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Surface hydrophilicity promotes bacterial twitching motility

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ABSTRACT Twitching motility is a form of bacterial surface translocation powered by the type IV pilus (T4P). It is frequently analyzed by interstitial colony expansion between agar and the polystyrene surfaces of petri dishes. In such assays, the twitching motility of *Acinetobacter nosocomialis* was observed with MacConkey but not Luria-Bertani (LB) agar media. One difference between these two media is the presence of bile salts as a selective agent in MacConkey but not in LB. Here, we demonstrate that the addition of bile salts to LB allowed *A. nosocomialis* to display twitching. Similarly, bile salts enhanced the twitching of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in LB. These observations suggest that there is a common mechanism, whereby bile salts enhance bacterial twitching and promote interstitial colony expansion. Bile salts disrupt lipid membranes and apply envelope stress as detergents. Surprisingly, their stimulatory effect on twitching appears not to be related to a bacterial physiological response to stressors. Rather, it is due to their ability to alter the physicochemical properties of a twitching surface. We observed that while other detergents promoted twitching like bile salts, stresses applied by antibiotics, including the outer membrane-targeting polymyxin B, did not enhance twitching motility. More importantly, bacteria displayed increased twitching on hydrophilic surfaces such as those of glass and tissue culture-treated polystyrene plastics, and bile salts no longer stimulated twitching on these surfaces. Together, our results show that altering the hydrophilicity of a twitching surface significantly impacts T4P functionality.

IMPORTANCE The bacterial type IV pilus (T4P) is a critical virulence factor for many medically important pathogens, some of which are prioritized by the World Health Organization for their high levels of antibiotic resistance. The T4P is known to propel bacterial twitching motility, the analysis of which provides a convenient assay for T4P functionality. Here, we show that bile salts and other detergents augment the twitching of multiple bacterial pathogens. We identified the underlying mechanism as the alteration of surface hydrophilicity by detergents. Consequently, hydrophilic surfaces like those of glass or plasma-treated polystyrene promote bacterial twitching, bypassing the requirement for detergents. The implication is that surface properties, such as those of tissues and medical implants, significantly impact the functionality of bacterial T4P as a virulence determinant. This offers valuable insights for developing countermeasures against the colonization and infection by bacterial pathogens of critical importance to human health on a global scale.

KEYWORDS twitching motility, *Pseudomonas aeruginosa*, *Acinetobacter*, bile salts, detergents, surface property, hydrophilicity

Twitching motility is a form of non-flagellated bacterial locomotion that allows bacteria to move on or between solid surfaces (1–4). It is powered by the bacterial type IV pilus (T4P), which can be assembled and disassembled by the supramolecular T4P machinery (T4PM) (1, 5–7). The current model proposes that it is the recurrent cycles of T4P assembly and disassembly, or extension and retraction, that powers this form of

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bacterial surface motility (8, 9). The T4PM assembles the long T4P filament that protrudes from a cell into its surroundings. When the tip of an extended T4P attaches to a solid substratum, the retraction of the T4P by the T4PM moves a bacterium toward the point of attachment. This translocation of bacterial cells on or between solid surfaces results in bacterial twitching motility.

Of relevance to human health, the T4P plays a crucial role in the pathogenesis of many important bacterial pathogens (10–15). These include *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* both on the list of priority pathogens per the World Health Organization (WHO) (16). One of the primary functions of the T4P as a virulence factor is for adherence to human cells or tissues to initiate colonization and invasion (4, 17, 18). *Acinetobacter nosocomialis,* a close relative of *A. baumannii,* is an opportunistic pathogen primarily causing nosocomial or hospital-acquired infections (19). The *A. nosocomialis* M2 strain has been used as a model for studies of *Acinetobacter* pathogenesis and T4P functionality (18, 20–22). Despite the lack of flagella and the acineto- or non-motile designation for this genus, many *Acinetobacter* species are, in fact, motile by T4P-dependent twitching motility (23–26). As such, the analysis of twitching motility provides a convenient assay for investigating the functionality of the bacterial T4P in these medically important pathogens.

Twitching motility is routinely analyzed by observing interstitial colony expansion between the lower surface of solidified nutrient agar and that of plastic petri dishes made of polystyrene (25, 27). Such stab assays involve the inoculation of the interstitial space by stabbing through the agar, and this method has been used for the identification of T4P or *pil* genes by the isolation of *P. aeruginosa* mutants that were defective in twitching motility (3). T4P genes encode the core components of the T4PM, and their functions in twitching motility are conserved among *P. aeruginosa* and many gram-negative and gram-positive bacteria (2, 10, 28–31). These include PilA, the major pilin, as well as PilB, the T4P extension ATPase, and PilT, the T4P retraction ATPase. Along with other T4P proteins, the PilB and the PilT ATPases polymerize and depolymerize pilins into or from the T4P filament, respectively. Bacterial translocation by twitching motility over distances longer than the length of an extended pilus depends on the dynamic nature of T4P assembly and disassembly coordinated by the T4PM (8, 9).

