Surface Hydrophilicity Promotes Bacterial Twitching Motility

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Abstract

The bacterial type IV pilus (T4P) is a critical virulence factor for many pathogens. These include *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, which are WHO priority pathogens because of their increasing antibiotic resistance. The T4P powers bacterial twitching motility which is frequently analyzed by interstitial colony expansion between agar and the polystyrene surfaces of plastic Petri dishes. In such assays with Petri dishes, the twitching motility of *Acinetobacter nosocomialis*, an opportunistic human pathogen, was observed with MacConkey but not Luria-Bertani (LB) agar media. One difference between these two media is that bile salts are present as a selective agent in MacConkey but not in LB. We found here that that the addition of bile salts to LB allowed *A. nosocomialis* to display twitching. Similarly, supplementation of bile salts to LB enhanced the twitching of *A. baumannii* and *P. aeruginosa*. These observations suggest that there is a common mechanism for bile salts to enhance bacterial twitching to promote interstitial colony expansion. Bile salts are used to inhibit the growth of Gram^{+} bacteria in MacConkey as they are detergents that can disrupt lipid membranes. Surprisingly, their effects on twitching motility are more related to the alteration of physicochemical properties of twitching surfaces rather than a change in bacterial physiology. This is because bacteria displayed increased twitching motility on hydrophilic surfaces such as those of glass and plasma-treated polystyrene plastics. On such surfaces, twitching motility is no longer stimulated by bile salts. We conclude that the hydrophilicity of surfaces, such as those of animal tissues as well as of medical implants and devices, may significantly impact the functionality of T4P as a virulence factor and its interaction with hosts in the establishment and persistence of a bacterial infection.

36 **Introduction**

37 Twitching motility is a form of non-flagellated bacterial translocation that allows bacteria
38 to move on or between solid surfaces [1]. This type of motility is powered by the bacterial type
39 IV pilus (T4P) and the supramolecular T4P machinery (T4PM) [2-5]. In the current model, it is
40 the recurrent cycles of T4P extension and retraction that powers this bacterial surface motility
41 [6]. The T4PM assembles the long T4P protein filament that protrudes from a cell into its
42 surroundings. When the tip of an assembled or extended T4P attaches to a solid substratum, the
43 retraction of the T4P by the T4PM moves a bacterium directionally. This results in the
44 translocation of bacterial cells on or between solid surfaces, resulting in bacterial twitching
45 motility [7].

46 More important to human health, T4P plays a crucial role in the pathogenesis of many
47 important bacterial pathogens [8-13]. These include World Health Organization (WHO) priority
48 pathogens [14], *Pseudomonas aeruginosa* and *Acinetobacter baumannii* which use T4P to adhere
49 to human cells or tissues to initiate colonization and invasion [1, 15, 16]. *Acinetobacter*
50 *nosocomialis*, a close relative of *A. baumannii*, is itself an opportunistic pathogen primarily
51 causing nosocomial or hospital-acquired infections [17]. Additionally, the *A. nosocomialis* M2
52 strain has been used as a model system for *Acinetobacter* studies of pathogenesis [16, 18, 19].
53 Although the lack of flagellated motility led to the acineto- or non-motile designation for this
54 genus, many *Acinetobacter* species, in fact, possess T4P and exhibit the T4P-dependent
55 twitching motility [20]. As such, twitching motility provides a convenient assay for bacterial T4P
56 in these medically important pathogens.

