Genetic Analysis of *Vibrio cholerae* monolayer formation reveals a key role for \( \Delta \Psi \) in the transition to permanent attachment

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Abstract

A bacterial monolayer biofilm is a collection of cells attached to a surface but not to each other. Monolayer formation is initiated when a bacterial cell forms a transient attachment to a surface. While some transient attachments are broken, others transition into the permanent attachments that define the monolayer biofilm. In this work, we describe the results of a large-scale, microscopy-based genetic screen for *Vibrio cholerae* mutants that are defective in formation of the monolayer biofilm. This screen identified mutations that alter both transient and permanent attachment. Transient attachment was somewhat slower in the absence of flagellar motility. However, flagellar mutants eventually formed a robust monolayer. In contrast, in the absence of the flagellar motor, monolayer formation was severely impaired. A number of proteins that modulate the *V. cholerae* ion motive force were also found to affect the transition from transient to permanent attachment. Using chemicals that dissipate various components of the ion motive force, we discovered that dissipation of the membrane potential $\Delta \Psi$ completely blocks the transition from transient to permanent attachment. We propose that, as a bacterium approaches a surface, the interaction of the flagellum with the surface leads to a transient hyperpolarization of the bacterial cell membrane. This, in turn, initiates the transition to permanent attachment.
Introduction

*V. cholerae* colonizes surfaces in marine environments, freshwater environments, and the mammalian small intestine (59). Surface colonization can take the form of a monolayer, which is the result of interactions between individual bacteria and the surface, or of a multilayer, which is the result of interactions between neighboring bacteria and between these bacteria and the colonized surface. Three types of *V. cholerae* multilayer biofilms have been defined, each of which is activated by particular environmental conditions (Figure 1). The seawater biofilm requires only environmental Ca\(^{2+}\), which is thought to form bridges between negatively charged *V. cholerae* O-antigen surface polysaccharides on neighboring cells (25, 26). When expressed *in vitro* and in the mammalian intestine, toxin co-regulated pili on neighboring cells bundle to form the cellular aggregates that define the TCP-dependent biofilm (27, 55, 57). Finally, environments containing monosaccharides, bile, certain polyamines, or quorum sensing autoinducers stimulate the *Vibrio* polysaccharide (VPS)-dependent biofilm (15, 18, 24, 25, 37). There are many interesting aspects of the multilayer biofilm, including regulation of matrix synthesis, matrix structure and function, differentiation of bacteria within the biofilm, and resistance of biofilm-associated bacteria to antibiotics. As a result, these three-dimensional bacterial collections have been intensely scrutinized.

While the monolayer biofilm has been less well studied, it may well be the more relevant form of surface attachment in many interactions of *V. cholerae* and other bacteria with a host. In both commensal and pathogenic interactions with epithelial surfaces, adhesion may never progress beyond the monolayer stage either because of
bacterial internalization, harsh environmental conditions that preclude bacterial
replication, or the absence of environmental signals that promote intercellular
interactions. If a multilayer biofilm does develop, the monolayer biofilm is likely to serve
as a catalytic intermediate in this process.

Visual inspection of the process of monolayer formation by a motile bacterium
suggests that cells first form a loose association with the surface. This is termed
transient attachment because cells frequently escape the surface to become free-
swimming again. Loosely attached cells, however, may transition to a more stable
association with the surface known as permanent attachment. It is these permanently
attached cells that comprise the monolayer biofilm (Figure 1).

In *V. cholerae*, transient attachment is primarily mediated by the mannose-
sensitive hemagglutinin (MSHA) type IV pilus (37). However, in chitin-rich
environments, the chitin-regulated (ChiRP) type IV pilus also plays a role in transient
attachment (33). Arrest of flagellar motility appears to be the defining feature of the *V.
cholerae* transition to permanent attachment. First of all, flagellar filament mutants form
a denser monolayer biofilm (39). Secondly, transcription of flagellar genes is repressed
in monolayer-associated cells (37, 38). Interestingly, in *Bacillus subtilis*, a molecular
clutch that disengages the flagellum from its rotor has recently been identified. This
clutch, which is encoded within an operon containing genes required for biofilm
formation, has been proposed to facilitate the transition of free-swimming bacteria into
the biofilm-associated state (2). As of yet, no such structure has been identified in *V.
cholerae*. 
We previously reported that, in a minimal medium containing only amino acids as a carbon source, wild-type *V. cholerae* form a monolayer biofilm (37). Furthermore, by studying a limited number of genes, we established unique genetic requirements for monolayer biofilm formation as compared with formation of the multilayer biofilm (37, 38). In the present study, we have undertaken a large-scale, microscopy-based, genetic screen to identify novel mutations that alter *V. cholerae* monolayer formation. This screen identified structures previously known to participate in monolayer formation such as the mannose-sensitive hemagglutinin pilus (MSHA) and the flagellum. In addition, we identified a number of proteins with diverse functions whose common theme is the ability to modulate the ion motive force of *V. cholerae*. To further elucidate the role of the ion motive force in monolayer formation, we utilized ionophores to demonstrate that $\Delta \Psi$ plays a critical role in the transition from transient to permanent attachment. Based on our results, we propose a model in which arrest of the flagellar motor during transient attachment leads to hyperpolarization of the cell membrane. This, in turn, initiates the transition to permanent attachment.
Materials and Methods

