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 enhanced 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.

39 **Importance**

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

52 **Introduction**

53 Twitching motility is a form of non-flagellated bacterial locomotion that allows bacteria
54 to move on or between solid surfaces (1-4). It is powered by the bacterial type IV pilus (T4P)
55 which can be assembled and disassembled by the supramolecular T4P machinery (T4PM) (1, 5-
56 7). The current model proposes that it is the recurrent cycles of T4P assembly and disassembly,
57 or extension and retraction, that powers this form of bacterial surface motility (8, 9). The T4PM
58 assembles the long T4P filament that protrudes from a cell into its surroundings. When the tip of
59 an extended T4P attaches to a solid substratum, the retraction of the T4P by the T4PM moves a
60 bacterium toward the point of attachment. This translocation of bacterial cells on or between
61 solid surfaces, results in bacterial twitching motility.

62 Of relevance to human health, the T4P plays a crucial role in the pathogenesis of many
63 important bacterial pathogens (10-15). These include *Pseudomonas aeruginosa* and
64 *Acinetobacter baumannii,* both on the list of priority pathogens per the World Health
65 Organization (WHO) (16). One of the primary functions of the T4P as a virulence factor is for
66 adherence to human cells or tissues to initiate colonization and invasion (4, 17, 18).
67 *Acinetobacter nosocomialis,* a close relative of *A. baumannii,* is an opportunistic pathogen
68 primarily causing nosocomial or hospital-acquired infections (19). The *A. nosocomialis* M2
69 strain has been used as a model for studies of *Acinetobacter* pathogenesis and T4P functionality
70 (18, 20-22). Despite the lack of flagella and the acineto- or non-motile designation for this genus,
71 many *Acinetobacter* species are, in fact, motile by T4P-dependent twitching motility (23-26). As
72 such, twitching motility provides a convenient assay for investigating the functionality of the
73 bacterial T4P in these medically important pathogens.

74 Twitching motility is routinely analyzed by observing interstitial colony expansion
75 between the lower surface of solidified nutrient agar and that of plastic Petri dishes made of
76 polystyrene (25, 27). Such stab assays involve the inoculation of the interstitial space by stabbing
77 through the agar, and this method has been used for the identification of T4P or *pil* genes by the
78 isolation of *P. aeruginosa* mutants that were defective in twitching motility (3). T4P genes
79 encode the core components of the T4PM and their functions in twitching motility are conserved
80 among *P. aeruginosa* and many Gram-negative and Gram-positive bacteria (2, 10, 28-31). These
81 include PilA, the major pilin, as well as PilB, the T4P extension ATPase, and PilT, the T4P
82 retraction ATPase. Along with other T4P proteins, the PilB and the PilT ATPases polymerize
83 and depolymerize pilins into or from the T4P filament, respectively. Bacterial translocation by
84 twitching motility over distances longer than the length of an extended pilus depends on the
85 dynamic nature of T4P assembly and disassembly coordinated by the T4PM (8, 9).
86 The regulation of bacterial motility by environmental cues has been studied most
87 extensively in flagellated bacteria (32-35). Besides chemotactic responses (36), the biogenesis of
88 bacterial flagella is modulated through gene expression and flagellar assembly by signals such as
89 nutrient and surface availability (32-35). In addition, an alternative sigma factor, which is
90 responsive to envelope and other environmental stressors, transcriptionally regulates the
91 expression of flagellar genes in many bacteria (37-39). Although the T4P has been investigated
92 to a lesser extent, there is clear evidence that its biogenesis and function are influenced by
93 regulatory mechanisms and environmental factors. In many T4P or *pil* gene clusters, there are
94 conserved two-component systems including PilS and PilR (2, 31). In selected organisms, these
95 regulators have been demonstrated to affect the expression of T4P genes (40-43). Signals of both
96 chemical and physical nature are known to influence T4P-mediated motility (44-46). For

97 example, lactate can induce PilT-dependent T4P retraction in *Neisseria meningitidis* whereas
98 both temperature and blue light were shown to influence bacterial twitching motility (47, 48).
99 It was observed previously that *A. nosocomialis* exhibited twitching motility only with
100 MacConkey but not with Luria-Bertani (LB) agar media on polystyrene Petri dishes (22, 49). In
101 our current study, we investigated the underlying reasons for the observed differences in
102 bacterial twitching between these two media. We determined that bile salts are the key
103 component that allows *A. nosocomialis* to twitch in MacConkey media. This is because the
104 addition of bile salts to LB allowed *A. nosocomialis* to twitch to a similar extent as with
105 MacConkey. We also observed similar stimulatory effects of bile salts on the twitching of *P.*
106 *aeruginosa* and *A. baumannii*. Bile salts are anionic detergents that can apply membrane stress to
107 bacteria (50-53). Our results further demonstrate that other detergents likewise can enhance
108 bacterial twitching on polystyrene surfaces. Antibiotics, including the outer membrane-targeting
109 polymyxin B, do not increase *P. aeruginosa* twitching motility. This suggests that the
110 mechanism for the stimulatory effect of bile salts is unlikely related to a bacterial response to the
111 presence of a general or envelope stressor. Instead, we suspected that bile salts and other
112 detergents increased the hydrophilicity of polystyrene surfaces, and it was this increase in
113 hydrophilicity that promoted bacterial twitching. Indeed, we observed that glass surfaces, which
114 are more hydrophilic than polystyrene, significantly promoted twitching motility. In contrast,
115 increasing the hydrophobicity of glass surfaces by coating attenuated twitching. Like glass,
116 plasma-treatment of polystyrene surfaces is known to increase their hydrophilicity for culturing
117 tissues or cells. We observed that tissue culture (TC)-treated polystyrene surfaces significantly
118 increased bacterial twitching. Moreover, the addition of bile salts no longer stimulated twitching
119 on glass or TC-treated polystyrene surfaces. Our results here suggest that bacterial pathogens

120 may have evolved mechanisms to differentially interact with surfaces that have varying

121 physicochemical properties to optimize host recognition, colonization, and infections.