57 Twitching motility is commonly analyzed by observing interstitial colony expansion
58 between the surfaces of solidified nutrient agar and of plastic Petri dishes made of polystyrene

59 [21]. It was observed previously that *A. nosocomialis* exhibited twitching only with MacConkey
60 agar, but not Luria-Burtani (LB) agar on these polystyrene petri dishes [22]. Notwithstanding
61 their commonalities, LB lacks peptone, lactose and bile salts that are present in MacConkey.
62 Peptone is a proteinous nutrient source and lactose is a carbon and energy source. Bile salts,
63 primarily sodium glycocholate and sodium taurocholate, are detergents produced from
64 cholesterol degradation in humans and other vertebrates [23]. They are included in MacConkey
65 as a selective agent against Gram$^+$ bacteria and for enteric bacteria. The amphipathic nature of
66 bile salts allows them to interact with hydrophilic and hydrophobic molecules, thus enabling
67 them to result in envelope stress on bacteria [24] or alter the physicochemical properties of solid
68 surfaces they are applied to [25].
69 In this study, we examined the composition of MacConkey media and determined that
70 bile salts are stimulatory of *A. nosocomialis* twitching motility on polystyrene Petri dishes. We
71 also observed similar stimulatory effects of bile salts on the twitching of *P. aeruginosa* and *A.*
72 *baumannii*. Our results further demonstrate that other detergents likewise can enhance bacterial
73 twitching on polystyrene surfaces. Antibiotics, including outer membrane-targeting polymyxin
74 B, do not increase *P. aeruginosa* twitching motility, suggesting that the mechanism for the
75 stimulatory effect of bile salts is unlikely related to a bacterial response to envelope or
76 antimicrobial stress. Instead, we discovered that it was the hydrophilicity of surfaces that
77 promotes bacterial twitching. Plasma-treated polystyrene or glass surfaces, which are more
78 hydrophilic, for example, promote twitching motility significantly without detergents. The
79 results here suggest that bacterial pathogens may have evolved mechanisms to differentially
80 interact with surfaces with different physicochemical properties to optimize host recognition,
81 colonization and infections.

82 **Results**

83 **Bile salts enable *A. nosocomialis* twitching motility in agar plates.** It has been reported in the
84 literature that *Acinetobacter nosocomialis* displays twitching motility in agar plate assays with
85 MacConkey but not in LB [22]. In these assays, bacterial cells are stabbed through the agar to form an
86 interstitial colony on the Petri dish surface below agar [21]. The size of an interstitial colony can be
87 measured to quantify twitching motility in such assays. As shown in Figure 1, the *A. nosocomialis* M2
88 strain shows clear twitching with MacConkey, but not with LB agar. These results here confirmed the
89 effect of agar media on *A. nosocomialis* twitching motility in the literature.

90 To investigate the underlying reason for this observation, the composition of these two
91 commonly used growth media were compared. As shown in Table S1, both media use the same
92 concentration of agar and sodium chloride, 1.2% and 0.5% respectively. The key components in
93 MacConkey that are absent in LB include peptone, lactose, and bile salts. Neutral red, another
94 component that differed between them, is a pH indicator dye for the detection of lactose
95 fermentation and we considered it unlikely to be responsible for the differences in twitching. We
96 supplemented the LB agar with peptone, lactose or bile salts at the same concentration as in
97 MacConkey agar to determine if one of these could enable *A. nosocomialis* to twitch. As shown
98 in Figure 1A, bile salts, but not the others, were found to permit *A. nosocomialis* twitching
99 motility in LB agar plates. This result suggests a bile salt-specific stimulation of twitching
100 motility in *A. nosocomialis*.

101 Twitching motility as analyzed by the agar stabbing method has been observed in
102 *Acinetobacter baumannii,* a closely related *Acinetobacter* species and WHO priority pathogen
103 [14]. The twitching motility of this bacterium is similarly noted in MacConkey agar and not LB
104 agar. We tested two *A. baumannii* strains, AYE and AB0057, in LB supplemented with bile salts

105 in comparison with *A. nosocomialis* M2. As shown in Figure 1C, bile salts stimulated the
106 twitching motility of the two *A. baumannii* strains similar to the observation of *A. nosocomialis*.
107 These results suggested that the stimulatory effects of bile salts on twitching motility occurred in
108 *Acinetobacter* species as a general phenomenon.