Bacterial strains and media. Relevant bacterial strains and plasmids used in these studies and the primers used to generate them are listed in Tables 1 and 2, respectively. Strains were propagated in Luria-Bertani broth and then diluted into the previously described monolayer-specific minimal medium (MM) for quantification of monolayer formation (37). For quantification of total attached cells, monolayers were rinsed with phosphate-buffered saline, pH 7.4 (PBS). For quantification of permanently attached cells only, monolayers were rinsed with PBS supplemented with 0.1 % α-methylmannoside (PBS/AMM). Where indicated, the medium was supplemented with streptomycin (100 µg/ml, Sigma), ampicillin (150 µg/ml, Sigma) or kanamycin (50 µg/ml, Sigma). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma) and valinomycin (Sigma) were used to test the effects of ionophores on monolayer formation. A 500 µM stock solution of CCCP was prepared in dimethyl sulfoxide (DMSO). For all experiments, this solution was diluted in MM to yield a final concentration of 0.5 µM. A 10 mM stock solution of valinomycin was prepared in ethanol and diluted to yield the indicated concentration. For monolayer experiments, a stock solution of 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO, Alexis Biochemicals), an inhibitor of Na⁺-NQR, was prepared in ethanol and then diluted 1:1000 in MM to yield a final concentration of 50 µM.

Transposon mutagenesis. In addition to a previously generated library of mini-Tn10 transposon-insertion mutants (17), a new library of Himar transposon-insertion mutants (53) was constructed as follows. Wild-type V. cholerae (Sm') and the E. coli strain SM10λpir harboring the conditionally replicating plasmid pFD1 (Ap', Km') were cultured
on LB-agar plates containing either streptomycin (100 µg/ml) or ampicillin (150 µg/ml), respectively. These strains were then mated on an LB agar plate for 2 h at 37°C. *V. cholerae* carrying transposon-insertions were isolated by growth on LB agar containing streptomycin (100 µg/ml) and kanamycin (50 µg/ml). Mutants were transferred to fresh LB agar containing streptomycin and kanamycin, grown overnight at 27 °C, and used as described below to test monolayer formation.

Screen for monolayer-altered mutants. Transposon-insertion mutants were isolated, transferred to the wells of a 96-well plate containing 100 µl of LB broth, and incubated for 6-8 hrs at 27 °C. One µl of each culture was transferred to a well of a 24 well, non-tissue culture treated, polystyrene plate (Falcon) containing 300 µl of MM. Plates were incubated overnight (16h) at 27 °C with gentle agitation to allow monolayers to form. At the end of the incubation, planktonic cells were removed. Remaining planktonic and transiently attached cells were separated from monolayers by vigorous agitation for 15 minutes in the presence of 300 µl of PBS/AMM. This procedure was repeated two times. Washed monolayers were assessed qualitatively by visualization with an Eclipse TE200-E microscope (Nikon) equipped with an IEEE1394 Digital CCD camera (Hamamatsu), and mutants forming altered monolayers were stored at -80 °C for further study.

Secondary screens. Selected mutants found to be defective for monolayer formation were evaluated in the following secondary screens. Growth curves were measured by inoculating the strains of interest into the wells of 96 well plates containing 100 µl of MM. Cultures were incubated at 27 °C with gentle agitation, and an OD$_{655}$ was measured at various times over a 24 hour period. Motility and biofilm formation in LB
broth were assayed as previously described (63). Mannose-sensitive hemagglutination assays were performed as previously described (63) with the exception that red blood cells harvested from sheep (Sigma) were used.

**Arbitrary PCR.** For selected mutants demonstrating altered monolayer formation, arbitrary PCR (17, 42) was used to amplify genomic DNA neighboring the inserted transposon. In this technique, the DNA sequence surrounding the transposon insertion site is amplified by two rounds of PCR. The first round uses a primer specific to the transposon and one or more primers that are designed to anneal to arbitrary sites on the chromosomal DNA. The second round uses a nested primer unique to the transposon and a primer that is identical to the 5' end of the arbitrary primers used in the first round. For transposon-insertion mutants generated using the mini-Tn10 transposon, insertion junctions were amplified using primers P20, ARB1, and ARB6 in round 1, followed by P2 with ARB2 in round 2 (17). For transposon-insertion mutants generated using the Himar transposon, insertion junctions were amplified using primers Mar5, ARB1 and ARB6 in round 1, followed by Mar3 and ARB2 in round 2. The sequences of these primers are listed in Table 2. Arbitrary PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and then sequenced using transposon-specific primers.

**Construction of deletion mutants.** *V. cholerae* mutants carrying in-frame deletions in ΔflaA and ΔmotX were made as previously described using available plasmids (11, 17, 37). *V. cholerae* mutants carrying in-frame deletions within the genes encoded at loci VC2704, VC2705, and VCA0667 were constructed as previously described (17). Briefly, the PCR primers listed in Table 2 were used to amplify two non-contiguous
approximately 500-bp fragments including a small portion of the N-terminal and C-terminal sequence of the gene of interest, respectively. The two fragments were joined using the gene splicing by overlap extension (SOE) technique (Horton et al 1990 and Lefebvre et al 1995). The resulting deletion fragment was gel purified and cloned into the pCR2.1 TOPO vector (Invitrogen). Amplification of the correct fragments was confirmed by sequence analysis of the pCR2.1 insertion. The fragment was then removed from pCR2.1 by digestion with SpeI and XhoI and ligated into the suicide plasmid pWM91 to yield the plasmids listed in Table 1. The plasmids were transformed into the E. coli strain SM10λpir and transferred into V. cholerae by conjugation. V. cholerae strains harboring a deletion of the gene of interest were created by double homologous recombination as described previously (17). The deletions of VC2704, VC2705, and VCA0667 removed 198, 1599, and 1040 nucleotides from the coding sequences of the respective genes.