The regulation of bacterial motility by environmental cues has been studied most extensively in flagellated bacteria (32–35). Besides chemotactic responses (36), the biogenesis of bacterial flagella is modulated through gene expression and flagellar assembly by signals such as nutrient and surface availability (32–35). In addition, an alternative sigma factor, which is responsive to envelope and other environmental stressors, transcriptionally regulates the expression of flagellar genes in many bacteria (37–39). Although the T4P has been investigated to a lesser extent, there is clear evidence that its biogenesis and function are influenced by regulatory mechanisms and environmental factors. In many T4P or *pil* gene clusters, there are conserved two-component systems, including PilS and PilR (2, 31). In selected organisms, these regulators have been demonstrated to affect the expression of T4P genes (40–43). Signals of both chemical and physical nature are known to influence T4P-mediated motility (44–46). For example, lactate can induce PilT-dependent T4P retraction in *Neisseria meningitidis,* whereas both temperature and blue light were shown to influence bacterial twitching motility (47, 48).

It was observed previously that *A. nosocomialis* exhibited significant twitching motility with MacConkey but minimum or severely diminished twitching with Luria-Bertani (LB) agar media on polystyrene petri dishes (22, 49). In our current study, we investigated the underlying reasons for the observed differences in bacterial twitching between these two media. We determined that bile salts are the key component that allows *A. nosocomialis* to twitch in MacConkey media. This is because the addition of bile salts to LB allowed *A. nosocomialis* to twitch to a similar extent as with MacConkey. We also observed similar stimulatory effects of bile salts on the twitching of *P. aeruginosa* and *A. baumannii*. Bile salts are anionic detergents that can apply membrane stress to

bacteria (50–53). Our results further demonstrate that other detergents likewise can enhance bacterial twitching on polystyrene surfaces. Antibiotics, including the outer membrane-targeting polymyxin B, do not increase *P. aeruginosa* twitching motility. This suggests that the mechanism for the stimulatory effect of bile salts is unlikely related to a bacterial response to the presence of a general or envelope stressor. Instead, we suspected that bile salts and other detergents increased the hydrophilicity of polystyrene surfaces, and it was this increase in hydrophilicity that promoted bacterial twitching. Indeed, we observed that glass surfaces, which are more hydrophilic than polystyrene, significantly promoted twitching motility. In contrast, increasing the hydrophobicity of glass surfaces with a hydrophobic coating attenuated twitching. Like glass, plasma treatment of polystyrene surfaces is known to increase their hydrophilicity for culturing tissues or cells. We observed that tissue culture (TC)-treated polystyrene surfaces significantly increased bacterial twitching. Moreover, the addition of bile salts no longer stimulated twitching on glass or TC-treated polystyrene surfaces. Our results here suggest that bacterial pathogens may have evolved mechanisms to differentially interact with surfaces that have varying physicochemical properties to optimize host recognition, colonization, and infections.

RESULTS

Bile salts enable *Acinetobacter* twitching motility in stab assays

It has been reported in the literature that *A. nosocomialis* displays significantly more twitching motility with MacConkey than with LB agar in stab assays (22, 49). In these assays, bacterial cells are stab inoculated through the agar to form an interstitial colony between the petri dish and the agar media (25, 27). After a period of incubation, the size of an interstitial colony can be measured to quantify twitching motility. As shown in Fig. 1A, the *A. nosocomialis* M2 strain shows clear twitching with MacConkey but not with LB agar. In comparison with a *pilA* mutant as the negative control, M2 appeared to possibly twitch more than with LB (Fig. S1) (22, 49). However, under our experimental conditions, there is no statistical difference between these two strains. These results confirmed that MacConkey media allow *A. nosocomialis* to display quantitatively and qualitatively more significant twitching motility than LB as observed previously (22, 49).

We compared the composition of these two commonly used bacterial growth media (Table S1). Notwithstanding their commonalities, LB lacks peptone, lactose, and bile salts that are present in MacConkey. Peptone is a proteinous nutrient source, and lactose is a carbon and energy source. Bile salts are cholesterol derivatives with aliphatic side chains (51, 54) that regulate various biological processes in vertebrates and their microbiomes (50, 54–57). The amphipathic nature of bile salts allows them to interact with and disrupt membranes, resulting in envelope stress in bacteria as detergents (51, 52). Both gram-positive and gram-negative bacteria can respond to the presence of bile salts, leading to changes in gene expression and cellular physiology (52, 58, 59). In the formulation of MacConkey, bile salts are included as a selective agent for enteric bacteria (60).

We supplemented LB agar with peptone, lactose, or bile salts at the same concentration present in MacConkey agar to determine if one of these could enable *A. nosocomialis* to twitch in LB. As shown in Fig. 1A, the addition of neither lactose nor peptone led to any discernible twitching motility in *A. nosocomialis*. In contrast, the supplementation of bile salts resulted in *A. nosocomialis* twitching motility in LB comparable to what was observed with MacConkey agar. These results indicated that bile salts are the component that specifically stimulates twitching motility of *A. nosocomialis* as analyzed by stab assays with polystyrene petri dishes.

