached to the surface as a monolayer. Taken together, these results indicate (i) that pili and flagella facilitate but are not absolutely required for attachment to the surface; (ii) that flagella are required for spread along the surface; and (iii) that EPS is necessary for formation of the three-dimensional biofilm structure.

Discussion

We are interested in the role of biofilm formation in the emergence of new pathogens from aquatic environments. In order to understand the genetic basis of biofilm formation in our prototype, *V. cholerae*, we have isolated and characterized transposon insertion mutants that are defective in biofilm development. The mutants identified can be divided into three groups: (i) those with insertions in genes involved in type IV pili biogenesis and function; (ii) those with insertions in genes involved in flagellar motility; and (iii) those with insertions in genes involved in EPS synthesis.

Two external structures, the flagellum and MSHA type IV pili, are involved in attachment to the abiotic surface. These two structures may promote attachment to a surface either by acting as specific adhesins or through the generation of force. Time course studies of biofilm formation by the relevant mutants demonstrate that flagella and MSHA pili accelerate, but are not required for, attachment to abiotic surfaces. Furthermore, there is no difference in the rate of biofilm formation by mutants that have no flagellum and mutants with a paralyzed flagellum. We also see no difference in the rate of biofilm formation between *mshA* mutants

and *pilT* mutants, suggesting, by analogy with the *pilT* gene of *P. aeruginosa*, that the presence of a non-functional pili does not enhance the ability of the bacterium to attach to a surface. These two observations (i) that both pili and flagellar mutants eventually attach to abiotic surfaces; and (ii) that the rates of biofilm formation by mutants in which the flagellum and the MSHA type IV pili are either paralyzed or absent are indistinguishable, suggest that the generation of force is the essential property of these structures in accelerating attachment to an abiotic surface.

The requirement for the generation of force in attachment of *V. cholerae* to an abiotic surface is consistent with the existence of a repulsion between the bacterium and the abiotic surface. Thus, if the flagellum and pili are absent, there is still a finite probability that the bacterium will collide with the surface with enough force to overcome the repulsion, and attachment to the surface will occur, albeit more slowly. Electrostatic interactions have been hypothesized to be responsible for a repulsion between the bacterium and the surface that is sensed over a distance of 10–20 nm (Fletcher, 1996). Once the bacterium overcomes this repulsion, it may reach a distance of < 1 nm from the abiotic surface, where specific interactions are thought to be responsible for the strong attraction between the bacterium and the surface that results in attachment (Gristina, 1987; Fletcher, 1996). Experimental measurements of these types of forces have only been undertaken recently (Razatos *et al*., 1998). More detailed studies are required to understand the nature and magnitude of these forces and their effect on attachment to a surface.

We have also isolated biofilm-defective mutants that initially attach to abiotic surfaces in a manner that is indistinguishable from the wild type but are then unable to develop into a three-dimensional biofilm. These are the mutants in EPS production. EPS has been implicated in the distinctive colony morphology of the rugose variant of *V. cholerae* El Tor (Yildiz and Schoolnik, 1999). Interestingly, the work of Yildiz and Schoolnik (1999) has shown that EPS was absent from colonies of wild-type *V. cholerae* El Tor. Our studies imply, however, that EPS is expressed in biofilm-associated cells of wild-type *V. cholerae* El Tor. This suggests that EPS may be expressed under different conditions in the wild type and rugose variant and, perhaps, that a part of the regulatory machinery that controls EPS production in the wild type is bypassed in the rugose variant. Exopolysaccharide production has been implicated in biofilm formation previously (Davies *et al*., 1993; Hoyle *et al*., 1993; Yildiz and Schoolnik, 1999). Our finding supports a role for exopolysaccharide in stabilizing the three-dimensional biofilm structure. Possible mechanisms for this stabilization include physical constraint and minimization of intercellular repulsions both by maintenance of an intercellular distance and by shielding of the electrostatic charges on the bacterial surface.

We have isolated and characterized *V. cholerae* mutants that are defective in attachment, surface motility and formation of a three-dimensional structure. These mutants define a developmental pathway for biofilm formation by *V. cholerae* El Tor. Figure 7 depicts our current model of the role that these gene products play in the development of a three-dimensional biofilm. Flagellar motility allows the bacterium to swim through a repulsive potential towards the abiotic surface. As the bacterium nears the surface, the MSHA pilus is able to tether the bacterium and pull it onto the surface, where attractive interactions with the surface lead to attachment. Movement along the surface again involves the generation of force by flagella, because the attraction between the bacterium and the surface must be overcome in order to move. Finally, EPS is required to stabilize cell–cell interactions that are integral to the formation of a three-dimensional biofilm.