Construction of motX rescue plasmid: The gene at locus VC2601, which encodes motX, was amplified using the polymerase chain reaction. PCR primers were designed to remove the N-terminal leader sequence and C-terminal V5 epitope tag and polyhistidine region flanking the pBAD vector cloning site (Invitrogen). Instead, a 6X-His sequence was included in the C-terminal primer. Amplified products were cloned into a pBAD-TOPO expression vector to yield the rescue construct pBAD-TOPO-motX. The insertion was confirmed by DNA sequence analysis. In addition, the pBAD-TOPO-motX plasmid was introduced into the V. choleraeΔmotX mutant by electroporation, and rescue of motility was confirmed by inoculation into swarm agar (data not shown).

Analysis of monolayer formation. For formation of monolayer biofilms, the strains of interest were cultured in LB broth or MM overnight. In the morning, these cultures were
diluted into 300 µl of MM in 24 well, non-tissue culture treated, polystyrene plates
(Falcon) to yield the starting OD₆₅₅ as noted. Monolayers were formed by incubation at
27 °C with gentle agitation. At the end of the incubation, planktonic cells were removed.
Remaining planktonic and transiently attached cells were separated from monolayer-
associated cells by vigorous agitation for 15 minutes in the presence of 300 µl of PBS or
PBS/AMM as noted. This procedure was repeated two times. Washed monolayers
were visualized with an Eclipse TE200-E microscope (Nikon) equipped with an
IEEE1394 Digital CCD camera (Hamamatsu). IPLab software (Scanalytics, Inc.) was
used for image acquisition and quantification. Where noted, images were collected at
400X magnification, and the total area of the surface covered by cells within each image
was quantified. All measurements included at least three biological replicates and were
repeated several times.

Measurement of monolayer formation and membrane potential in the presence of
chemicals. For monolayer formation and membrane potential measurements with
chemicals, wild-type V. cholerae was cultured overnight in MM at 37 °C. The cultures
were diluted 1:10 into 1 ml of fresh MM prepared with the indicated chemical. These
cells were allowed to adjust to the presence of the chemical for 30 minutes and then
transferred into six 24 well plates in 300 µl aliquots. Monolayers were allowed to form
over 1 hour and were then rinsed three times by vigorous agitation for 5 minutes in the
presence of 300 µl of PBS/AMM. Surface coverage was evaluated as described above.

For membrane potential measurements, 3,3'-diethyloxacyrbocyanine iodide
(DiOC₂(3), Molecular Probes) was added to a final concentration of 30 µM after
incubation of cells with the indicated chemical for 1 hr at 27 °C. The plates were
incubated for an additional 30 minutes in the dark. The fluorescent intensity in each well was then measured using a HTS7000 spectrophotometer (Perkin Elmer) with an excitation filter of 485 nm and an emission filter of 535 nm.

Quantitative Reverse Transcription-PCR (qRT-PCR). After growth of *V. cholerae* to exponential phase in MM, valinomycin was added to reach a final concentration of 10 µM, and the cells were incubated with gentle shaking for 90 minutes more at 27 °C. Cells were then pelleted by centrifugation, and total RNA was isolated using the RNeasy kit (Qiagen). The qRT-PCR reaction was performed using 1 ng of total RNA in a 25 µl volume, the relevant primer pair as noted in Table 2, and the QuantiTect SYBR Green RT-PCR kit (Qiagen). The level of the *clpX* (VC1921) transcript was used to normalize all qRT-PCR reactions. Template-free and reverse transcriptase-free reactions were included to verify the absence of contaminants. The experiments were conducted on a ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following steps: (i) 50 °C for 30 min; (ii) 95 °C for 15 min; and (iii) 40 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C. A dissociation curve was performed for each primer pair. Data was analyzed using the 7000 system software (Applied Biosystems). Measurements were performed in triplicate.
Results

A genetic screen to identify monolayer mutants. While multiple forward genetic screens designed to identify biofilm-altered mutants have been performed in a variety of microbes including *V. cholerae* (5-8, 16, 30, 40, 41, 49, 50, 52, 58, 60, 61, 63-65), this is one of the first reports of a screen for monolayer-altered mutants. Our goal was to identify mutants that formed altered transient or permanent attachments. We previously showed that, while α-methylmannoside (AMM) blocks re-attachment of transiently attached cells, permanently attached cells are immune to its action. Therefore, in order to detect mutants that were unable to form either transient or permanent attachments, monolayers were rinsed as described above with PBS/AMM.

Mutant monolayers were examined visually by phase-contrast microscopy. In total, 5,617 transposon-insertion mutants were screened. In the initial screen, we identified three-hundred and twenty-seven mutants forming monolayer biofilms that were different from those formed by wild-type *V. cholerae*. Monolayer formation by these mutants was retested in triplicate. Two hundred and fifty-nine mutants demonstrated altered monolayer formation upon repeat testing. The types of monolayers identified and the scoring system utilized are shown in Figure 2. In secondary screens, we tested mutants for growth in MM, for motility in swarm agar, and for hemagglutination. Mutants with similar phenotypes in secondary screens were grouped, and the transposon-insertion sites of several mutants in each group were identified by sequence analysis of arbitrary PCR products. In total, the transposon-insertion sites of 109 mutants (40%) were identified. Forty-seven transposon-insertion mutants (18%) remained uncharacterized because their behavior was indistinguishable
from that of wild-type *V. cholerae* in secondary screens for growth, hemagglutination, motility, and we were unable to identify the transposon insertion site by arbitrary PCR. Table 3 represents a functional categorization of genes whose mutation altered monolayer formation but not growth rate. While the gene carrying the transposon insertion is listed, polar effects on neighboring genes may be responsible for the observed monolayer phenotypes of these transposon-insertion mutants. For this reason, to study the monolayer phenotype in more detail, we subsequently constructed mutants carrying in-frame deletions in genes of interest.