Twitching motility has been observed in *A. baumannii* (24, 61), a closely related *Acinetobacter* species and a WHO priority pathogen (16). The twitching motility of this bacterium is similarly noted in MacConkey agar as analyzed by a similar assay (22, 49). However, variable motility phenotypes were observed with LB media for different clinical isolates (61), suggesting an effect of media composition on *A. baumannii* twitching. We tested two *A. baumannii* strains, AYE and AB0057 (62, 63), in LB supplemented with bile

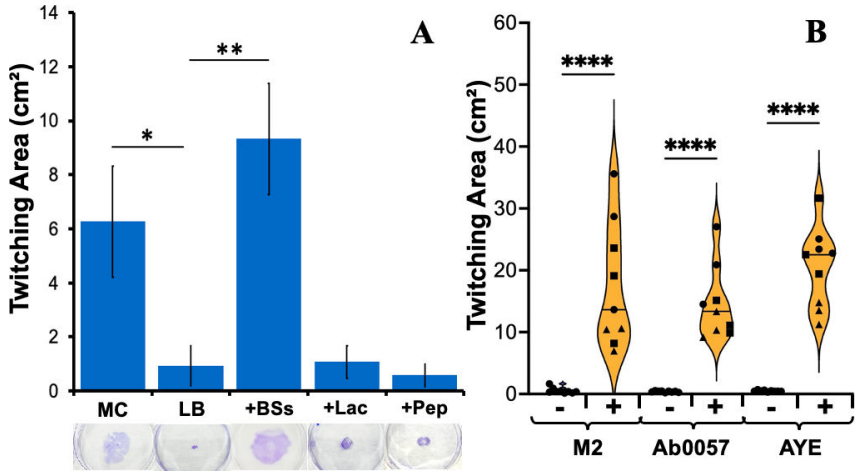

FIG 1 Bile salts enable *Acinetobacter* to twitch. (A) Bile salts allow *A. nosocomialis* M2 to twitch in LB media. The twitching motility of *A. nosocomialis* M2 was analyzed with MacConkey (MC) or Luria-Bertani media without or with 0.5% bile salts (+BSs), 1% lactose (+Lac), or 2% peptone (+Pep) with standard polystyrene petri dishes as described in Materials and Methods. Data shown are the averages from three biological experiments each performed in triplicate. Representative images of twitching motility are shown below their respective categories. (B) Bile salts provoke *A. baumannii* twitching in LB media. *A. baumannii* strains AB0057 and AYE were analyzed for twitching motility in LB agar without (–) or with (+) 0.5% bile salts on standard polystyrene petri dishes with *A. nosocomialis* (M2) as a control. Data shown are from three biological experiments, represented by different symbols, each performed in triplicate. The violin plot shows the frequency distribution curves of the data, where the horizontal line indicates the median. Single, double, and quadruple asterisks indicate two values are statistically different with $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively.

salts in comparison with *A. nosocomialis* M2. As shown in Fig. 1B, while none of these strains displayed twitching motility with the LB agar, supplementation of bile salts elicited twitching motility of both *A. baumannii* strains similar to *A. nosocomialis*. These results suggest that the stimulatory effects of bile salts on twitching motility are a more general phenomenon in the *Acinetobacter* genus.

Bile salts stimulated *P. aeruginosa* twitching motility

The above observations prompted us to investigate if bile salts enhanced bacterial twitching in other bacteria. *P. aeruginosa*, another WHO priority pathogen (16), has been used as a model for studies of bacterial twitching (8, 64, 65). Its twitching motility has been routinely analyzed using stab assays with LB instead of MacConkey agar plates (66–69). *P. aeruginosa* PAO1, a frequently used laboratory strain, exhibits twitching motility in LB agar (70, 71). However, the addition of bile salts to LB significantly increased its twitching motility (Fig. 2A). Furthermore, we examined the dose response of PAO1 twitching to bile salts. As shown in Fig. 2B, the stimulation of twitching motility shows concentration dependency, with a plateau between 0.1% and 0.4% of bile salts. At higher concentrations, bile salts start to inhibit *P. aeruginosa* growth and reduce its twitching motility in this assay (data not shown). We additionally examined the twitching motility of PA14, another commonly used *P. aeruginosa* strain in the literature (72–74). It was observed that the twitching motility of PA14 was stimulated by bile salts in LB media like that of PAO1 (Fig. S1). These results demonstrate that the stimulatory effect of bile salts on twitching is applicable to both *Acinetobacter* species and *P. aeruginosa* isolates. For the remainder of this study, we primarily used *P. aeruginosa* PAO1 as the model organism to investigate the mechanisms by which bile salts stimulate bacterial twitching motility.