V. cholerae El Tor is the predominant cause of cholera in the world today. This suggests that it has taken over the niche of classical *V. cholerae* in the environment. The biofilms

produced by *V. cholerae* El Tor are thicker and more densely packed than those produced by classical *V. cholerae* (P. I. Watnick and R. Kolter, unpublished results). Because biofilm formation is such an important part of survival in the aquatic environment, we hypothesize that its superior ability to form biofilms may impart a survival advantage to *V. cholerae* El Tor. This has perhaps contributed to its worldwide predominance. We plan to address this hypothesis in future studies.

Experimental procedures

Bacterial strains and plasmids

N16961Sm, a streptomycin-resistant mutant of a *V. cholerae* El Tor strain isolated from the seventh pandemic, was used for all biofilm studies. KFV11 (Watnick *et al*., 1999), a mutant of N16961Sm containing a large, in frame deletion in the *mshA* gene, was used as the prototype for studies of biofilm formation by all mutants blocked in MSHA secretion and biogenesis. Transposon mutagenesis was carried out by mating with the *E. coli* strain β-2155, which is a diaminopimelate (DAP) auxotroph and *λpir* lysogen (Kolter *et al*., 1978), containing pBSL180, a suicide plasmid carrying a mini-Tn*10* transposon derivative marked with a kanamycin (Km) cassette (Pridmore, 1987; Alexeyev and Shokolenko, 1995). For fluorescent labelling of biofilms, *V. cholerae* was transformed with pSMC2 (Bloemberg *et al*., 1997), a plasmid containing a constitutively expressed gene for the green fluorescent protein (GFP). Approximately 60% of the cells lose plasmid-mediated ampicillin resistance during 24 h of growth unless antibiotic selection is provided. Thus, GFP-labelled *V. cholerae* biofilms were formed in the presence of antibiotic selection.

Transposon mutagenesis and screen for defects in biofilm formation

Matings of *V. cholerae* (strain N16961Sm) with *E. coli* strain β-2155 (pBSL180) were carried out for 2 h at 37°C on Luria–Bertani agar plates containing 0.3 mM DAP. Transposon insertion mutants of *V. cholerae* were then selected by plating on LB agar containing 50 μg ml^{-1} added Km. The screen for biofilm-defective mutants was performed in a fashion similar to that described previously for *P. aeruginosa* and *E. coli* (O'Toole and Kolter, 1998b; Pratt and Kolter, 1998). Briefly, Km-resistant colonies were transferred to polyvinylchloride (PVC) microtitre dish wells filled with 100 μl of LB broth. The dishes were allowed to incubate at room temperature for 24 h. At this point, the wells were rinsed and stained with crystal violet as described previously (O'Toole and Kolter, 1998b). Mutants that were unable to produce a biofilm in the microtitre dish assay were retrieved from the master plate and retested in triplicate. Mutants that failed to make biofilms on the secondary screen were stored in glycerol at −80°C for further study.

Arbitrary PCR

For sequence analysis of transposon junctions, an arbitrary PCR protocol was used as described previously (O'Toole and Kolter, 1998b). Arbitrary PCR involves two rounds of PCR. The first round includes a primer unique to the transposon and, in this case, two arbitrary primers that are designed to hybridize to an arbitrary sequence on the chromosome. The second round includes a nested primer unique to the transposon as well as a primer that is identical to the 5′ end of the arbitrary primer. This round is designed specifically to amplify PCR products obtained in the first round. Arbitrary PCR products were obtained using the following two sets of primers: (i) P12 with ARB1 and ARB6 (round 1), P11 with ARB2 (round 2); or (ii) P20 with ARB1 and ARB6 (round 1) followed by P2 with ARB2 (round 2). The sequences of these primers are listed below. Both these sets of primers were intended for sequence analysis upstream of the left transposon junction.

Transposon primers—P12: 5′-CAGCGCATCGCCTTCTAT CGC-3′; P11: 5′-CTTGACGAGTTCTGAGCGGG-3′; P20: 5′-CCGCGGTGGAGCTCC-3′; P2: 5′-ATGACAAG ATGTG-TATCCACC-3′.

Arbitrary primers—ARB1: 5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNNNGATAT-3′; ARB6: 5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNNNACGCC-3′; ARB2: 5′-GGCCACGCGTCGACTAGTAC-3′.

Motility and haemagglutination assays

Motility and haemagglutination assays with human red blood cells were performed as described previously (Gardel and Mekalanos, 1996; Pratt and Kolter, 1998).

Time course assays

Sterile, covered polystyrene microtitre dish wells were filled with 100 μl of LB broth with 100 μg ml^{-1} added streptomycin. Mutants were inoculated in triplicate and allowed to incubate for times varying from 8 h to 72 h. Before biofilm quantification, the growth rate of mutant strains was assessed by measuring the OD_{630} of all wells using a microtitre plate reader. Growth defects were not noted for any of the mutants selected for further study. Wells were subsequently rinsed, adherent bacteria were stained with crystal violet, and 300 μl of dimethylsulphoxide (DMSO) was added to each well to resuspend attached crystal violet. Biofilm formation was then quantified by measuring an OD_{570} directly for each well using a microtitre plate reader.