109 **Bile salt-stimulated twitching motility of *P. aeruginosa*.** These above observations prompted us to
110 investigate if bile salts enhance bacterial twitching by a more general mechanism in other bacteria.
111 *Pseudomonas aeruginosa*, a WHO priority pathogen [14], has been used as a model for studies of
112 twitching motility [6, 26, 27]. Its twitching motility has been routinely analyzed with LB instead of
113 MacConkey agar plates. As shown in Figure 2A, bile salts supplemented at 0.5%, the same
114 concentration present in MacConkey, significantly increased the twitching motility of the *P. aeruginosa*
115 strain PAO1, the frequently used strain for the studies of twitching motility in this bacterium [28, 29].
116 The dose response of bile salts on PAO1 twitching was examined and as shown in Fig. 2B, there is a
117 concentration-dependent response, reaching a maximum stimulation around 0.4% bile salts. At higher
118 concentrations, bile salts start to inhibit *P. aeruginosa* growth and reduce its twitching area in this assay.
119 We also observed that the twitching motility of PA14, another common *P. aeruginosa* strain in research
120 laboratories [30-32], was stimulated by bile salts in LB media (Figure S2). These results demonstrate
121 that the stimulatory effect of bile salts on twitching is applicable to both *Acinetobacter* species and *P.*
122 *aeruginosa*. For the remaining part of this study, we mostly used *P. aeruginosa* PAO1 as the model
123 organism to investigate the mechanisms by which bile salts stimulate bacterial twitching motility.

124 **Detergents stimulate twitching motility.** Bile salts are anionic detergents, produced from
125 cholesterol degradation [33], that can elicit a lipid membrane stress response in bacteria [34]. Thus, it is
126 possible that bile salts function as a detergent and apply envelope stress to stimulate bacterial twitching
127 [34]. To examine this possibility, we investigated the effect of other detergents on the twitching motility

128 of *P. aeruginosa.* To avoid crosstalk between growth inhibition and twitching motility, non-inhibitory
129 concentrations of detergent were determined to guide their use in the twitching motility assays (Table
130 S2). For this experiment, we tested sodium dodecyl sulfate (SDS), an anionic detergent, as well as Triton
131 X-100 and Triton X-114, which are non-ionic detergents (Table S2). As shown in Fig. 3A, all the
132 detergents examined, whether anionic or non-ionic, significantly stimulated the twitching motility of *P.*
133 *aeruginosa* much like bile salts. These results suggest that the stimulatory effects of bile salts on
134 twitching is related to their chemicophysical properties as detergents.

135 The stimulatory effects of detergent on twitching motility could be explained by a physiological
136 response of a bacterium to envelope stress applied by these amphipathic molecules or a general stress
137 applied by other stressors [35]. To investigate this, we tested a few antibiotics with different targets at
138 non-inhibitory concentrations as stressors distinct from detergents. These included ampicillin,
139 gentamicin, and ciprofloxacin, which target cell wall biosynthesis, ribosome function and DNA
140 topology, respectively. Maximum non-inhibitory concentrations (MaxNICs) of these antibiotics were
141 determined by testing the effect of different concentrations on *P. aeruginosa* growth in liquid culture
142 (Table S3). Antibiotics at their respective MaxNICs were tested for their effect on *P. aeruginosa*
143 twitching. As shown in Figure 3B, none of these three antibiotics increased *P. aeruginosa* twitching
144 motility. This suggested that the stimulation of twitching was unlikely the result of a general
145 physiological response to stressors. We additional tested the effect of polymyxin B, which targets the
146 outer membrane and would apply envelop stress similar to bile salts and the detergents. Somewhat
147 surprisingly, this antibiotic at its MaxNIC (Table S3) showed no stimulatory effect on *P. aeruginosa*
148 twitching (Figure 3B). These results suggested that the observed stimulation of twitching motility by
149 bile salts and other detergents in Figures 1 and 2 was not a response to envelope stress.