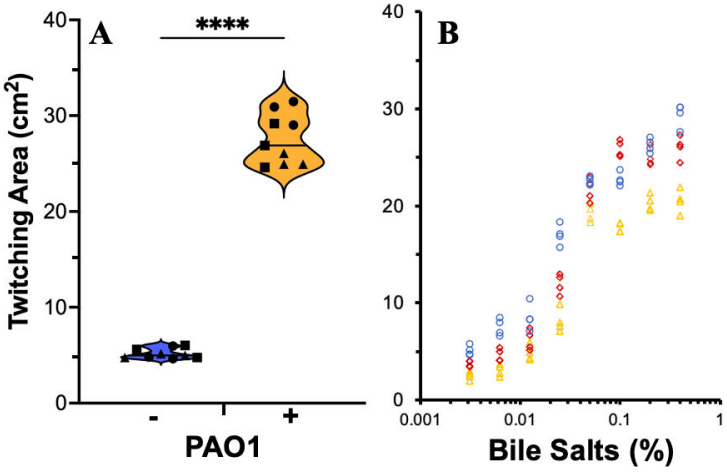

FIG 2 Bile salts enhance *P. aeruginosa* twitching motility. (A) Bile salts increase *P. aeruginosa* twitching. Twitching motility of PAO1 was analyzed without (–) or with (+) 0.5% bile salts as in Fig. 1B with data similarly presented. Quadruple asterisks indicate two values are statistically different with $P < 0.0001$. (B) Dose effect of bile salts on PAO1 twitching. PAO1 twitching was analyzed with the standard polystyrene petri dish protocol as in panel A with varying concentrations (% [wt/vol]) of bile salts as indicated. Data presented are from three biological experiments, each performed in quadruplicate with data points from the same experiment represented by the same symbols in color and shape.

Detergents stimulate bacterial twitching

Bile salts, produced from cholesterol metabolism, are anionic detergents (50–52, 55). They are known to apply membrane or envelope stress in bacteria (51, 52, 75). It is possible that bile salts function as a detergent to apply envelope or general stress to cells, and it was the cellular stress response that underlies the stimulatory effects of bile salts on bacterial twitching motility. To examine this possibility, we investigated the effect of other detergents on the twitching motility of *P. aeruginosa*. To avoid the complications between growth inhibition and twitching motility, we determined the maximum non-inhibitory concentrations of detergents experimentally (Table S2) to guide their use in our twitching motility assays. For this experiment, we supplemented the LB media with the anionic detergent sodium dodecyl sulfate (SDS) or the non-ionic detergents Triton X-100 and Triton X-114 (Table S2). As shown in Fig. 3A, all the detergents examined, whether anionic or non-ionic, significantly stimulated the twitching motility of *P. aeruginosa* much like bile salts. These results support the notion that the promotional effects of bile salts on twitching are related to their amphipathic properties as detergents.

The stimulatory effects of bile salts and other detergents on twitching motility could be explained by a physiological response of a bacterium to envelope stress applied by these amphipathic molecules (51, 52, 75, 76) or a general stress response to various environmental stressors (77). To investigate this, we tested antibiotics with different modes of action at their maximum non-inhibitory concentrations as stressors. These included ampicillin, gentamicin, and ciprofloxacin, which target cell wall biosynthesis, ribosome function, and DNA topology, respectively. We first determined the maximum non-inhibitory concentrations of these antibiotics by testing their effect on *P. aeruginosa* growth at different concentrations (Table S3). We then tested these antibiotics at their respective maximum non-inhibitory concentrations for their effect on *P. aeruginosa* twitching. As shown in Fig. 3B, none of these above antibiotics affected *P. aeruginosa* twitching motility significantly. This suggested that the stimulation of twitching by detergents was unlikely the result of a physiological response to general stressors. Moreover, we tested the effect of polymyxin B, which applies envelope stress as do bile salts, by targeting the outer membrane of gram-negative bacteria (53, 78). Somewhat