122

123 **Results**

124 **Bile salts enable *Acinetobacter* twitching motility in stab assays.** It has been reported in the
125 literature that *A. nosocomialis* displays twitching motility with MacConkey, but not LB agar, in stab
126 assays (22, 49). In these assays, bacterial cells are stab-inoculated through the agar to form an interstitial
127 colony between the Petri dish and the agar media (25, 27). After a period of incubation, the size of an
128 interstitial colony can be measured to quantify twitching motility. As shown in Figure 1A, the *A.*
129 *nosocomialis* M2 strain shows clear twitching with MacConkey, but not with LB agar. These results
130 confirmed that MacConkey but not LB media allows *A. nosocomialis* to display twitching motility as
131 observed previously (22, 49).

132 We compared the composition of these two commonly used bacterial growth media (Table
133 S1). Notwithstanding their commonalities, LB lacks peptone, lactose, and bile salts that are
134 present in MacConkey. Peptone is a proteinous nutrient source and lactose is a carbon and
135 energy source. Bile salts are cholesterol derivatives with aliphatic side chains (51, 54) that
136 regulate various biological processes in vertebrates and their microbiomes (50, 54-57). The
137 amphipathic nature of bile salts allows them to interact with and disrupt membranes, resulting in
138 envelope stress in bacteria as detergents (51, 52). Both Gram-positive and Gram-negative
139 bacteria can respond to the presence of bile salts, leading to changes in gene expression and
140 cellular physiology (52, 58, 59). Generally, bile salts are more inhibitory of Gram-positive
141 bacteria because of the lack of an outer membrane. Thus, they are included in MacConkey as a
142 selective agent against Gram-positive bacteria in favor of enteric bacteria.

143 We supplemented LB agar with peptone, lactose, or bile salts at the same concentration
144 present in MacConkey agar to determine if one of these could enable *A. nosocomialis* to twitch
145 in LB. As shown in Figure 1A, the addition of neither lactose nor peptone led to any discernible

146 twitching motility in *A. nosocomialis*. In contrast, the supplementation of bile salts resulted in *A.*
147 *nosocomialis* twitching motility in LB comparable to what was observed with MacConkey agar.
148 These results indicated that bile salts are the component that specifically stimulates twitching
149 motility of *A. nosocomialis* as analyzed by stab assays with polystyrene Petri dishes.

150 Twitching motility has been observed in *A. baumannii* (24, 60), a closely related
151 *Acinetobacter* species and a WHO priority pathogen (16). The twitching motility of this
152 bacterium is similarly noted in MacConkey agar as analyzed by a similar assay (22, 49).
153 However, variable motility phenotypes were observed with LB media for different clinical
154 isolates (60), suggesting an effect of media composition on *A. baumannii* twitching. We tested
155 two *A. baumannii* strains, AYE and AB0057 (61, 62), in LB supplemented with bile salts in
156 comparison with *A. nosocomialis* M2. As shown in Figure 1B, while none of these strains
157 displayed twitching motility with the LB agar, supplementation of bile salts elicited twitching
158 motility of both *A. baumannii* strains similarly to *A. nosocomialis*. These results suggest that the
159 stimulatory effects of bile salts on twitching motility is a more general phenomenon in the
160 *Acinetobacter* genus.

161 **Bile salts stimulated *P. aeruginosa* twitching motility.** The above observations prompted us to
162 investigate if bile salts enhanced bacterial twitching in other bacteria. *P. aeruginosa*, another WHO
163 priority pathogen (16), has been used as a model for studies of bacterial twitching (8, 63, 64). Its
164 twitching motility has been routinely analyzed using stab assays with LB instead of MacConkey agar
165 plates (65-68). *P. aeruginosa* PAO1, a frequently used laboratory strain, exhibits twitching motility in
166 LB agar (69, 70). However, the addition of bile salts to LB significantly increased its twitching motility
167 (Figure 2A). Further, we examined the dose response of PAO1 twitching to bile salts. As shown in
168 Figure 2B, the stimulation of twitching motility shows concentration dependency, with a plateau

169 between 0.1% and 0.4% of bile salts. At higher concentrations, bile salts start to inhibit *P. aeruginosa*
170 growth and reduce its twitching motility in this assay (data not shown). We additionally examined the
171 twitching motility of PA14, another commonly used *P. aeruginosa* strain in the literature (71-73). It was
172 observed that the twitching motility of PA14 was stimulated by bile salts in LB media like that of PAO1
173 (Figure S1). These results demonstrate that the stimulatory effect of bile salts on twitching is applicable
174 to both *Acinetobacter* species and *P. aeruginosa* isolates. For the remainder of this study, we mostly
175 used *P. aeruginosa* PAO1 as the model organism to investigate the mechanisms by which bile salts
176 stimulate bacterial twitching motility.

177 **Detergents stimulate bacterial twitching.** Bile salts, produced from cholesterol metabolism, are
178 anionic detergents (50-52, 55). They are known to apply membrane or envelope stress in bacteria (51,
179 52, 74). It is possible that bile salts function as a detergent to apply envelope or general stress to cells
180 and it was the cellular stress response that underlies the stimulatory effects of bile salts on bacterial
181 twitching motility. To examine this possibility, we investigated the effect of other detergents on the
182 twitching motility of *P. aeruginosa.* To avoid the complications between growth inhibition and
183 twitching motility, we determined the maximum non-inhibitory concentrations of detergents
184 experimentally (Table S2) to guide their use in our twitching motility assays. For this experiment, we
185 supplemented the LB media with the anionic detergent sodium dodecyl sulfate (SDS), or the non-ionic
186 detergents Triton X-100 and Triton X-114 (Table S2). As shown in Figure 3A, all the detergents
187 examined, whether anionic or non-ionic, significantly stimulated the twitching motility of *P. aeruginosa*
188 much like bile salts. These results support the notion that the promotional effects of bile salts on
189 twitching are related to their amphipathic properties as detergents.