Phase microscopy

For observation of initial attachment to a surface, biofilms were formed on the bottoms of sterile, 24-well polystyrene microtitre dishes. This was done by growing cultures to mid-log phase, adding 400 μl of the culture to a well and observing attachment to the bottom of the well over time using an inverted-phase microscope equipped with a CCD video camera system (Optronics Engineering) and computer interface (magnification 400×). For incubations of 24 h or more, biofilms of the *V. cholerae* strain of interest were formed at room temperature on borosilicate coverslips placed in 50 ml Falcon tubes that had been filled with 6 ml of LB broth with appropriate antibiotics added and a 1:100 dilution of an overnight *V. cholerae* culture. At the desired end-point, the coverslip was rinsed with LB broth or distilled water to remove non-adherent bacteria and placed over a concave microscope slide filled with LB broth. Bacteria associated with the biofilm were then observed at 600× magnification using an Optiphot-2 microscope (Nikon) equipped with a CCD video camera system (Optronics Engineering) and computer interface.

Confocal scanning laser microscopy

Biofilms of the GFP-expressing *V. cholerae* strain of interest were formed on borosilicate coverslips as described above. Confocal microscopy was performed on 72 h biofilms. A MRC-1024 confocal microscope (Bio-Rad) was used to collect z-sections through the biofilm of interest using a 488 nm excitation wavelength for visualization of the fluorescent bacteria (magnification 600×).