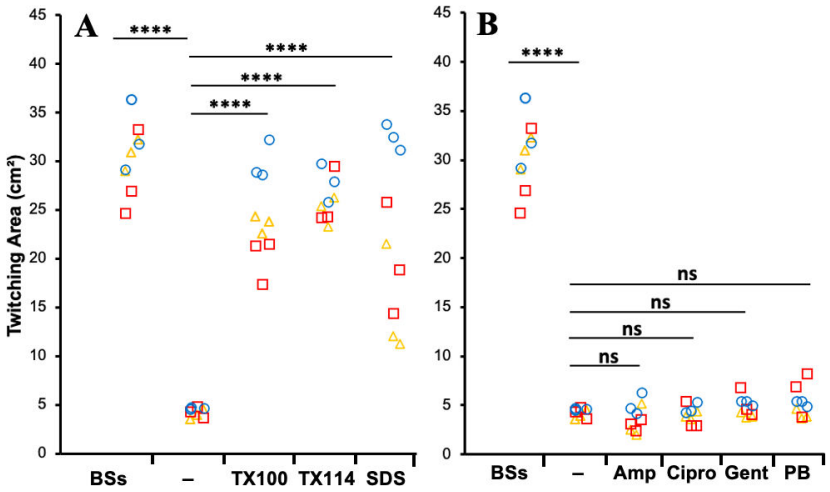

FIG 3 Detergents, but not antibiotics, promote *P. aeruginosa* twitching. (A) Effects of detergents. PAO1 twitching was analyzed with the standard petri dish protocol as in Fig. 2A with LB agar without modification (–) or with bile salts (BSs) (5 mg/mL), Triton X-100 (TX100) (75 µg/mL), Triton X-114 (TX114) (75 µg/mL), or SDS (850 µg/mL). (B) Effects of antibiotics. PAO1 twitching was analyzed as in (A) with ampicillin (Amp) (313 ng/mL), ciprofloxacin (Cipro) (31 ng/mL), gentamicin (Gent) (31 ng/mL), or polymyxin B (PB) (313 ng/mL). Data presented in both panels are from three biological experiments, each performed in triplicate. Data points from the same experiment are represented by the same symbols in color and shape. Results in both panels are from the same sets of biological replicates, and the controls (BSs and –) are from the same data sets as a result. Quadruple asterisks indicate two values are statistically different with $P < 0.0001$. Antibiotics resulted in values that are not significantly (ns) different with $P > 0.05$.

unexpectedly, this antibiotic showed no stimulatory effect on *P. aeruginosa* twitching (Fig. 3B). These results suggested that the observed stimulation of twitching motility by bile salts and other detergents (Fig. 2 and 3A) might not be related to a response to general or envelope stress.

Glass enhances *P. aeruginosa* twitching in comparison to polystyrene

Detergents such as bile salts are amphipathic molecules with both polar and non-polar moieties (50, 54, 56, 57, 79). As such, they can change the physicochemical properties of a surface (50, 80–82). In stab assays for twitching motility, bacteria cells translocate in the interstitial space between the solidified agar media and the hydrophobic surface of a polystyrene petri dish. We considered the possibility that bile salts in a growth media may interact with the hydrophobic surface of the polystyrene petri dishes to alter its physicochemical properties. Such interactions may allow bile salts to make the polystyrene surface more hydrophilic to possibly facilitate twitching motility. In comparison with polystyrene, glass petri dishes present a more hydrophilic surface. We therefore examined *P. aeruginosa* twitching with LB media using glass in comparison with polystyrene petri plates. As shown in Fig. S2, *P. aeruginosa* was observed to twitch significantly more on glass petri dishes than polystyrene ones in LB without the addition of bile salts. These results are consistent with the proposition that surface hydrophobicity or hydrophilicity plays crucial roles in bacterial twitching.

Bile salts do not enhance *P. aeruginosa* twitching on glass surfaces

We reproduced the above observation on glass (Fig. S2) with a modified twitching assay, where a glass or a polystyrene microscope slide was used as the twitching surface (see Materials and Methods). In this assay, the slides were cleaned and sterilized before they were placed in a polystyrene petri dish. Molten LB agar media were then poured into

the petri dish. Twitching motility was analyzed as before, except that the incubation time was shortened to limit the twitching zone to be within the boundaries of the width of the microscope slide. As shown in Fig. 4A, PAO1 twitched significantly more on glass slides than on polystyrene ones, as was observed with petri dishes (Fig. S2). As expected, the addition of bile salts significantly stimulated twitching on the polystyrene slide (Fig. 4A). In contrast, the supplementation of bile salts showed no promotional effect on twitching with the glass slide (Fig. 4A). A *pilA* mutant, which is non-piliated, was used as the non-twitching control, and it showed no twitching motility on all surfaces with or without bile salts (Fig. 4). It is also noteworthy that the twitching motility on the polystyrene slide in the presence of bile salts showed no statistical difference from that on the glass slides with or without bile salts. These results indicate that surface hydrophilicity likely enhances twitching motility, and the effects of bile salts on twitching could be attributed to their ability to change a hydrophobic surface to a more hydrophilic one. This is consistent with the observation that the stimulatory effect of bile salts is no longer observed on the more hydrophilic glass surface in contrast with polystyrene ones.

Increase in hydrophobicity of glass surfaces reduces *P. aeruginosa* twitching

Next, we modified the surface of the glass slides to be more hydrophobic using a chemical treatment. For this, we pretreated the glass slides with a polydimethylsiloxane (PDMS) solution before the analysis of twitching motility. PDMS is known to coat glass surfaces to make them more hydrophobic (83). As shown in Fig. 4B, the treatment of the glass surface with PDMS significantly reduced *P. aeruginosa* twitching to a level that is not significantly different from that on a polystyrene slide. In comparison, PDMS treatment did not impact twitching motility of *P. aeruginosa* on polystyrene slides (Fig. 4B), ruling out any inhibitory effects by PDMS. The *P. aeruginosa pilA* mutant showed no twitching under all experimental conditions as expected (Fig. 4). These results are consistent with the idea that hydrophilicity of surfaces enhances *P. aeruginosa* twitching and that bile salts and other detergents stimulate twitching motility on hydrophobic polystyrene surfaces by making them more hydrophilic.