190 The stimulatory effects of bile salts and other detergents on twitching motility could be explained by
191 a physiological response of a bacterium to envelope stress applied by these amphipathic molecules (51,

192 52, 74, 75) or a general stress response to various environmental stressors (76). To investigate this, we
193 tested antibiotics with different modes of action at their maximum non-inhibitory concentrations as
194 stressors. These included ampicillin, gentamicin, and ciprofloxacin, which target cell wall biosynthesis,
195 ribosome function and DNA topology, respectively. We first determined the maximum non-inhibitory
196 concentrations of these antibiotics by testing their effect on *P. aeruginosa* growth at different
197 concentrations (Table S3). We then tested these antibiotics at their respective maximum non-inhibitory
198 concentrations for their effect on *P. aeruginosa* twitching. As shown in Figure 3B, none of these above
199 antibiotics affected *P. aeruginosa* twitching motility significantly. This suggested that the stimulation of
200 twitching by detergents was unlikely the result of a physiological response to general stressors.
201 Moreover, we tested the effect of polymyxin B, which applies envelope stress, as do bile salts, by
202 targeting the outer membrane of Gram-negative bacteria (53). Somewhat unexpectedly, this antibiotic
203 showed no stimulatory effect on *P. aeruginosa* twitching (Figure 3B). These results suggested that the
204 observed stimulation of twitching motility by bile salts and other detergents (Figures 2 and 3A) might
205 not be related to a response to general or envelope stress.

206 **Glass enhances *P. aeruginosa* twitching in comparison to polystyrene.** Detergents such as bile
207 salts are amphipathic molecules with both polar and non-polar moieties (50, 54, 56, 57, 77). As such,
208 they can change the physicochemical properties of a surface (50, 78-80). In stab assays for twitching
209 motility, bacteria cells translocate in the interstitial space between the solidified agar media and the
210 hydrophobic surface of a polystyrene Petri dish. We considered the possibility that bile salts in a growth
211 media may interact with the hydrophobic surface of the polystyrene Petri dishes to alter its
212 physicochemical properties. Such interactions may allow bile salts to make the polystyrene surface more
213 hydrophilic to possibly facilitate twitching motility. In comparison with polystyrene, glass Petri dishes
214 present a more hydrophilic surface. We therefore examined *P. aeruginosa* twitching with LB media

215 using glass in comparison with polystyrene Petri plates. As shown in Figure S2, *P. aeruginosa* was
216 observed to twitch significantly more on glass Petri dishes than polystyrene ones in LB without the
217 addition of bile salts. These results are consistent with the proposition that surface hydrophobicity or
218 hydrophilicity plays crucial roles in bacterial twitching.

219 **Bile salts do not enhance *P. aeruginosa* twitching on glass surfaces.** We reproduced the above
220 observation on glass (Figure S2) with a modified twitching assay where a glass or a polystyrene
221 microscope slide was used as the twitching surface (see Materials and Methods). In this assay, the slides
222 were cleaned and sterilized before they were placed in a polystyrene Petri dish. Molten LB agar media
223 was then poured into the Petri dish. Twitching motility was analyzed as before, except that the
224 incubation time was shortened to limit the twitching zone to be within the boundaries of the width of the
225 microscope slide. As shown in Figure 4A, PAO1 twitched significantly more on glass slides than on
226 polystyrene ones as was observed with Petri dishes (Figure S2). As expected, the addition of bile salts
227 significantly stimulated twitching on the polystyrene slide (Figure 4A). In contrast, the supplementation
228 of bile salts showed no promotional effect on twitching with the glass slide (Figure 4A). A *pilA* mutant,
229 which is non-piliated, was used as the non-twitching control, and it showed no twitching motility on all
230 surfaces with or without bile salts (Figure 4). It is also noteworthy that the twitching motility on the
231 polystyrene slide in the presence of bile salts showed no statistical difference from that on the glass
232 slides with or without bile salts. These results indicate that surface hydrophilicity likely enhances
233 twitching motility, and the effects of bile salts on twitching could be attributed to their ability to change
234 a hydrophobic surface to a more hydrophilic one. This is consistent with the observation that the
235 stimulatory effect of bile salts is no longer observed on the more hydrophilic glass surface in contrast
236 with polystyrene ones.

237 **Increase in hydrophobicity of glass surfaces reduces *P. aeruginosa* twitching**. Next, we modified
238 the surface of the glass slides to be more hydrophobic using a chemical treatment. For this, we
239 pretreated the glass slides with a polydimethylsiloxane (PDMS) solution before the analysis of twitching
240 motility. PDMS is known to coat glass surfaces to make them more hydrophobic (81). As shown in
241 Figure 4B, the treatment of the glass surface with PDMS significantly reduced *P. aeruginosa* twitching
242 to a level that is not significantly different from that on a polystyrene slide. In comparison, PDMS
243 treatment did not impact twitching motility of *P. aeruginosa* on polystyrene slides (Figure 4B), ruling
244 out any inhibitory effects by PDMS. The *P. aeruginosa pilA* mutant showed no twitching under all
245 experimental conditions as expected (Figure 4). These results are consistent with the idea that
246 hydrophilicity of surfaces enhance *P. aeruginosa* twitching, and that bile salts and other detergents
247 stimulate twitching motility on hydrophobic polystyrene surfaces by making them more hydrophilic.
248 **Increase in hydrophilicity of polystyrene surfaces drastically enhances bacterial twitching**
249 **motility**. While natural polystyrene surfaces are hydrophobic, they can be treated with plasma gas to
250 increase their hydrophilicity for tissue culture (TC) purposes (82). The surfaces of plasma- or TC-treated
251 plates are therefore more hydrophilic than non-treated ones. We compared *P. aeruginosa* twitching
252 motility with 6-well polystyrene plates either TC-treated or non-treated (Figure 5A). The *P. aeruginosa*
253 PAO1 strain exhibited significantly increased twitching motility on plasma-treated surfaces over the
254 non-treated ones in LB media (Figure 5A). The magnitude of increase in this case is about 2- to 3-fold.
255 This increase is more pronounced than on glass surfaces which led to an increase of 1-fold or less
256 (Figures 4A). While the addition of bile salts significantly enhanced *P. aeruginosa* twitching on
257 untreated plates, it had no stimulatory effect on twitching with the TC-treated surfaces.
258 We examined whether the drastic increase in twitching motility with TC-treated polystyrene surfaces
259 with *P. aeruginosa* could be extended to *A. nosocomialis*. As shown in Figure 5B, *A. nosocomialis* M2