Acknowledgments

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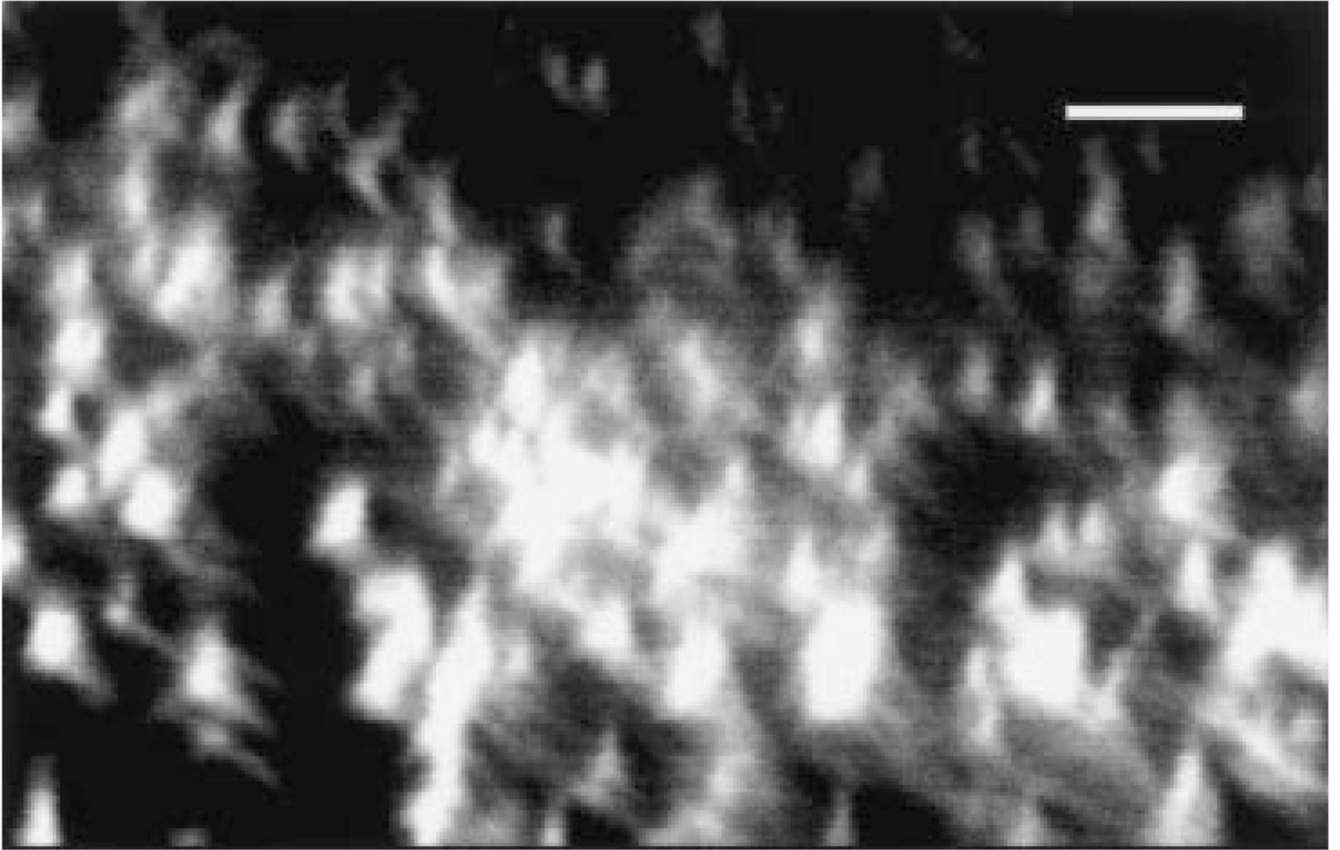
Fig. 1.
Confocal scanning laser micrograph through the xz-plane of a 5-day-old biofilm of *V. cholerae*. Biofilms were formed with *V. cholerae* that constitutively express GFP from a plasmid. Thus, bright areas represent biofilm-associated