A large number of the mutations we identified affected synthesis of the MSHA pilus and the O-antigen and O-antigen capsular polysaccharide. As has previously been shown (37), attachment was completely abolished by most mutations affecting MSHA pilus structure and function (Figure 2). Furthermore, mutants harboring transposon insertions in genes encoding O-antigen synthesis proteins formed surface-attached bacterial aggregates that met the definition of a multilayer biofilm. In *V. cholerae* O139, the O-antigen is a component of both the lipopolysaccharide in the bacterial outer membrane and the capsular polysaccharide (62). We previously documented that the O-antigen and O-antigen capsule are able to mediate intercellular interactions when negative charges are neutralized by Ca\(^{2+}\) (26). The present findings suggest that mutation of the O-antigen also enables intercellular interactions. We hypothesize that, in the absence of intercellular repulsion mediated by the *V. cholerae* O-antigen, *V. cholerae* forms a multilayer rather than a monolayer biofilm.

A number of mutants identified in our screen had in common transposon insertions in genes encoding proteins with a role in generation or utilization of the *V. cholerae*
*V. cholerae* ion motive force (Table 3). The ion motive force maintained by *V. cholerae* is comprised of the sodium motive force and the proton motive force. Taken collectively, the ion motive force has three components, namely (i) ΔpH, which reflects the proton gradient maintained across the inner membrane, (ii) ΔpNa, which reflects the sodium gradient maintained across the inner membrane, and (iii) the membrane potential denoted ΔΨ, which reflects the charge difference maintained across the inner membrane. The sodium and proton motive forces both contribute to ΔΨ. Proteins identified in our screen that play a role in generation of the ion motive force included subunits of the sodium pumping NADH–quinone oxidoreductase Na\(^+\)-NQR, NAD\(^+\) synthesis enzymes, ubiquinone synthesis enzymes, and DsbD. Proteins identified in our screen that utilize the ion motive force included subunits of the Na\(^+\)-powered flagellar motor (4, 10, 19) and possibly the protein encoded at locus VC2705, which is annotated as a sodium-solute symporter. The experiments described below focused on the role of these two groups of proteins in formation of the monolayer biofilm.

**Evidence that Na\(^+\)-NQR impacts monolayer formation.** Na\(^+\)-NQR, the primary Na\(^+\) pump in *V. cholerae*, uses the energy released as a result of electron transfer from NADH to ubiquinone to power the translocation of Na\(^+\) across the cell membrane, generating both ΔpNa and ΔΨ. Our primary monolayer screen identified two monolayer-defective mutants harboring insertions in the genes *nqrB* and *nqrD*, which encode two of the six subunits of Na\(^+\)-NQR. Our *nqrD::TnMar* mutant grew as well as wild-type *V. cholerae*, while the *nqrB::TnMar* grew at a slower rate and was, therefore, excluded from further study. The monolayer defect of the *nqrD::TnMar* mutant was reproducible but small (Figure 3A).
NADH is an important co-factor in monolayer formation. In our screen, we identified monolayer-deficient mutants carrying insertions in the genes encoding the V. cholerae NadB and NadC homologs. NadB (L-aspartate oxidase) and NadC (quinolinic acid phosphoribosyltransferase) catalyze the first and third steps in the pathway for de novo synthesis of NAD⁺. NADH is an essential cofactor in myriad oxidation-reduction reactions throughout the cell including the action of Na⁺-NQR. The nadB and nadC mutants displayed a monolayer defect similar to that of the nqrD mutant (Figure 3A), suggesting that the primary role of their gene products in monolayer formation is provision of a cofactor for Na⁺-NQR.

In E. coli, NAD⁺ can be synthesized de novo from L-aspartate, or it can be scavenged from environmental sources, such as nicotinamide and nicotinic acid. We reasoned that if a defect in NAD⁺ synthesis were responsible for the observed defect in monolayer formation by the nadB and nadC mutants, then the monolayer-deficient phenotype of these mutants would be rescued by addition of nicotinic acid or nicotinamide to the culture medium. Because the nqrD mutant is unable to use NADH to form a Na⁺ gradient, supplementation of the growth medium with nicotinic acid or nicotinamide should not rescue the monolayer phenotype of the nqrD mutant. Indeed, as shown in Figure 3A, supplementation of the growth medium with nicotinic acid or nicotinamide rescued the monolayer defect of the nadB and nadC mutants but had no effect on the monolayer defect of the nqrD mutant. This supports the hypothesis that a paucity of NADH is responsible for the defect of the nadB and nadC mutants in monolayer formation.
The role of ubiquinone in monolayer formation. In our screen, we identified several
monolayer-defective mutants carrying transposon insertions in *ubiC*. In *E. coli*, UbiC
(chorismate pyruvate-lyase) catalyzes the first committed step in the ubiquinone
biosynthesis pathway, which converts chorismate into pyruvate and 4-hydroxybenzoate
(29). *ubiC* mutants had no observable growth defect in MM but formed a monolayer
that was less confluent than that of wild-type *V. cholerae* (Figure 3B). Quinones are
electron carriers that are essential for electron transport through respiratory chains.
Ubiquinone is a lipid-soluble, diffusible electron carrier that shuttles electrons and
protons between sites in electron transport proteins located near the cytoplasmic and
periplasmic face of the inner membrane, leading to generation of ΔpH and ΔΨ (3, 46).
Ubiquinone receives electrons from a number of enzymes including Na⁺-NQR.
Because the monolayer formed by the *ubiC* mutant is much less dense than that formed
by the *nqrD* mutant, we hypothesize that, in addition to Na⁺-NQR, ubiquinone may
receive electrons from other enzymes that are operative in monolayer formation.
When minimal medium is supplemented with 4-hydroxybenzoate, the
requirement for UbiC in the synthesis of ubiquinone is bypassed. We hypothesized
that, if the monolayer phenotype of the *ubiC* mutant were due to a defect in the
ubiquinone synthesis pathway, supplementation of the growth medium with 4-
hydroxybenzoate should rescue the monolayer defect of this mutant. To test this, we
compared monolayer formation by the *ubiC* mutant and wild-type *V. cholerae* in the
presence and absence of 4-hydroxybenzoate. As shown in Figure 3B, in the presence
of 4-hydroxybenzoate, monolayer formation by the *ubiC* mutant was indistinguishable
from that of wild-type _V. cholerae_. This result confirms that the monolayer defect of the
_ubiC_ mutant is, in fact, the result of a defect in ubiquinone synthesis.