260 displayed no twitching motility in LB media with the non-treated plates. This is expected because these
261 plates are made of polystyrene like the Petri dishes routinely used for twitching motility assays. TC-
262 treated plates drastically increased M2 twitching with LB media by almost 50-fold without the addition
263 of bile salts or detergents. An isogenic *pilA* mutant was used as a control, and it displayed no twitching
264 under all experimental treatments (Figure 5B). The results here indicate the enhancement of twitching
265 motility by hydrophilic surfaces is not confined to *P. aeruginosa.* Similar enhancement in *A.*
266 *nosocomialis* suggests a more general phenomenon where hydrophilic surfaces promote interactions that
267 are more favorable for bacterial twitching as mediated by the T4P as a motility apparatus.
268
269 **Discussion**
270 The interaction with surfaces is essential for the survival and proliferation of bacteria in
271 their natural environment as well as in health and disease. In their natural habitats, most bacteria
272 exist in multicellular ensembles known as biofilms, the establishment of which depends on
273 bacterial attachment to surfaces (83-86). During bacterial infection of a host, one of the earliest
274 steps in the process is the adhesion of a pathogen to the surfaces of host cells, tissues, and
275 medical implants. From a bacterial perspective, such interactions rely on the timely biogenesis
276 and proper functioning of adhesins on their surfaces. One of the structures critical for bacterial
277 adhesion to both biotic and abiotic surfaces is the bacterial T4P which is prevalent in both Gram-
278 positive and Gram-negative bacteria (2, 13, 28, 29, 31). It is an important virulence factor in
279 many pathogens including *P. aeruginosa* and *A. baumannii* which are both on the WHO priority
280 pathogens list (16). In these bacteria, as well as *A. nosocomialis* and others, the T4P is known to
281 power bacterial twitching motility, which provides a convenient assay for the investigation of
282 T4P biogenesis and function.

283 Here we described an unexpected mechanism by which bile salts and other detergents can
284 stimulate bacterial twitching motility. It was previously observed that *A. nosocomialis* exhibits
285 twitching motility with MacConkey but not Luria-Bertani (LB) agar media (16). This
286 phenomenon was observed using stab assays to visualize interstitial colony expansion with
287 polystyrene Petri dishes. After confirming this observation, we identified bile salts as the
288 component in MacConkey responsible for eliciting *A. nosocomialis* twitching motility (Figure
289 1A). The stimulatory effects of bile salts on twitching are not limited to *A. nosocomialis* as we
290 made similar observations with multiple strains of *A. baumannii* (Figure 1B) and *P. aeruginosa*
291 (Figures 2A and S1). Additionally, our results indicated that other detergents, whether anionic or
292 non-ionic, likewise promoted *P. aeruginosa* twitching motility (Figure 3A). Surprisingly, this
293 stimulation of twitching is likely not due to a physiological change in response to the presence of
294 bile salts and other detergents in the growth medium. Instead, it is the ability of detergents to
295 alter the physicochemical properties of a surface that enhances twitching motility in multiple
296 bacterial species.
297 The above conclusion is based on a few lines of experimental evidence from this study.
298 First, antibiotics with various modes of actions failed to enhance *P. aeruginosa* twitching
299 motility (Figure 3B). These included polymyxin B, which mimics the effects of bile salts and
300 other detergents in applying envelope stress. These results suggested that the enhancement of
301 twitching motility by detergents is likely not a bacterial response to a general or envelope
302 stressor in a growth media. Second, we observed that surfaces of glass, which are more
303 hydrophilic than that of polystyrene, significantly enhanced twitching motility (Figures S2 and
304 4A). We further demonstrated that the use of glass surfaces abrogated the stimulatory effect of
305 bile salts such that the addition of bile salts no longer promoted *P. aeruginosa* twitching motility

306 on these surfaces (Figure 4A). When the surface properties of glass were changed by the
307 application of a hydrophobic coating, the enhancement of bacterial twitching by glass was
308 reversed (Figure 4B). These results show that hydrophilic surfaces promote twitching, whereas
309 hydrophobic ones suppress it. Because bile salts in the growth media only enhance twitching
310 motility on polystyrene but not on glass surfaces, we conclude that bile salts likely function to
311 modify natural polystyrene surfaces to be more hydrophilic, which promote bacterial motility.
312 Lastly, we performed experiments with TC-treated and nontreated polystyrene surfaces (Figure
313 5). The TC-treated surfaces, which are more hydrophilic, significantly enhanced bacterial
314 twitching motility. As similarly observed on glass surfaces, the addition of bile salts no longer
315 displayed a stimulatory effect on *P. aeruginosa* and *A. nosocomialis* twitching on TC-treated
316 plates. These results support our conclusion that the physicochemical properties of a surface
317 significantly impact the effectiveness of T4P-powered twitching motility in bacteria. On
318 hydrophilic surfaces, bacteria twitch more, and on hydrophobic surfaces, they twitch less. We
319 further conclude that the promotional effects of bile salts and other detergents on bacterial
320 twitching are largely due to their ability to change the properties of a surface over which bacteria
321 translocate by twitching motility.

322 Categorically, there are two possible mechanistic explanations for the observed effects of
323 the physicochemical properties of a surface on bacterial twitching. There have been reports that
324 bacteria attach better to hydrophobic surfaces in the context of biofilm formation or otherwise
325 (87-90). It follows that reducing hydrophobicity may lead to alteration of the interactions of a
326 bacterial cell or its pilus with a subsurface over which a bacterium translocates by twitching
327 motility. Sustained twitching movement relies on the recurrence of a multi-step process. These
328 steps include the unobstructed extension of a T4P, the subsequent attachment of the pilus

329 through its distal end for anchoring, followed by a successful T4P retraction event. It is
330 conceivable that tampering with any of these steps through physicochemical interactions with a
331 surface can lead to changes in bacterial twitching behaviors. Alternatively, surface sensing has
332 been demonstrated to mediate changes in cell physiology and behavior (6, 91-96). It is possible
333 that the physicochemical properties of a surface may be detected by a bacterium through surface
334 sensing to modulate T4P biogenesis and its functions, leading to alterations in twitching motility.
335 Further investigation is necessary to determine if the above scenarios or others, either alone or in
336 combination, are the underlying reasons for the observed enhancement of T4P-powered bacterial
337 twitching by surface hydrophilicity.
338