150 **Hydrophilic surfaces promote twitching motility in *P. aeruginosa.*** Detergents are amphipathic
151 molecules that can mediate both hydrophobic and hydrophilic interactions [33]. Besides physiological
152 effects on bacteria as envelope stressors, they can also change properties of surfaces on which bacterial
153 twitching motility occurs [25]. In the stabbing assay, bacteria cells twitch in the interstitial space
154 between the agar media and the surface of the hydrophobic polystyrene Petri dish. We considered the
155 possibility that detergents such as bile salts may interact with the hydrophobic surface of the polystyrene
156 Petri dishes. Such interactions could coat the surface and make it more hydrophilic to facility twitching
157 motility. Glass is a hydrophilic surface, so we compared *P. aeruginosa* twitching on glass versus
158 polystyrene microscope slides placed within Petri dishes containing LB agar without bile salts or
159 detergents. As shown in Figure 4, twitching motility on glass is significantly higher than that on
160 polystyrene for the wildtype (WT) strain. The addition of bile salts had minimum effects on twitching on
161 glass surfaces, in contrast to the observations with polystyrene ones (Figure 4). These results indicate
162 that surface hydrophilicity likely enhances twitching motility and the effects of detergents and bile salts
163 on twitching could be attributed to their ability to change a hydrophobic surface to a more hydrophilic
164 one.

165 To further examine this possibility, we used a coating to change the surface hydrophilicity of glass
166 by pretreating it with a polydimethylsiloxane solution (PDMS). When the glass surface was layered with
167 PDMS to mimic the hydrophobicity of a polystyrene plate, *P. aeruginosa* twitched at similar levels as it
168 did with polystyrene Petri dishes in LB media without bile salts (Figure 4B). PDMS coating did not
169 impact twitching motility of *P. aeruginosa* on polystyrene surfaces which is already hydrophobic
170 (Figure 4B). A *P. aeruginosa pilA* mutant was used as control. It showed no twitching with any of the
171 surfaces with or without PDMS treatment. These results are consistent with the notion that hydrophilic

172 surfaces enhance *P. aeruginosa* twitching and that bile salts and other detergents stimulate twitching
173 motility on the hydrophobic polystyrene surfaces by making it more hydrophilic.
174 While natural polystyrene surfaces are hydrophobic, they can be treated with plasma gas to increase
175 their hydrophilicity for tissue culture purposes [36]. We therefore compared *P. aeruginosa* twitching
176 motility on 6-well polystyrene plates either untreated or treated by plasma gas for tissue culture. As
177 shown in Figure 5A, the wildtype *P. aeruginosa* PAO1 strain exhibited significantly increased twitching
178 motility on plasma-treated surfaces in LB media over those untreated surfaces. In contrast, while the
179 addition of bile salts significantly enhanced *P. aeruginosa* twitching on untreated plates, it had no
180 significant effect on PAO1 twitching on the treated surfaces. These results led us to conclude that the
181 stimulatory effect of bile salts on twitching motility is related to the changes in surface hydrophobicity
182 instead of a physiological response of a bacterium elicited by the presence of bile salts or other
183 detergents.
184 The increase in *P. aeruginosa* twitching motility without any media additives on hydrophilic
185 surfaces was striking, thus we wanted to confirm if this phenotype was consistent with *Acinetobacter*
186 *nosocomialis*, which is non-motile in LB media. Markedly, the wildtype *A. nosocomialis* M2 strain (M2
187 WT) also exhibited increased twitching motility on plasma-treated polystyrene in LB media and no
188 twitching on hydrophobic polystyrene in LB media (Figure 5B). The difference between these two
189 polystyrene structures is their hydrophilicity, therefore we provided significant evidence that surface
190 hydrophilicity promotes twitching motility in *P. aeruginosa* and *A. nosocomialis*.
191
192 **Discussion**
193 Here we describe the discovery of detergent-stimulated twitching motility and identify a
194 relationship between surface hydrophilicity and twitching motility of *Acinetobacter* species as
195 well as *P. aeruginosa*. It was previously observed that *A. nosocomialis* can perform twitching