**Sodium-solute symports in monolayer formation.** We identified two mutants in
our genetic screen with transposon insertions just upstream of and directly in the gene
at locus VC2705, encoding a putative sodium-solute symporter. We have named this
gene _sssA_ for _sodium-solute symporter A_. _SssA_ is homologous to the _Na⁺_-proline family
of symporters, which includes the _E. coli_ _Na⁺_-proline symporter _PutP_ (22). Because of
its similarity to sodium-proline symporters, we hypothesize that _SssA_ dissipates both
_ΔpNa_ and _ΔΨ_ when transporting its solute.

_SssA_ is in a putative operon with the gene at locus VC2704, encoding a
hypothetical 88 amino acid protein with two predicted transmembrane helices.
Homologs of this protein are found in a variety of bacterial species, encoded by genes
that are typically located adjacent to a gene encoding a sodium-solute symporter. We
hypothesized that _SssA_ might work in tandem with VC2704 to modulate monolayer
formation, and, therefore, named this protein _SssH_ for _sodium solute symporter helper_.

To study the role of _SssH_ and _SssA_ in monolayer formation, we first constructed
strains carrying in-frame deletions in the corresponding genes and tested their ability to
form monolayers. As shown in Figure 4A, monolayers formed by _ΔsssA_ and _ΔsssH_
mutants appeared similar to those formed by wild-type _V. cholerae_ after washing with
PBS, but a greater proportion of _ΔsssH_ and _ΔsssA_ monolayer cells detached following
washing in PBS/AMM. This suggests that fewer of the _ΔsssH_ and _ΔsssA_ mutant cells
had undergone the transition to permanent attachment as compared with wild-type _V.
cholerae. Thus, SssA and SssH play a role the transition from transient to permanent attachment.

The *V. cholerae* genome encodes three Na\(^+\)-solute symporters including SssA, the *V. cholerae* PutP homolog (VCA1071), and VCA0667. Our previous studies suggested that PutP and OpuD, a glycine betaine transporter encoded by VC1279, are the major transporters of proline in *V. cholerae* (23). Structure-function studies of *E. coli* PutP have demonstrated that the amino acid residues D55, S340, and T341 play a role in Na\(^+\) binding, while S57 plays a role in proline binding (21, 48, 51). S340, T341, and S57 are present in the three *V. cholerae* Na\(^+\)-solute symporters. However, D55 is displaced in SssA and absent in VCA0667.

Based on the homology of SssA to *E. coli* PutP, we hypothesized that other proline transporters might be important for monolayer formation. Therefore, we constructed strains carrying in-frame deletions in the genes encoding each of these proteins as well as OpuD and assessed the ability of these strains to form a monolayer (Figure 4A). As was observed for the ΔsssA mutant, the ΔputP mutant also was defective for monolayer formation. However, no additive effect was observed when a strain containing deletions of both *putP* and sssA was tested, suggesting that the proteins encoded by these genes function in the same signaling pathway or attachment mechanism. The ΔopuD and ΔVCA0667 mutants had no defect in monolayer formation. We questioned whether the function of VCA0667 and OpuD in monolayer formation might be uncovered in the background of a ΔputP mutation. However, even in the background of *putP* and sssA deletions, VCA0667 and OpuD played no role in monolayer formation (Figure 4A and data not shown).
Based on the homology of SssA to *V. cholerae* PutP and evidence that the latter transports proline, we considered the possibility that proline might be an environmental signal that enhances the transition from transient to permanent attachment. Because our minimal medium does not contain proline, we hypothesized that supplementation of this medium with proline might augment monolayer formation. To test this, we compared monolayer formation by wild-type *V. cholerae* and Na⁺-solute symporter mutants in the presence and absence of L-proline. As shown in Figure 4B, the presence of 0.1 mM L-proline in the medium had no effect on monolayer formation by wild-type *V. cholerae* or the ∆sssH, ∆sssA, ∆putP, or ∆opuD mutants. This suggested to us that proline transport is not the critical function of these proteins in monolayer formation.

**The *V. cholerae* flagellar motor but not flagellar motility is essential for monolayer formation.** In our screen, we identified two classes of flagellar motility mutants that altered monolayer formation in very different ways. While mutations in the flagellar structure generally produced a denser monolayer, mutations in the flagellar motor drastically decreased monolayer formation. This suggested to us that the flagellar motor might play an important role in monolayer formation.