339 **Materials and Methods**

340 **Strains and culture conditions.** The bacterial strains used in this study are listed in Table 1. These
341 include *A. nosocomialis* M2, *A. baumannii* strains Ab0057 and AYE, and *P. aeruginosa* strains PAO1
342 and PA14. When appropriate, isogenic *pilA* mutants were used as controls. *P. aeruginosa* strains were
343 grown at 37°C on 1.5% Luria-Bertani agar (LBA) while *A. nosocomialis* and *A. baumannii* strains were
344 grown at 37°C on 1.5% MacConkey agar (Oxoid). Oxoid bile acids were used in this study when
345 indicated.
346

347 **Twitching motility assays.** Twitching motility was analyzed by three different protocols using the agar
348 stab methods (27) (97) with 1.2% agar media. For all stab assays, the inoculum was prepared by
349 spreading a loopful of bacteria from the outside edge of an overnight plate culture onto a fresh 1.5%
350 LBA plate to create a thin layer of cells. Using a toothpick, bacteria were picked up to inoculate a plate
351 by stabbing through the agar to touch either the polystyrene or glass surface below.

352 The first protocol uses a standard 100 mm × 15 mm polystyrene or glass Petri dish (Fisher
353 Scientific) as previously described (27, 97). In brief, plates with 25 ml agar media were prepared a day
354 before the assays. These plates were then dried in a biosafety cabinet for 20 minutes before stab
355 inoculation. After 48 hours of incubation at 37°C in a humidity chamber, the agar media was removed,
356 and the twitching zone was visualized by staining with 1% crystal violet. Twitching areas were
357 determined using the NIH ImageJ software (98).
358 The second protocol uses either a glass (Opto-Edu) or a polystyrene (VWR International)
359 microscope slide (1″ × 3″) inside a standard polystyrene Petri dish for analyzing twitching motility. The
360 microscope slides were first submerged in a filter-sterilized polydimethylsiloxane (PDMS) solution
361 (RainX) (99) or in 70% ethanol as a control. These slides were air dried on a rack at 40°C before being
362 placed at the bottom of a polystyrene Petri dish. 25 ml of molten agar media was poured over the
363 microscope slides into the Petri dish a day before. A twitching assay is initiated by stab-inoculation as
364 described above except that the incubation is shortened to 18 hours to limit the twitching zone within the
365 boundaries of the microscope slide. One additional modification is that the twitching zone in this case
366 was visualized by incident light and traced with a permanent marker without removing the agar from the
367 Petri dish. Twitching area was determined using ImageJ as above.
368 The third protocol uses 6-well polystyrene plates with or without TC-treatment (Falcon). Each
369 well of a plate contains 2 ml agar media and twitching motility assays were initiated by stab inoculation
370 as described above. The twitching area was analyzed as described for the microscope slide-based assay
371 except that the incubation time was extended to 24 hours.
372

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657

658 **Table 1. Bacterial strains used in this study.**

Strain		Description	Reference
Acinetobacter nosocomialis	M2	Clinical isolate	(100)
	M2 Δ*pilA*	*pilA* deletion and insertion of kanamycin resistant cassette (Δ*pilA*::kan)	(23)
Acinetobacter baumannii	Ab0057	Clinical isolate	(61)
	AYE	Clinical isolate	(62)
Pseudomonas aeruginosa	PAO1	Wildtype	(101)
	PW8622	PAO1 *pilA*-H02::ISphoA/hah	(102)

659

660 **Figure Legends**

661

662 **Figure 1. Bile salts enable *Acinetobacter* to twitch. A.** Bile salts allow *A. nosocomialis* M2 to
663 twitch in LB media. The twitching motility of *A. nosocomialis* M2 was analyzed with
664 MacConkey (MC) or Luria-Bertani (LB) media without or with 0.5% bile salts (+BSs), 1%
665 lactose (+Lac), or 2% peptone (+Pep) with standard polystyrene Petri dishes as described in
666 Materials and Methods. Data shown are the averages from three biological experiments each
667 performed in triplicate. **B.** Bile salts provoke *A. baumannii* twitching in LB media. *A. baumannii*
668 strains AB0057 and AYE were analyzed for twitching motility in LB agar without (-) or with (+)
669 0.5% bile salts on standard polystyrene Petri dishes with *A. nosocomialis* (M2) as a control. Data
670 shown are from three biological experiments, represented by different symbols, each performed
671 in triplicate. Single (*), double (**), and quadruple (****) asterisks indicate two values are
672 statistically different with $P<0.05$, $P<0.01$, and $P<0.0001$ by the Student's T-test, respectively.

673

674 **Figure 2. Bile salts enhance *P. aeruginosa* twitching motility. A.** Bile salts increase *P.*
675 *aeruginosa* twitching. Twitching motility of PAO1 was analyzed without (-) or with (+) 0.5%
676 bile salts as in Figure 1B with data similarly presented. Quadruple asterisks (****) indicates
677 values that are statistically different with $P<0.0001$ by the Student's T-test. **B.** Dose effect of bile
678 salts on PAO1 twitching. PAO1 twitching was analyzed with the standard polystyrene Petri dish
679 protocol as in (A) with varying concentrations (w/v %) of bile salts as indicated. Data presented
680 in both panels are from three biological experiments each performed in triplicate. Data points
681 from the same experiment are represented by the same symbols in color and shape.