196 motility on MacConkey agar but not Luria-Bertani (LB) agar [22]. We discovered that bile salts
197 were the component in MacConkey agar that facilitated the twitching motility of *A. nosocomialis*
198 as well as *A. baumannii* on polystyrene surfaces. This stimulatory effect further extended to *P.*
199 *aeruginosa*. We observed that other ionic and anionic detergents, such as Triton X-100, Triton
200 X-114 and sodium dodecyl sulfate (SDS) also promoted *P. aeruginosa* twitching motility. The
201 stimulatory effect of bile salts and other detergents on bacterial twitching is likely due to their
202 effects on physicochemical properties of surfaces instead of a physiological response of the
203 bacteria to detergents. This conclusion is supported by our experimental results with antibiotics
204 that have various modes of actions, as they all failed to enhance twitching motility. Rather, we
205 found that physicochemical properties of surfaces play a critical role in modulating the level of
206 bacterial twitching motility: surface hydrophilicity promotes whereas hydrophobicity suppresses
207 bacterial twitching.

208 The interaction between pathogens and host surfaces is essential for the establishment of a
209 bacterial infection. Additionally, interactions with the surfaces of medical implants and devices play a
210 major role in the establishment of hospital-acquired bacterial infections [15, 37-40]. The bacterial type
211 IV pilus (T4P) is an important virulence factor needed for the establishment of infection for many WHO
212 priority pathogens [14], such as *P. aeruginosa* and *A. baumannii* [15]. Thus, studying the T4P as a
213 motility apparatus is essential to better understanding the first step of infection. Interested in
214 understanding how MacConkey agar could enable the T4P-mediated twitching motility of *A.*
215 *nosocomialis*, we discovered an important relationship between surface chemistry and twitching
216 motility. Not only did hydrophilic surfaces, such as glass and plasma-treated polystyrene increase
217 bacterial twitching motility, but hydrophobic surfaces, like polystyrene, completely disabled twitching
218 motility of *A. nosocomialis*. This result highlights a powerful control point for T4P functionality via

219 surface polymer construction. Hence, we propose that the hydrophilicity of surfaces, such as those of
220 host tissues or medical implants, may play an important role in the activity of the T4P as a virulence
221 factor responsible for the establishment and persistence of a bacterial infection.

222 While the implications of this discovery are a much-needed consideration in future research, we
223 note that the mechanism of surface hydrophilicity-promoted twitching motility remains unclear.
224 Hydrophobic surfaces allow for better attachment by *P. aeruginosa* for biofilm formation, a nonmotile
225 or sessile state [41]. Thus, it is possible that the interaction between the T4P head and surfaces during
226 twitching motility has better attachment to hydrophilic surfaces than hydrophobic surfaces. Additionally,
227 hydrophilicity may play a role in reducing the friction between the cell surface during translocation over
228 the surface via pilus retraction. In general, there are like-to-like observations for surface attachment, in
229 which bacteria with hydrophobic cell surfaces adhere better to hydrophobic surface materials [42]. Both
230 *P. aeruginosa* and *A. baumannii* have high surface hydrophobicity [43, 44], thus adhering better to
231 hydrophobic twitching surfaces. When these hydrophobic cells move over hydrophilic surfaces, it is
232 expected that the cells will adhere less which may promote increased motility. Although we are unsure
233 of the mechanism of surface hydrophilicity-promoted twitching, it is reasonable to conclude that
234 increased hydrophilicity of a material surface would stimulate twitching motility.