Flagellar motility in *V. cholerae* is dependent on the sodium motive force, which powers the Na⁺-dependent flagellar motor responsible for turning the helical flagellar filament. Mutation of the *motXY* and *pom AB* genes, which encode components of the flagellar motor of *Vibrios*, results in a paralyzed flagellum (9, 31, 32, 43, 44, 54, 56, 66, 67). Mutation of *flaA*, encoding the building blocks of the flagellar filament, results in a bacterium with no flagellar filament. To further characterize the role of the flagellar
filament and flagellar motor in monolayer formation, we constructed mutants carrying in-
frame deletions of flaA, motX, or both. We then studied their ability to form a
monolayer. As shown in Figure 5A, we observed that surface attachment by a ΔflaA
mutant was robust, while surface attachment by the ΔmotX and ΔmotX ΔflaA mutants
was severely impaired. As shown in Figure 5B, the monolayer defect of the ΔmotX
mutant was completely rescued by providing the motX gene in trans. Partial rescue of
monolayer formation was also observed for the ΔflaAΔmotX mutant. This suggests that
the flagellar motor plays a critical role in monolayer formation that is distinct from that
played by the flagellar filament.

Flagellar motility is believed to enable bacteria to approach surfaces closely
enough to initiate transient attachment. To evaluate the role of flagellar motility early in
the course of monolayer formation, we compared total surface attachment by wild-type
V. cholerae and a ΔflaA mutant over time (Figure 5C). We observed that surface
attachment by a ΔflaA mutant lagged behind that of wild-type V. cholerae at early time
points. After ten hours of exposure to the surface, however, a PBS-rinsed ΔflaA mutant
monolayer had a level of surface coverage that was equivalent to that of wild-type V.
cholerae. This suggests that, although surface attachment of ΔflaA mutant cells occurs
more slowly than that of wild-type V. cholerae, over time, the numbers of wild-type and
mutant surface-attached cells equalize.

We conclude that flagellar motility accelerates early interactions with the surface
but is not essential for transient attachment. Furthermore, we hypothesize that,
independent of its role in motility, the flagellar motor plays an important role in the
transition from transient to permanent attachment. We propose that this role is to modulate the ion motive force.

**Chemical analysis of the role of $\Delta pH$ and $\Delta pNa$ in monolayer formation.** In the preceding experiments, we have presented genetic evidence that proteins involved in generation and utilization of the *V. cholerae* ion motive force play a role in the transition from transient to permanent attachment. However, because so many proteins and pathways contribute to generation and utilization of the ion motive force, the monolayer phenotype caused by any single mutation is likely to be small. To circumvent the issue of functional redundancy, we took a chemical approach. We first examined the effect of HQNO (2-n-Heptyl-4-hydroxyquinoline N-oxide) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) on monolayer formation. HQNO is a specific inhibitor of Na$^+$-NQR (20, 39), and, therefore, blocks generation of $\Delta pNa$ and $\Delta \Psi$ by Na$^+$-NQR. CCCP$^-$ is a weak lipophilic acid that crosses the inner membrane as CCCPH, deposits a proton in the cytoplasm, and returns to the periplasm in response to the membrane potential, thereby completely dissipating the proton motive force, which includes $\Delta pH$ and a portion of $\Delta \Psi$.

We first confirmed that these chemicals had the expected effect on $\Delta \Psi$. $\Delta \Psi$ is most conveniently measured by the use of voltage-sensitive dyes. Voltage-sensitive dyes are lipophilic, cationic fluorescent dyes that are attracted to and concentrate in the negatively charged cytoplasm of polarized cells. Aggregation of the dye in the cytoplasm alters the fluorescence of the dye. We used 3,3'-diethyloxacarbocyanine iodide [DiOC$_2$(3)], which fluoresces at 535 nm in the non-aggregated state, to compare the membrane potential of untreated wild-type *V. cholerae* and *V. cholerae* treated with
CCCP, HQNO, or both. The data are reported as the difference between the fluorescence emitted by the dye in the presence of the chemical and that emitted in the absence of chemical. Therefore, a more positive measurement indicates a greater degree of membrane depolarization. As shown in Figure 6A, CCCP and HQNO, when added individually, moderately reduced $\Delta \Psi$, while addition of both had an additive effect on $\Delta \Psi$.

We then quantified wild-type *V. cholerae* monolayer formation in the presence of CCCP and HQNO (Figure 6B). In these experiments, incubations with chemicals were short to avoid a reduction in viability. Incubation with CCCP alone had only a modest effect on wild-type *V. cholerae* monolayer formation. A slight decrease in swimming speed was also noted. From these observations, we concluded (i) that $\Delta pH$ is not essential for monolayer formation and (ii) that *V. cholerae* is able to maintain $\Delta pNa$ in the absence of $\Delta pH$. Incubation with HQNO also had a modest effect on monolayer formation and caused a slight decrease in swimming speed. Because motility was preserved in the presence of HQNO, we concluded that HQNO does not fully dissipate $\Delta pNa$. Two possible explanations for this are (i) that another Na$^+$ transporter contributes to generation of $\Delta pNa$ in *V. cholerae* or (ii) that *V. cholerae* is able to convert proton motive force into sodium motive force. To distinguish between these two possibilities, we measured motility and monolayer formation in the presence of both CCCP and HQNO. We reasoned that if *V. cholerae* maintained $\Delta pNa$ in the presence of HQNO by converting proton motive force into sodium motive force, addition of CCCP would decrease flagellar motility and monolayer formation. In contrast, if another Na$^+$ transporter were contributing to $\Delta pNa$ in the presence of HQNO, addition of CCCP
would not have this effect on flagellar motility and monolayer formation. In fact, we observed that incubation with HQNO and CCCP greatly decreased monolayer formation and abolished flagellar motility. From these observations, we conclude that *V. cholerae* maintains ΔpNa in the presence of HQNO by converting proton motive force into sodium motive force. As a result, using this approach, we were unable to determine the role of ΔpNa in monolayer formation independently of ΔpH and ΔΨ. Instead, we elected to look directly at the role of ΔΨ in monolayer formation.