682

683 **Figure 3. Detergents, but not antibiotics, promote *P. aeruginos*a twitching. A.** Effects of
684 detergents. PAO1 twitching was analyzed with the standard Petri dish protocol as in Figure 2A
685 with LB agar without modification (-) or with bile salts (BSs) (5 mg/ml), Triton X-100 (TX100)
686 (75 μg/ml), Triton X-114 (TX114) (75 μg/ml), or sodium dodecyl sulfate (SDS) (850 μg/ml). **B.**
687 Effects of antibiotics. PAO1 twitching was analyzed as in (A) with ampicillin (Amp) (313
688 ng/ml), ciprofloxacin (Cipro) (31 ng/ml), gentamicin (Gent) (31 ng/ml), or polymyxin B (PB)
689 (313 ng/ml). Data presented in both panels are from three biological experiments each
690 performed in triplicate. Data points from the same experiment are represented by the same
691 symbols in color and shape. Triple (***) and quadruple (****) asterisks indicate two values are
692 statistically different with P<0.001 and P<0.0001 by the Student's T-test, respectively.
693 Antibiotics resulted in values that are not significantly (ns) different with P>0.05.
694
695 **Figure 4. Glass surfaces increase *P. aeruginosa* twitching motility. A.** Glass surfaces
696 stimulate PAO1 twitching motility and eliminate the stimulatory effect of bile salts. The
697 twitching motility of PAO1 and its isogenic *pilA* mutant were analyzed with polystyrene (PS) or
698 glass microscope slides in LB without or with 0.5% bile salts (BSs) after 18 hours of incubation
699 (see Materials and Methods). **B.** Hydrophobic coating of glass reduces twitching motility.
700 Experiments were performed as in A, except that the microscope slides were coated without or
701 with polydimethylsiloxane (PDMS) (see Materials and Methods). Data presented in both panels
702 are from three biological experiments each performed in triplicate. Double asterisks (**) indicate
703 two values are statistically different with P<0.01 by the Student's T-test.
704
705 **Figure 5. TC-treated plates ameliorate twitching and abolish the effects of bile salts. A.** *P.*
706 *aeruginosa* twitching on TC-treated polystyrene. The twitching motility of *P. aeruginosa* PAO1

707 and its isogenic *pilA* mutant strain was analyzed with 6-well polystyrene plates (see Materials
708 and Methods) either TC-treated or non-treated in LB agar without or with 0.5% bile salts (BSs).
709 **B.** *A. nosocomialis* twitching on TC-treated polystyrene. The twitching motility of *A.*
710 *nosocomialis* M2 and its isogenic *pilA* deletion mutant was analyzed as in A. Data presented in
711 both panels are from three biological experiments each performed in triplicate. Double (**) and
712 quadruple (****) asterisks indicate that two values are statistically different with $P<0.01$ and
713 $P<0.0001$ by the Student's T-test, respectively.

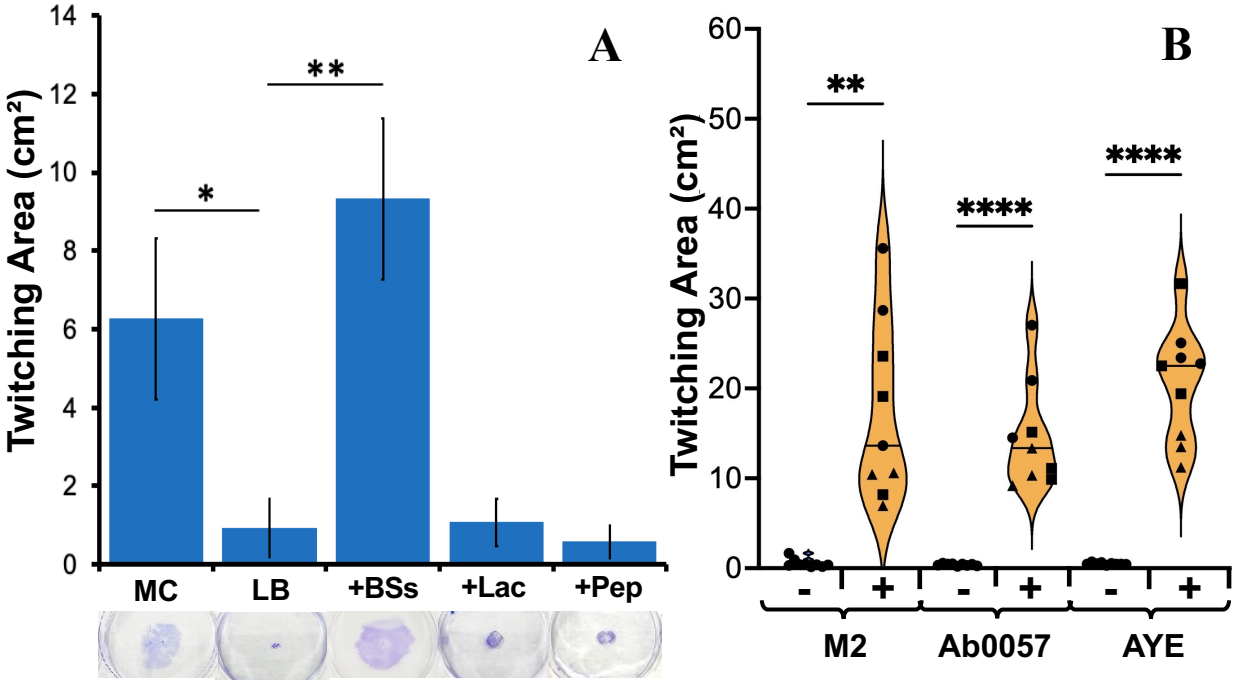

Figure 1. Bile salts enable *Acinetobacter* to twitch. A. Bile salts allow *A. nosocomialis* M2 to twitch in LB media. The twitching motility of *A. nosocomialis* M2 was analyzed with MacConkey (MC) or Luria-Bertani (LB) media without or with 0.5% bile salts (+BSs), 1% lactose (+Lac), or 2% peptone (+Pep) with standard polystyrene Petri dishes as described in Materials and Methods. Data shown are the averages from three biological experiments each performed in triplicate. **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. Single (*), double (**), and quadruple (****) asterisks indicate two values are statistically different with $P<0.05$, $P<0.01$, and $P<0.0001$ by the Student's T-test, respectively.