bacteria. The substratum is located at the bottom of the micrograph. Bar = 5 μm.
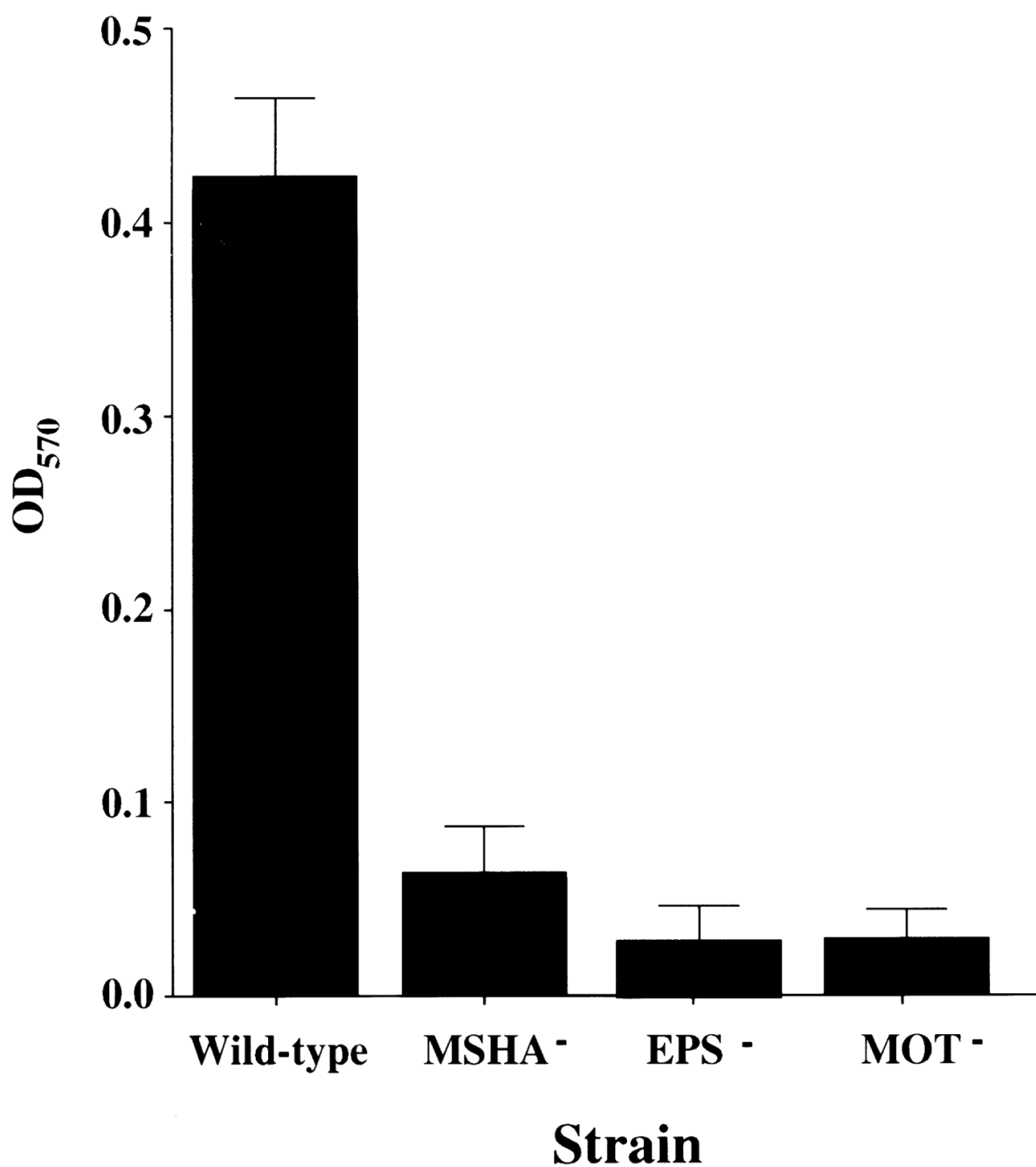
Fig. 2.
Quantification of bacteria in biofilms formed by wild-type *V. cholerae*, an MSHA mutant (MSHA), an exopolysaccharide mutant (EPS) and a motility mutant (MOT). The biofilms were stained with crystal violet. Crystal violet was