235 While our observations are consistent with surface hydrophilicity promoted twitching
236 motility, it is also possible the changes in motility are due to the surface sensing capacity of the
237 T4P. The T4P has been implicated as a surface sensor in *Caulobacter crescentus* [45-47]. The
238 T4P retraction motor and minor pilins were also found to be involved in sensing surface
239 association necessary for quorum sensing in *P. aeruginosa* [48]. Additionally, twitching motility
240 has been shown to require bacteria to sense and respond to surfaces [49]. Thus, it is possible that
241 bacteria are sensing the hydrophilicity of a surface, and this results in the increased twitching

242 motility phenotype we describe in this study. Since surface hydrophilicity is less conducive for
243 hydrophobic cell attachment [42], it is possible that surface sensing of hydrophobicity would
244 result in increased twitching via cell signaling.

245 A few lines of evidence suggest that hydrophilic surfaces increase the twitching motility of *P.*
246 *aeruginosa* and *A. nosocomialis*. Mainly, two hydrophobic surfaces, glass and plasma-treated
247 polystyrene, resulted in significantly increased twitching. On the other hand, hydrophobic surfaces
248 decreased twitching motility. Using detergents and antibiotics we also supported the physicochemical
249 twitching response and demonstrated that bacterial physiology was not the mechanism of detergent-
250 stimulated motility. While this result highlights the discovery of surface hydrophilicity-promoted
251 motility, future work is necessary to elucidate the mechanisms of this phenomenon. Additionally, it
252 would be interesting to investigate the extents of this phenomenon as it may play a role in other T4P-
253 dependent motilities, such as social gliding motility in *Myxococcus xanthus* [50], or other pathogens that
254 utilize twitching motility, like *Neisseria gonorrhoeae* [1, 51]. We suggest that the hydrophilicity of
255 surfaces, such as those of animal tissues as well as of medical implants and devices, may significantly
256 impact the functionality of T4P as a virulence factor and its interaction with hosts in the establishment
257 and persistence of a bacterial infection.

258

259 **Materials and Methods**

260 **Strains and culture conditions.** The bacterial strains used in this study are detailed in Table 1. These
261 include wildtype and *pilA* mutant *Acinetobacter nosocomialis* M2 and *Pseudomonas aeruginosa* PAO1.
262 Additionally, wildtype *Acinetobacter baumannii* strains, Ab0057 and AYE, were used. *Pseudomonas*
263 *aeruginosa* PA14 was used in supplemental analysis. MacConkey broth media from the manufacturer,
264 Oxoid, supplemented with 1.2% granulated agar, was used for twitching assays. The components of this

265 media include 1% lactose, 2% peptone, 0.5% sodium chloride, 0.5% bile salts, and 0.075% neutral red.
266 LB broth supplemented with 1.2% granulated agar was also utilized for twitching assays throughout this
267 study. *P. aeruginosa* strains were grown and maintained on 1.5% Luria-Bertani agar (LBA) at 37°C,
268 while *A. nosocomialis* and *A. baumannii* strains were grown and maintained on 1.5% MacConkey agar
269 at 37°C.
270
271 **Polystyrene Petri dish twitching motility assay.** Twitching assays were performed on fresh 25 mL
272 1.2% LBA plates prepared the day before. When additives were added to the media, antibiotics or
273 detergents were utilized at their maximum non-inhibitory concentration. Before performing the assay,
274 the 1.2% LBA plates were dried face up without their lids on in the biosafety cabinet for 20 minutes.
275 Condensation was wiped off the Petri dish lids, and after 20 minutes of drying, the plates and the lids
276 were sterilized under UV light for 2 minutes. The inoculum was prepared by spreading a loopful of
277 bacteria from the outside edge of the overnight culture onto an untouched 1.5% LBA plate to create a
278 thin layer of culture. Using a toothpick, a unified amount of inoculum was picked up and inoculated the
279 1.2% LBA plates by stabbing through the agar, making sure to touch the bottom of the plate. The
280 inoculated 1.2% LBA plates were incubated in stacks at 37°C for 48 hours in a humidity chamber. The
281 humidity chamber was prepared by placing a dampened paper towel to cover the bottom of a plastic box.
282 Every 24 hours the paper towel was removed and replaced with a fresh one. After 48 hours of
283 incubation, the twitching motility was visualized by removing the agar from the Petri dish and stained
284 with 1% crystal violet. Twitching area was determined by analyzing the stained area of the plate with
285 ImageJ software. This protocol was modified from a previously described twitching motility assay [21].
286