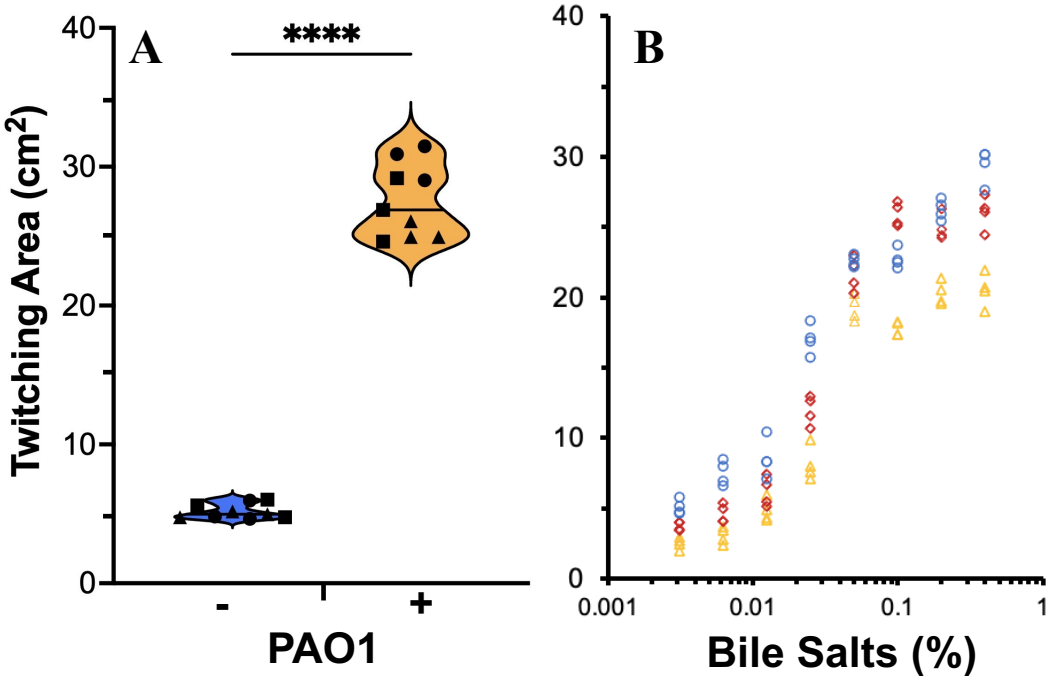

Figure 2. Bile salts enhance *P. aeruginosa* twitching motility. A. Bile salts increase *P. aeruginosa* twitching. Twitching motility of PAO1 was analyzed without (-) or with (+) 0.5% bile salts as in Figure 1B with data similarly presented. Quadruple asterisks (****) indicates values that are statistically different with P<0.0001 by the Student's T-test. **B.** Dose effect of bile salts on PAO1 twitching. PAO1 twitching was analyzed with the standard polystyrene Petri dish protocol as in (A) with varying concentrations (w/v %) of bile salts as indicated. 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.

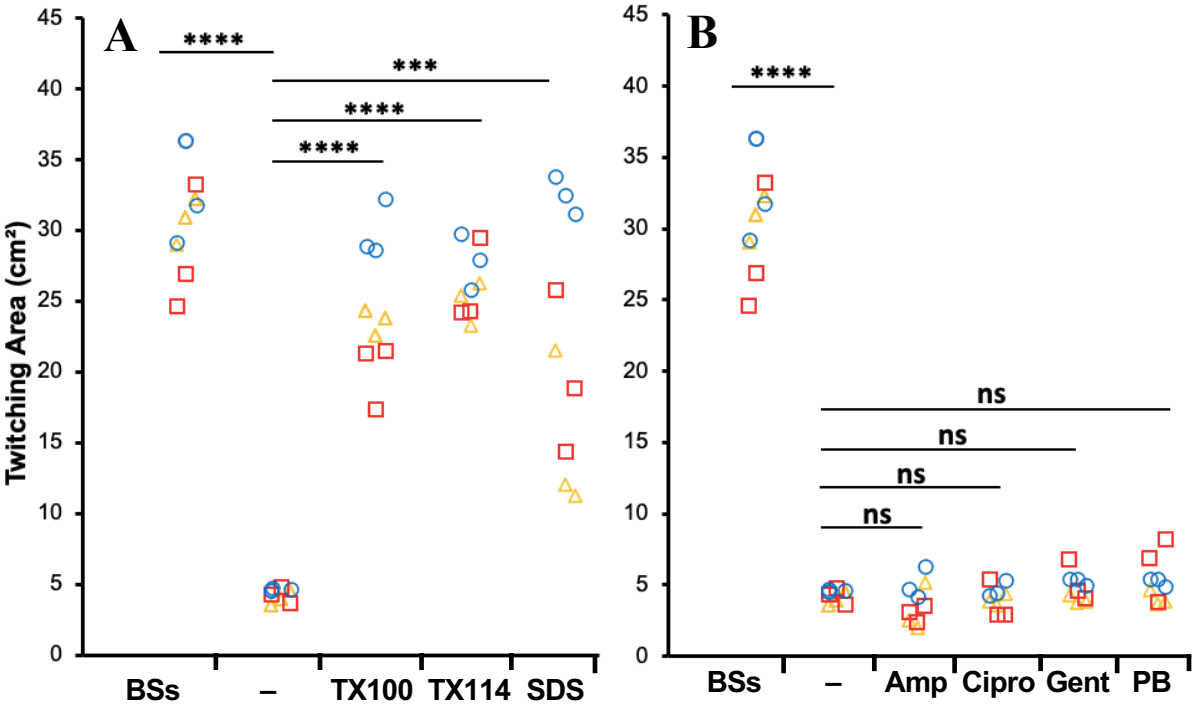

Figure 3. Detergents, but not antibiotics, promote *P. aeruginosa* twitching. A. Effects of detergents. PAO1 twitching was analyzed with the standard Petri dish protocol as in Figure 2A with LB agar without modification (-) or with bile salts (BSs) (5 mg/ml), Triton X-100 (TX100) (75 µg/ml), Triton X-114 (TX114) (75 µg/ml), or sodium dodecyl sulfate (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. Triple (***) and quadruple (****) asterisks indicate two values are statistically different with $P<0.001$ and $P<0.0001$ by the Student's T-test, respectively. Antibiotics resulted in values that are not significantly (ns) different with $P>0.05$.