solubilized, and the optical density of the resultant solution was measured.
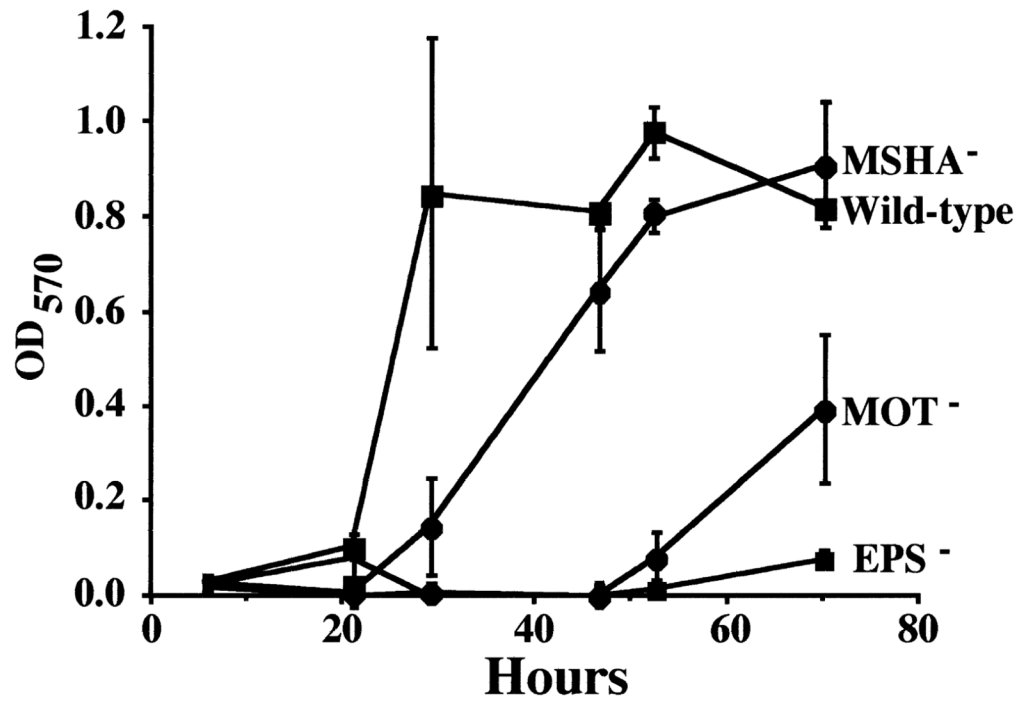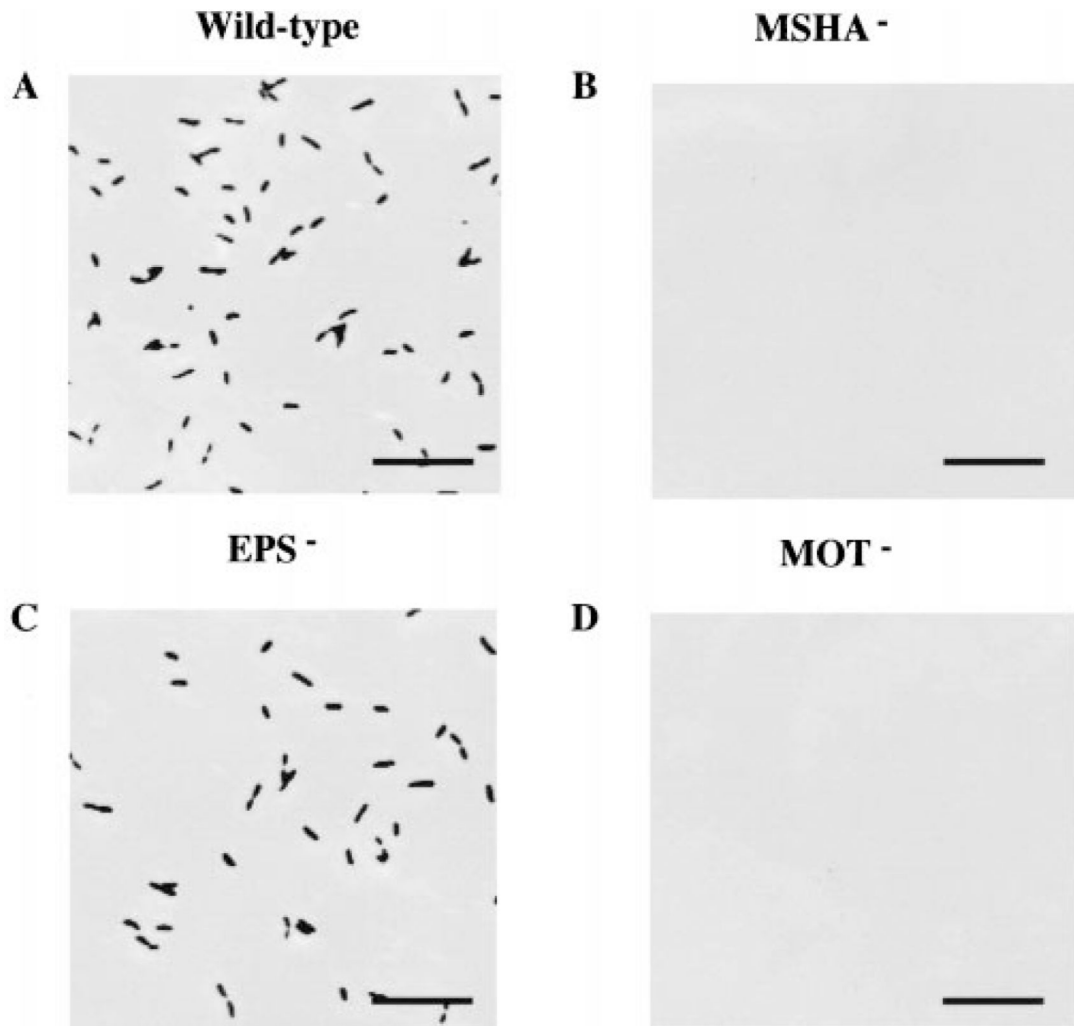
Fig. 3.
Biofilm development over 72 h for *V. cholerae* El Tor and representative derivative mutants.
OD_{570} quantifies the amount of crystal violet associated with the biofilm after staining.