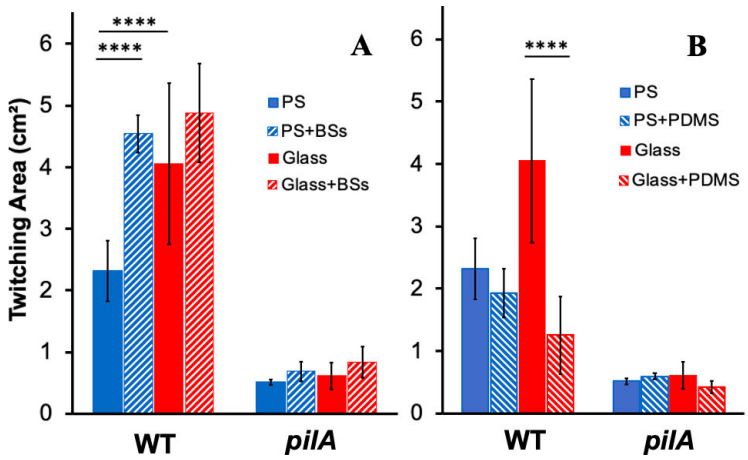

FIG 4 Glass surfaces increase *P. aeruginosa* twitching motility. (A) Glass surfaces stimulate PAO1 twitching motility, and bile salts no longer enhance it. The twitching motility of PAO1 and its isogenic *pilA* mutant was analyzed with polystyrene (PS) or glass microscope slides in LB without or with 0.5% bile salts (BSs) after 18 hours of incubation (see Materials and Methods). (B) Hydrophobic coating of glass reduces twitching motility. Experiments were performed as in A, except that the microscope slides were coated without or with polydimethylsiloxane (PDMS) (see Materials and Methods). Data presented in both panels are from three biological experiments each performed in triplicate. The data for the controls (PS and Glass) are the same controls in both panels as they are from the same sets of biological replicates. Quadruple asterisks indicating two values are statistically different with $P < 0.0001$.

Increase in hydrophilicity of polystyrene surfaces drastically enhances bacterial twitching motility

While natural polystyrene surfaces are hydrophobic, they can be treated with plasma gas to increase their hydrophilicity for tissue culture purposes (84). The surfaces of plasma- or TC-treated plates are therefore more hydrophilic than non-treated ones. We compared *P. aeruginosa* twitching motility with six-well polystyrene plates either TC treated or non-treated (Fig. 5A). The *P. aeruginosa* PAO1 strain exhibited significantly increased twitching motility on plasma-treated surfaces over the non-treated ones in LB media (Fig. 5A). The magnitude of increase in this case is about two- to threefold. This increase is more pronounced than on glass surfaces, which led to an increase of onefold or less (Fig. 4A). While the addition of bile salts significantly enhanced *P. aeruginosa* twitching on untreated plates, with the TC-treated surfaces, it appeared to diminish twitching, albeit to an extent not statistically significant.

We examined whether the drastic increase in twitching motility with TC-treated polystyrene surfaces with *P. aeruginosa* could be extended to *A. nosocomialis*. As shown in Fig. 5B, *A. nosocomialis* M2 displayed no twitching motility in LB media with the non-treated plates. This is expected because these plates are made of polystyrene like the petri dishes routinely used for twitching motility assays. TC-treated plates drastically increased M2 twitching with LB media by almost 50-fold without the addition of bile salts or detergents. Interestingly, the addition of bile salts to TC-treated plates significantly decreased twitching motility by *A. nosocomialis*, much more so than *P. aeruginosa* (Fig. 5A). The comparable levels of M2 twitching on TC-treated and untreated plates in the presence of bile salts are consistent with bile salts modifying both surfaces to similar levels of hydrophobicity or hydrophilicity. An isogenic *pilA* mutant was used as a control, and it displayed no twitching under all experimental treatments (Fig. 5B). The results here indicate the enhancement of twitching motility by hydrophilic surfaces is not confined to *P. aeruginosa*. Similar enhancement in *A. nosocomialis* suggests a more general phenomenon where hydrophilic surfaces promote interactions that are more favorable for bacterial twitching as mediated by the T4P as a motility apparatus.

DISCUSSION

The interaction with surfaces is essential for the survival and proliferation of bacteria in their natural environment as well as in health and disease. In their natural habitats, most bacteria exist in multicellular ensembles known as biofilms, the establishment of which depends on bacterial attachment to surfaces (85–88). During bacterial infection of a host, one of the earliest steps in the process is the adhesion of a pathogen to the surfaces of host cells, tissues, and medical implants. From a bacterial perspective, such interactions rely on the timely biogenesis and proper functioning of adhesins on their surfaces. One of the structures critical for bacterial adhesion to both biotic and abiotic surfaces is the bacterial T4P, which is prevalent in both gram-positive and gram-negative bacteria (2, 13, 28, 29, 31). It is an important virulence factor in many pathogens, including *P. aeruginosa* and *A. baumannii,* which are both on the WHO priority pathogens list (16). In these bacteria, as well as *A. nosocomialis* and others, the T4P is known to power bacterial twitching motility, which provides a convenient assay for the investigation of T4P biogenesis and function.