**ΔΨ blocks the transition from transient to permanent attachment.** To evaluate the role of ΔΨ, we examined the effect of valinomycin on monolayer formation. Valinomycin is predicted to zero ΔΨ but not ΔpNa or ΔpH (1). We first measured the effect of valinomycin on the membrane potential as described above. We found that addition of valinomycin depolarized the membrane in a concentration-dependent manner (Figure 7A). Furthermore, motility and transient attachment were unaffected by the presence of valinomycin (data not shown). In spite of this, valinomycin blocked permanent attachment by wild-type *V. cholerae* in a concentration-dependent manner (Figure 7B). We then questioned whether ΔΨ might be required not only for the transition from transient to permanent attachment but also for maintenance of permanent attachment. To test this, we incubated preformed monolayers with valinomycin in PBS or PBS/AMM. As shown in Figure 7C, addition of this drug to pre-formed monolayers did not result in dissolution of the monolayer. Therefore, we conclude that ΔΨ is required for the transition from transient to permanent attachment. Furthermore, ΔΨ is not required for maintenance of the monolayer biofilm.
In previous work, we showed that transcription of flagellar genes was repressed in the monolayer biofilm and transcription of sssA was activated (37, 38). To test whether modulation of ΔΨ by valinomycin might directly affect transcription of these monolayer-specific genes, we measured transcript levels of flaA and sssA by quantitative RT-PCR in the presence and absence of valinomycin. However, addition of valinomycin did not have a significant effect on the transcription of these two monolayer-specific genes, suggesting that the transcriptional program of monolayer-associated cells is not directly linked to modulation of ΔΨ (data not shown).
Discussion

In this work, we report the results of a screen for *V. cholerae* genes encoding proteins that play a role in formation of the monolayer biofilm. A large number of genes identified in this screen encoded proteins that are involved in generation and dissipation of the ion motive force of *V. cholerae*. In particular, mutations in subunits of the flagellar motor substantially decreased monolayer formation. We used chemicals to further dissect the effect of the ion motive force on monolayer formation. These studies demonstrate that the membrane potential $\Delta \Psi$ is required for monolayer formation but not monolayer maintenance and are consistent with a model in which modulation of $\Delta \Psi$ initiates the transition to permanent attachment.

The concept of a transient increase in $\Delta \Psi$ as a bacterial signal has precedent in the microbiological literature. Transient increases in $\Delta \Psi$ following exposure to a chemoattractant such as glucose were documented almost thirty years ago (12-14, 35, 36). This was postulated to be part of a bacterial signal transduction mechanism that involved the chemotactic apparatus but was not essential for chemotaxis. More recently, $\Delta \Psi$ has been implicated in regulation of the *cidABC* and *lrgAB* operons of *Staphylococcus aureus*, which control autolysis (47). Thus, we propose that control of bacterial signal transduction by $\Delta \Psi$ may be a widespread and underappreciated phenomenon.

A possible model connecting the flagellar motor to modulation of $\Delta \Psi$ during surface attachment is depicted in Figure 8. In planktonic and monolayer-associated cells, the flow of ions across the inner membrane is in steady state, and $\Delta \Psi$ is maintained at a constant level. When *V. cholerae* approaches a surface, it may attach
by a single tether such as the flagellum. In this case, the bacterium is noted to spin in place. However, if it attaches by more than one tether such as the flagellum and the cell body, the flagellar motor arrests, the flow of ions through the motor ceases, and $\Delta \Psi$ is transiently increased. This transient increase in $\Delta \Psi$ signals to the cell that it is on a surface and initiates the transition to permanent attachment. Our experimental results support this model in that (i) cells that are defective in generation of $\Delta \Psi$, such as those we have isolated in our screen, also display reduced monolayer formation; (ii) cells with no flagellar motor are severely impaired in monolayer formation, and (iii) zeroing of the membrane potential by valinomycin completely blocks the transition to permanent attachment without inhibiting flagellar motility or transient attachment. In each of these cases, hyperpolarization of the cell membrane due to flagellar motor arrest should occur slowly or not at all. Recently, EpsE, a protein described as a molecular clutch, has been identified in *Bacillus subtilis*. EpsE is proposed to disengage the flagellar rotor from its motor in biofilm-associated cells (2). It is interesting to speculate that engagement of a clutch might be triggered by a transient increase in $\Delta \Psi$. However, a detailed evaluation of this model will require the development of reagents that allow real-time measurements of $\Delta \Psi$ in single cells. Furthermore, we do not rule out the possibility that the rotational speed of flagellar motor regulates transcription of an as yet identified adhesion.

While similar studies of multilayer biofilm formation have yielded very few proteins that are intestinal colonization factors in the infant mouse model, many of the proteins we have identified in our monolayer screen have previously been identified as colonization factors in the infant mouse intestine. Similarly to the results we present
here, the flagellum was not found to be necessary for colonization of the infant mouse intestine, while a flagellar motor mutant had a ten-fold decrease in colonization relative to wild-type *V. cholerae* (28, 64). In a separate study, genes encoding Na⁺-NQR as well as UbiC were identified in a signature-tagged mutagenesis screen targeting factors required for colonization of the infant mouse intestine (34). Lastly, a screen for genes that are preferentially expressed in the infant mouse intestine uncovered SssA (45). In competition with wild-type *V. cholerae*, SssA was found to have a ten-fold disadvantage in colonization. These findings suggest to us that the monolayer biofilm studied in this work provides a useful *in vitro* model for biofilm formation in the mammalian intestine.
Acknowledgements

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References


47. **Patton, T. G., S. J. Yang, and K. W. Bayles.** 2006. The role of proton motive force in expression of the *Staphylococcus aureus* *cid* and *lrg* operons. Mol Microbiol **59**:1395-404.


assay using a microtiter plate to screen for enteroaggregative *Escherichia coli.*


Figure Legends

Figure 1: A depiction of the four types of *V. cholerae* biofilms that have been defined. The inset shows the stages in formation of the monolayer biofilm, which is the focus of this study.