Wild-type

MSHA -

A

B

EPS -

MOT -

C

D

Fig. 4.
Phase-contrast micrographs comparing the attachment of wild-type *V. cholerae* and
representative mutants after 15 min of incubation with a polystyrene surface. Bar = 5 μm.
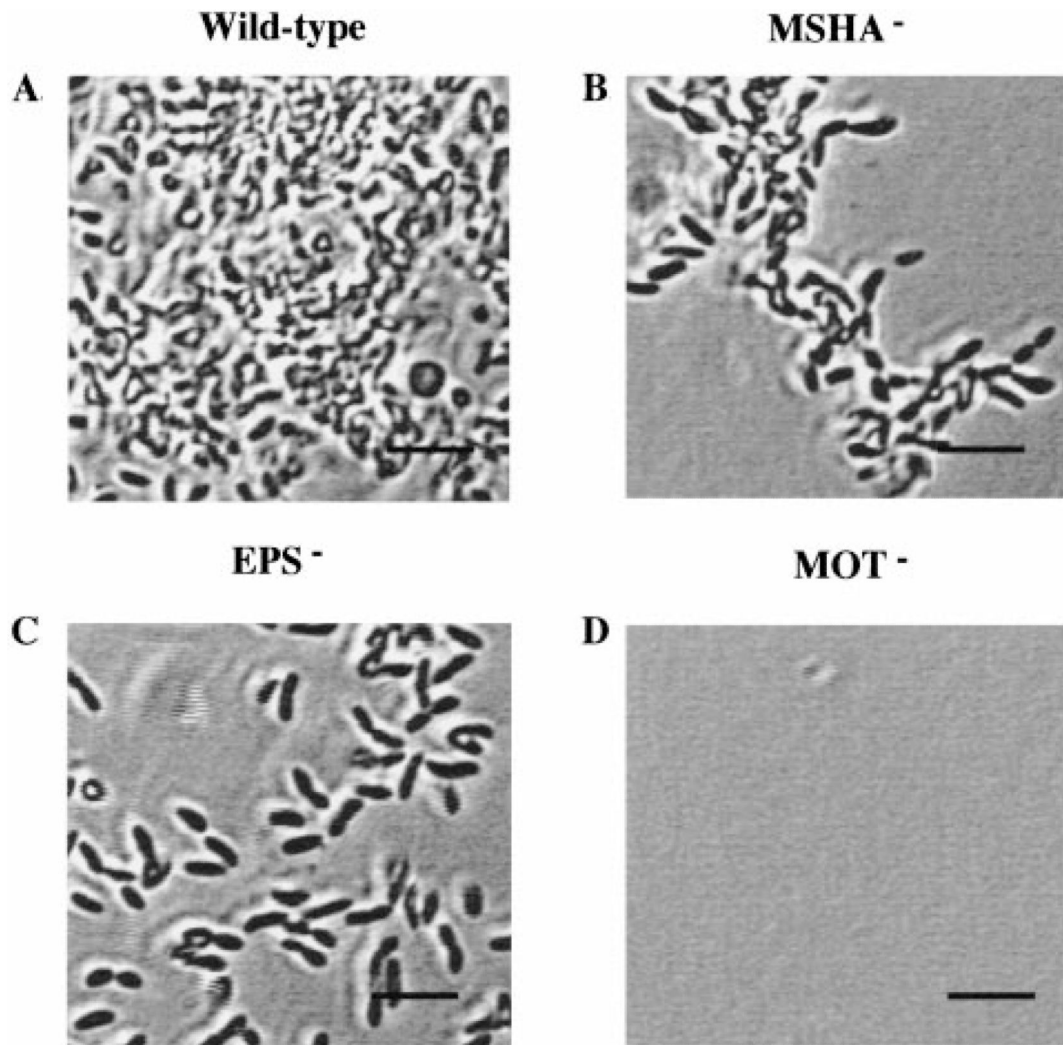
Fig. 5.
Phase-contrast micrographs comparing the attachment of wild-type *V. cholerae* and representative mutants after 24 h of incubation with a borosilicate coverslip. Bar = 2 μm.
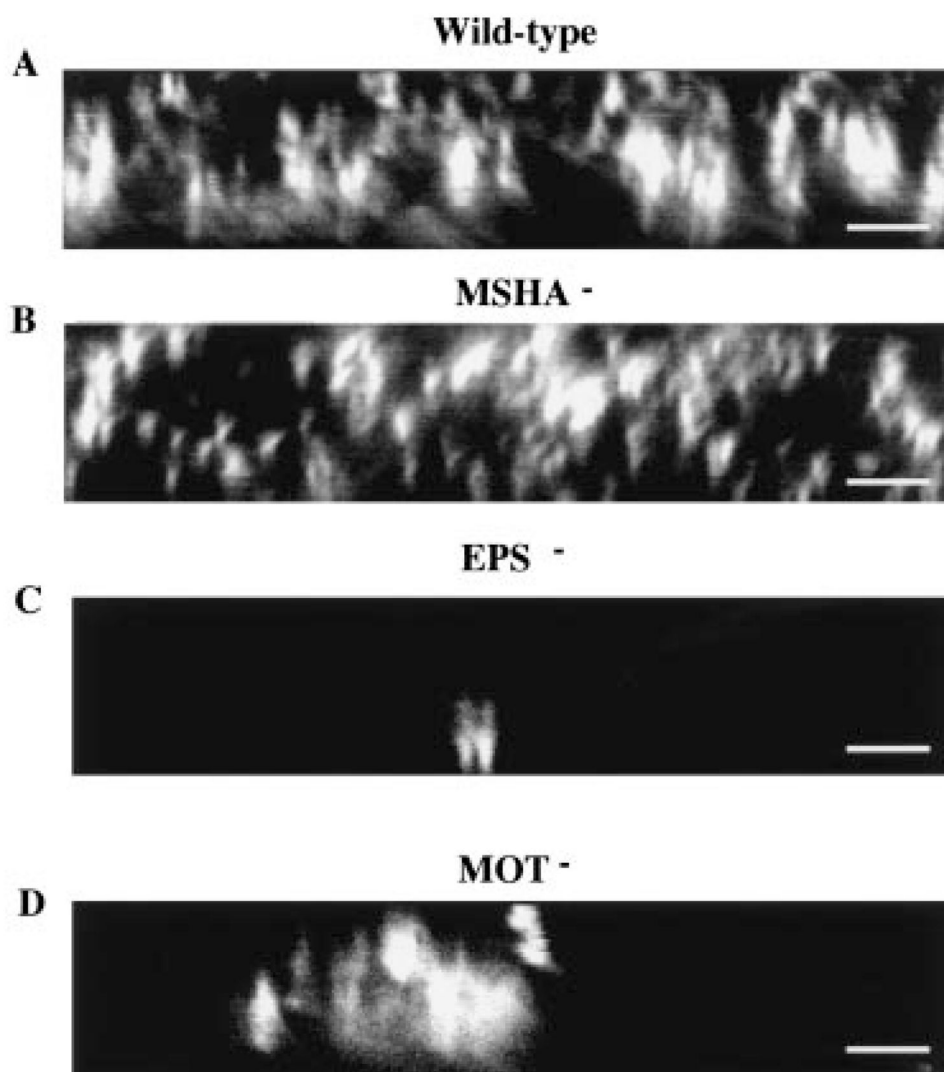
Wild-type