Here, we described an unexpected mechanism by which bile salts and other detergents can stimulate bacterial twitching motility. It was previously observed that *A. nosocomialis* exhibits significant twitching motility with MacConkey but not with Luria-Bertani agar media (22, 49). This phenomenon was observed using stab assays to visualize interstitial colony expansion with polystyrene petri dishes. After confirming this observation, we identified bile salts as the component in MacConkey responsible for eliciting *A. nosocomialis* twitching motility (Fig. 1A). The stimulatory effects of bile salts on twitching are not limited to *A. nosocomialis,* as we made similar observations with multiple strains of *A. baumannii* (Fig. 1B) and *P. aeruginosa* (Fig. 2A; Fig. S1). Additionally, our results indicated that other detergents, whether anionic or non-ionic, likewise

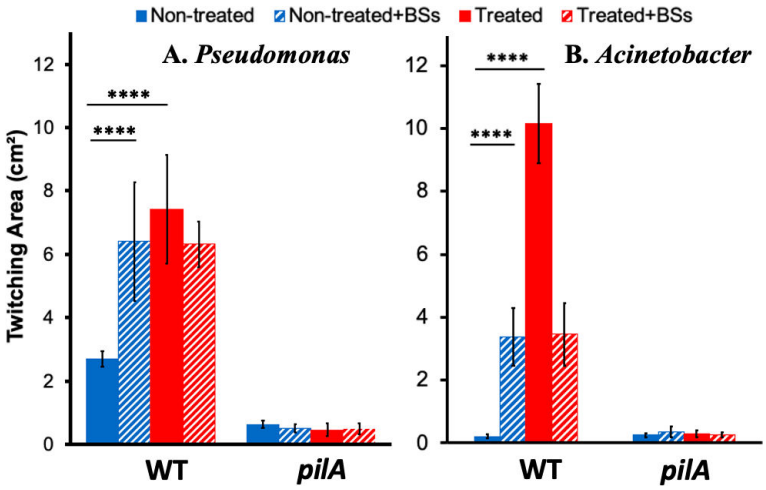

FIG 5 TC-treated plates ameliorate twitching and abolish the effects of bile salts. (A) *P. aeruginosa* twitching on TC-treated polystyrene. The twitching motility of *P. aeruginosa* PAO1 and its isogenic *pilA* mutant strain was analyzed with six-well polystyrene plates (see Materials and Methods) either TC treated or non-treated in LB agar without or with 0.5% bile salts (BSs). (B) *A. nosocomialis* twitching on TC-treated polystyrene. The twitching motility of *A. nosocomialis* M2 and its isogenic *pilA* deletion mutant was analyzed as in panel A. Data presented in both panels are from three biological experiments each performed in triplicate. Quadruple asterisks indicate that two values are statistically different with $P < 0.0001$.

promoted *P. aeruginosa* twitching motility (Fig. 3A). Surprisingly, this stimulation of twitching is likely not due to a physiological change in response to the presence of bile salts and other detergents in the growth medium. Instead, it is the ability of detergents to alter the physicochemical properties of a surface that enhances twitching motility in multiple bacterial species.

The above conclusion is based on a few lines of experimental evidence from this study. First, antibiotics with various modes of actions failed to enhance *P. aeruginosa* twitching motility (Fig. 3B). These included polymyxin B, which can apply envelope stress (78), as do bile salts and other detergents. These results suggested that the enhancement of twitching motility by detergents is likely not a bacterial response to a general or envelope stressor in a growth media. Second, we observed that surfaces of glass, which are more hydrophilic than that of polystyrene, significantly enhanced twitching motility (Fig. S2; Fig. 4A). We further demonstrated that the use of glass surfaces abrogated the stimulatory effect of bile salts such that the addition of bile salts no longer promoted *P. aeruginosa* twitching motility on these surfaces (Fig. 4A). When the surface properties of glass were changed by the application of a hydrophobic coating, the enhancement of bacterial twitching by glass was reversed (Fig. 4B). These results show that hydrophilic surfaces promote twitching, whereas hydrophobic ones suppress it. Because bile salts in the growth media only enhance twitching motility on polystyrene but not on glass surfaces, we conclude that bile salts likely function to modify natural polystyrene surfaces to be more hydrophilic, which promote bacterial motility. Lastly, we performed experiments with TC-treated and non-treated polystyrene surfaces (Fig. 5). The TC-treated surfaces, which are more hydrophilic, significantly enhanced bacterial twitching motility. As similarly observed on glass surfaces, the addition of bile salts no longer displayed a stimulatory effect on *P. aeruginosa* and *A. nosocomialis* twitching on TC-treated plates. These results support our conclusion that the physicochemical properties of a surface significantly impact the effectiveness of T4P-powered twitching motility in bacteria. On hydrophilic surfaces, bacteria twitch more, and on hydrophobic surfaces, they twitch less. We further conclude that the promotional effects of bile salts

and other detergents on bacterial twitching are largely due to their ability to change the properties of a surface over which bacteria translocate by twitching motility.