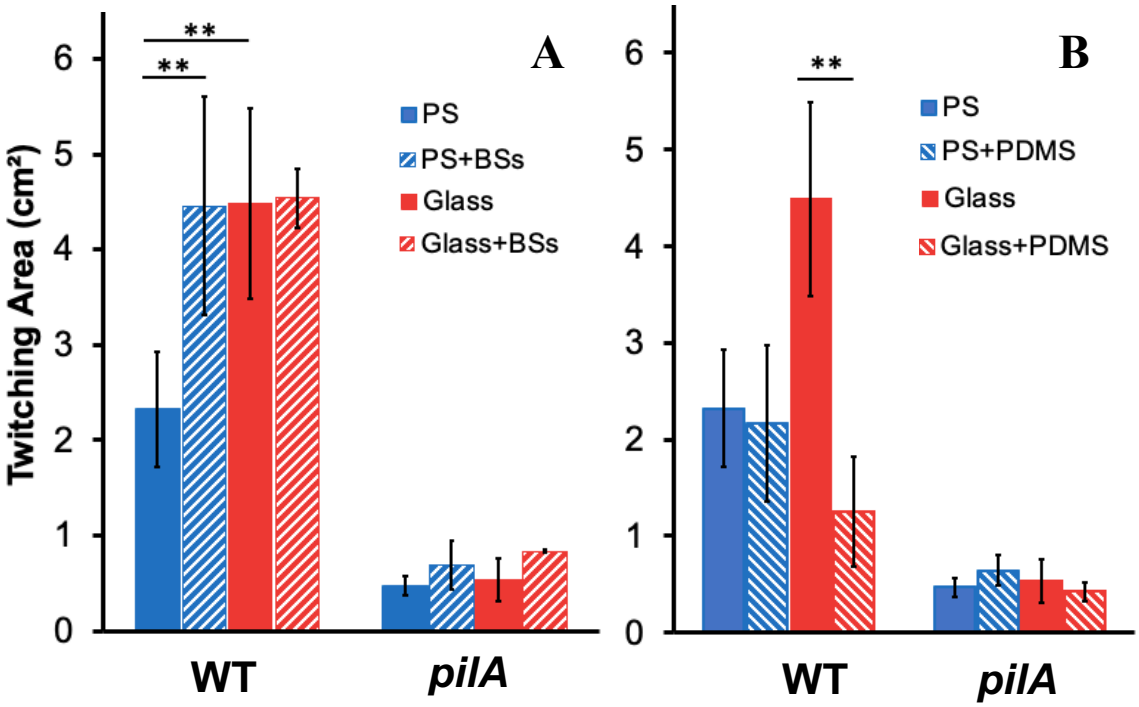

Figure 4. Glass surfaces increase *P. aeruginosa* twitching motility. A. Glass surfaces stimulate PAO1 twitching motility and eliminate the stimulatory effect of bile salts. The twitching motility of PAO1 and its isogenic *pilA* mutant were analyzed with polystyrene (PS) or glass microscope slides in 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. Double asterisks (**) indicate two values are statistically different with $P<0.01$ by the Student's T-test.

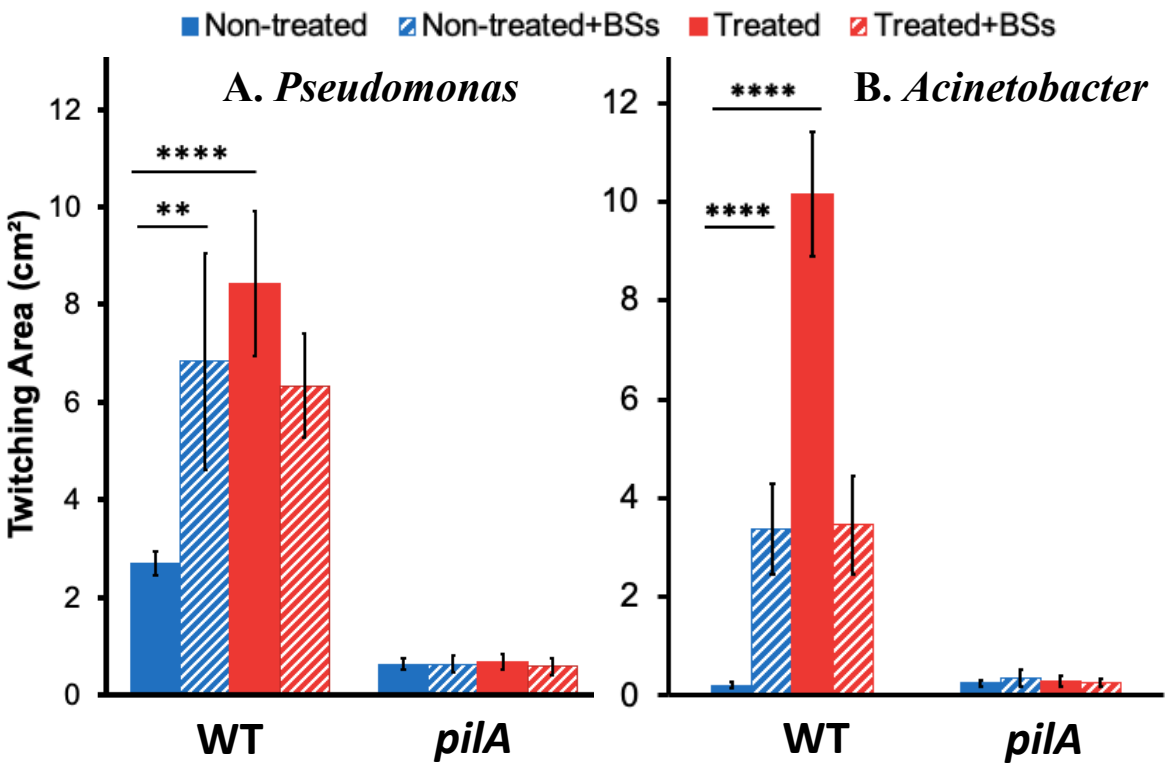

Figure 5. TC-treated plates ameliorate twitching and abolish the effects of bile salts. **A.** *P. aeruginosa* twitching on TC-treated polystyrene. The twitching motility of *P. aeruginosa* PAO1 and its isogenic *pilA* mutant strain was analyzed with 6-well polystyrene plates (see Materials and Methods) 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 A. Data presented in both panels are from three biological experiments each performed in triplicate. Double (**) and quadruple (****) asterisks indicate that two values are statistically different with P<0.01 and P<0.0001 by the Student's T-test, respectively.