Fig. 6.
Confocal scanning laser micrographs comparing the attachment of wild-type *V. cholerae* and representative mutants after 72 h of incubation with a borosilicate coverslip. Micrographs represent optical sections in the xz-plane with the substratum located at the bottom of the micrograph. Bar = 5 μm.
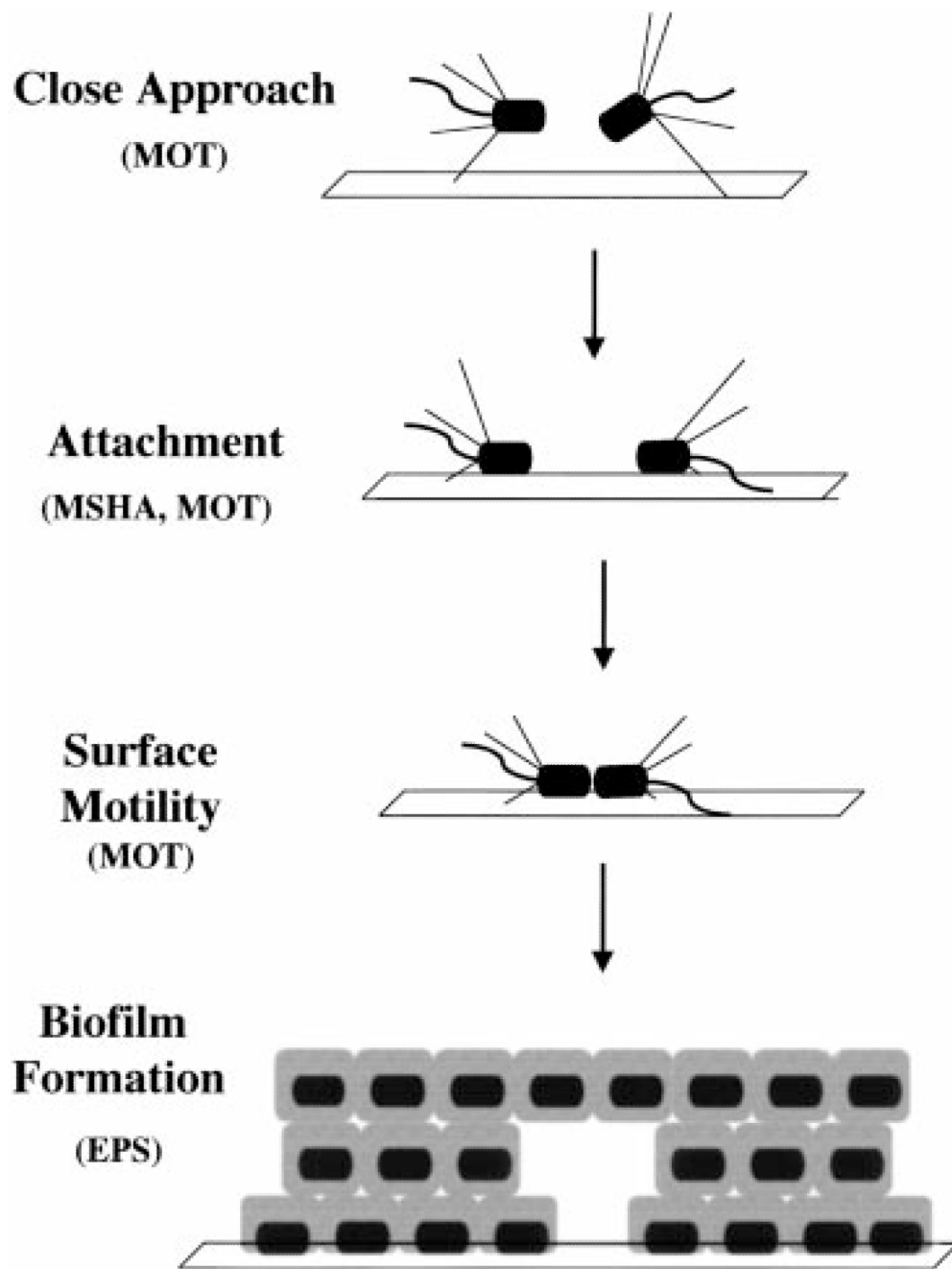
Fig. 7.
Proposed steps in the formation of a three-dimensional *V. cholerae* biofilm on an abiotic surface. Rods are *V. cholerae* with polar type IV MSHA pili and one polar flagellum. The bacteria swim towards the surface using flagellar motility. The bacteria attach to the surface using type IV pili, which then retract to draw the bacterium onto the surface. Flagella allow movement along the surface and microcolony formation, and EPS is important for the three-dimensional arrangement of bacteria.

Table 1

Representative biofilm mutants.

Mutant group	Identity/similarity (organism)	Function
Mannose-sensitive haemagglutination	MshA (*V. cholerae*)	Attachment to abiotic surfaces (Watnick *et al.*, 1999)
	PilT (*P. aeruginosa*)	Twitching motility (Whitchurch *et al.*, 1991)
Exopolysaccharide production	WcaJ (*E. coli*)	Colanic acid biosynthesis (Stevenson *et al.*, 1996)
	EpsD (*Ralstonia solanacearum*)	Exopolysaccharide biosynthesis (Huang and Schell, 1995)
	Cps19fF (*Streptococcus pneumoniae*)	Putative UDP-N-acetyl-D-mannosamine transferase (Morona *et al.*, 1997)
Motility	FlrC (*V. cholerae*)	σ^{54} transcriptional activator (Klose and Mekalanos, 1998)
	MotY (*V. alginolyticus*)	Sodium-driven flagellar motor (Okunishi *et al.*, 1996)
	FlgF (*E. coli*)	Flagellar basal body rod protein (Homma *et al.*, 1990; Blattner *et al.*, 1997)