Categorically, there are two possible mechanistic explanations for the observed effects of the physicochemical properties of a surface on bacterial twitching. There have been reports that bacteria attach better to hydrophobic surfaces in the context of biofilm formation or otherwise (89–92). It follows that reducing hydrophobicity may lead to alteration of the interactions of a bacterial cell or its pilus with a subsurface over which a bacterium translocates by twitching motility. Sustained twitching movement relies on the recurrence of a multi-step process. These steps include the unobstructed extension of a T4P, the subsequent attachment of the pilus through its distal end for anchoring, followed by a successful T4P retraction event. It is conceivable that tampering with any of these steps through physicochemical interactions with a surface can lead to changes in bacterial twitching behaviors. The effects of surface properties on bacterial surface motility were discussed in a recent review article (45). Alternatively, surface sensing has been demonstrated to mediate changes in cell physiology and behavior (6, 93–98). It is possible that the physicochemical properties of a surface may be detected by a bacterium through surface sensing to modulate T4P biogenesis and its functions, leading to alterations in twitching motility. Further investigation is necessary to determine if the above scenarios or others, either alone or in combination, are the underlying reasons for the observed enhancement of T4P-powered bacterial twitching by surface hydrophilicity.

MATERIALS AND METHODS

Strains and culture conditions

The bacterial strains used in this study are listed in Table 1. These include *A. nosocomialis* M2, *A. baumannii* strains Ab0057 and AYE, and *P. aeruginosa* strains PAO1 and PA14. When appropriate, isogenic *pilA* mutants were used as controls. *P. aeruginosa* strains were grown at 37˚C on 1.5% Luria-Bertani agar, while *A. nosocomialis* and *A. baumannii* strains were grown at 37˚C on 1.5% MacConkey agar (Oxoid). Oxoid bile acids were used in this study when indicated.

Twitching motility assays

Twitching motility was analyzed by three different protocols using the agar stab methods (22, 27, 49) with 1.2% agar media (102). The first protocol uses a standard 100 mm × 15 mm polystyrene or glass petri dish (Fisher Scientific) as previously described (22, 27, 49, 102). In brief, plates with 25 mL agar media were prepared a day before the assays and allowed to sit on the benchtop overnight. These plates were then dried in a biosafety cabinet for 20 minutes before stab inoculation. After 48 hours of incubation at 37˚C in a humidity chamber, the agar media were removed, and the twitching zone was visualized by staining with 1% crystal violet. Twitching areas were determined using the NIH ImageJ software (103).

TABLE 1 Bacterial strains used in this study

Species	Strain	Description	Reference
Acinetobacter nosocomialis	M2	Clinical isolate	(99)
	M2 Δ*pilA*	*pilA* deletion and insertion of kanamycin-resistant cassette (Δ*pilA*::kan)	(23)
Acinetobacter baumannii	Ab0057	Clinical isolate	(62)
	AYE	Clinical isolate	(63)
Pseudomonas aeruginosa	PAO1	Wild type	(100)
	PW8622	PAO1 *pilA*-H02::IS*phoA*/hah	(101)

The second protocol uses either a glass (Opto-Edu) or a polystyrene (VWR International) microscope slide (1, 3) inside a standard polystyrene petri dish for analyzing twitching motility. The microscope slides were first submerged in a filter-sterilized polydimethylsiloxane solution (RainX) (104) or in 70% ethanol as a control. These slides were air dried on a rack at 40˚C before being placed at the bottom of a polystyrene petri dish. They were then covered by 25 mL of molten agar, which was poured into the petri dish a day before. A twitching assay is initiated by stab inoculation as described previously (22, 27, 49, 102), except that the incubation is shortened to 18 hours to limit the twitching zone within the boundaries of the microscope slide. One additional modification is that the twitching zone in this case was visualized by incident lighting and traced with a permanent marker without removing the agar from the petri dish. Twitching area was determined using ImageJ as above.

The third protocol uses six-well polystyrene plates with or without TC treatment (Falcon). Each well of a plate contains 2 mL agar media, and twitching motility assays were initiated by stab inoculation as described above. The twitching area was analyzed as described for the microscope slide-based assay, except that the incubation time was extended to 24 hours.

Two-factor ANOVA with replication in Microsoft Excel was used for statistical analysis when appropriate. Error bars in figures represent standard derivations calculated from the means of three biological replicates.

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M.T.O., K.J.D., and Z.Y. designed research and analyzed data. M.T.O. and Z.Y. wrote the manuscript. M.T.O., T.M.S., and D.H. performed experiments. T.M.S. aided in figure preparation and protocol development.

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Megan T. O'Hara, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing | Tori M. Shimozono, Methodology, Visualization, Writing – review and editing | Keane J. Dye, Conceptualization, Investigation, Methodology, Supervision, Writing – review and editing | David Harris, Data curation, Investigation, Writing – review and editing | Zhaomin Yang, Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (mSphere00390-24-S0001.pdf). Tables S1 to S3; Fig. S1 to S3.

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