Figure 2: Micrographs of monolayers formed by representative transposon insertion mutants from each phenotypic category seen in the genetic screen. The interrupted gene and phenotypic classification of each mutant appears above its micrograph. Upward and downward arrows represent increased or decreased monolayers, respectively, and the number of arrows corresponds to the severity of the defect. The aggregative phenotype is discussed in the text. (Bar = 10 µM)

Figure 3: Monolayer formation by wild-type *V. cholerae* and mutants harboring transposon insertions in the NADH:ubiquinone oxidoreductase gene *nqrD*, the NAD$^+$ synthesis genes *nadB* and *nadC*, and the ubiquinone biosynthesis gene *ubiC*. Cultures were incubated overnight in LB broth and then used to inoculate MM at a starting OD$_{655}$ of 0.0005. (A) Quantification of monolayers formed by wild-type *V. cholerae*, an *nqrD::TnMar* mutant, a *nadB* mutant or a *nadC* mutant in the presence or absence of 1 µg/ml nicotinic acid or nicotinamide (B) Quantification of monolayers formed by wild-type *V. cholerae*, an *nqrD::TnMar* mutant, or a *ubiC* mutant in the presence or absence of 1 µg/ml 4-hydroxybenzoate. (*) signifies P<0.0001 when compared with wild-type *V. cholerae* monolayers measured under similar growth conditions.

Figure 4: Monolayer formation by wild-type *V. cholerae* and sodium-solute symporter mutants. Cells cultured in LB broth overnight were used to inoculate MM at a starting OD of 0.0005. (A) Quantification of monolayers formed by wild-type *V.cholerae* as well
as a number of sodium-solute symporter mutants. Monolayers were rinsed either with PBS or PBS/AMM. (*) signifies P<0.0001 when compared with wild-type V. cholerae monolayers. (B) Quantification of monolayers formed by wild-type V. cholerae and sodium-solute symporter mutants in the presence and absence of 0.1 mM L-proline.

**Figure 5:** Monolayer formation by wild-type V. cholerae (MO10) and ΔflaA, ΔmotX, and ΔflaAΔmotX mutants and rescue with the pBAD-TOPO-motX plasmid. Cells from an overnight culture in LB broth were used to inoculate MM at a starting OD of 0.0005. After incubation at 27°C for the indicated length of time, monolayers were washed three times with PBS. (A) Quantification of monolayer formation by wild-type V. cholerae (WT) and ΔflaA, ΔmotX, and ΔflaAΔmotX mutants. Micrographs were quantified 24 hours after exposure to a surface. (*) signifies P<0.0001 as compared with wild-type V. cholerae monolayers. (B) Quantification of monolayer formation by wild-type V. cholerae (WT) or ΔmotX and ΔflaAΔmotX mutants rescued either with the control plasmid pBAD-TOPO/lacZ/V5-His plasmid (placZ) or with the rescue plasmid pBAD-TOPO-motX carrying a wild-type copy of the motX gene (pmotX). Micrographs were quantified 18 hours after exposure to a surface. (C) Quantification of monolayer formation by wild-type V. cholerae (WT) and a ΔflaA mutant (FLA) at various times after exposure to a surface.

**Figure 6:** Effect of CCCP and HQNO on V. cholerae membrane potential and monolayer formation. (A) Quantification of wild-type V. cholerae membrane potential after 0.5 µM CCCP, 50 µM HQNO, or both. Increased ∆Fluorescence indicates a decrease in membrane polarization. (*) signifies P<0.009 as compared with untreated monolayers. (B) Quantification of monolayers formed over 1 hour by wild-type V.
*V. cholerae* in the presence or absence of 0.5 µM CCCP, 50 µM HQNO, or both. (*) signifies P<0.0005 as compared with untreated wild-type *V. cholerae* monolayers.

**Figure 7:** Effect of valinomycin on wild-type *V. cholerae* membrane potential and monolayer formation. (A) Membrane potential measurements of wild-type *V. cholerae* cells in the presence and absence of varying concentrations of valinomycin. Increased ∆Fluorescence is reflects a decrease in membrane potential. (*) signifies P<0.006 when compared with untreated wild-type *V. cholerae* monolayers. (B) Quantification of wild-type *V. cholerae* monolayers formed over 1 hour in the presence or absence of varying concentrations of valinomycin. (*) signifies P<0.0001 when compared with untreated wild-type *V. cholerae* monolayers. (C) Quantification of wild-type *V. cholerae* monolayers formed over 8 hours and then washed with MM or MM/AMM in the presence or absence of 10 µM valinomycin.

**Figure 8:** A model for the role of the flagellar motor and ∆Ψ in the transition from transient to permanent attachment. In the planktonic state, the flagellar motor is active, and the membrane potential is maintained at a constant level. When the cell attaches to the surface by more than one tether such as the flagellum and the cell body, flagellar motor arrests, the flow of ions through the motor ceases, and ∆Ψ is transiently increased. This initiates the transition to permanent attachment.
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**Construction of V. cholerae pBADmotX for rescue**
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- pBADMotXC C-terminal primer: TCAATGGTGATGATGATGATCCAGAACGCTTTCTCGGCGTCTT

**Arbitrary PCR**
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<td>Hypothetical protein</td>
</tr>
</tbody>
</table>
A.

Bar graph showing fluorescence levels for different treatments:
- COCP
- HGNO
- COCP/HGNO

B.

Bar graph showing area covered:
- No Drug
- COCP
- HGNO
- HGNO/COCP