287 **Twitching motility on glass and polystyrene microscope slides coated with a polydimethylsiloxane**
288 **solution.** Glass or polystyrene microscope slides were submerged in either a filter-sterilized
289 polydimethylsiloxane solution or 70% ethanol as a control. These slides were left on a rack to dry for a
290 few hours at 40°C. Once dry, the microscope slides were laid inside polystyrene Petri dishes, and 25 mL
291 of 1.2% LBA was poured on top of them. The modified plates were inoculated as described above. The
292 inoculated 1.2% LBA plates were incubated in stacks at 37°C for 18 hours in a humidity chamber. After
293 18 hours of incubation, the twitching motility was visualized without removing the agar from the Petri
294 dish by holding the plates up to the light and tracing the visible twitching area with a permanent marker.
295 Twitching area was determined by analyzing the traced area of the plate with ImageJ software.
296
297 **Optimized 6-well twitching motility assay on polystyrene and plasma-treated polystyrene.**
298 Twitching motility assays were performed as described above, with modifications to the volume of
299 media placed in each well and incubation times. 2 mL of 1.2% LBA, with or without 0.5% bile salts,
300 was dispensed into each well of a 6-well polystyrene or plasma-treated polystyrene plate. Inoculated
301 plates are placed in a humidity chamber and incubated for 24 hours at 37°C. Rather than staining the
302 plate with 1% crystal violet, once the agar is removed from the wells, the twitching area was traced with
303 a permanent marker. The twitching area was determined by analyzing the marked area with ImageJ
304 software.
305
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320

321

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451 **Figure Legends**

452

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

454

455 **Figure 1. Bile salts alone enable the twitching motility of *A. nosocomialis* in LB medium.**
456 **(A)** Subsurface twitching motility assays inoculated with *A. nosocomialis* were performed in
457 triplicate on MacConkey agar, Luria-Bertani (LB) agar, and LB agar supplemented with each
458 component of MacConkey agar, not found in LB media: 0.5% bile salts, 1% lactose, and 2%
459 peptone. Plates were incubated for 48 hours in a humidity chamber at 37°C. Asterisks (*) specify
460 that the twitching area values at the indicated additives are statistically different ($P<0.05$) from
461 those of the no-additive LB control. **(B)** A single colony of *A. baumannii* strains (AB0057 and
462 AYE) and *A. nosocomialis* (M2), were inoculated in triplicate into LB agar plates supplemented
463 with or without 0.5% w/v bile salts (BSs). After 48 hours of incubation, the agar was removed,
464 and cells adhered to the plate were stained with 1% crystal violet. Twitching area was quantified
465 using image analysis of stained plate region. Asterisks (*) specify that the twitching area values
466 with bile salts for the indicated strains are statistically different ($P<0.05$) from those without bile
467 salts.

468

469 **Figure 2. Twitching motility analysis of *Pseudomonas aeruginosa* in the presence of bile**
470 **salts. (A)** Subsurface twitching motility assays inoculated with *P. aeruginosa* (PAO1) were
471 performed in triplicate on LB agar supplemented with or without 0.5% bile salts (BSs). Asterisks
472 (*) specify that the twitching area values with bile salts for the indicated strains are statistically
473 different ($P<0.05$) from those without bile salts. **(B)** *P. aeruginosa* (PAO1) subsurface twitching

474 assays on LB agar with varying bile salt concentrations. After 48 hours of incubation, the agar
475 was removed, and cells adhered to the plate were stained with 1% crystal violet. Twitching area
476 (cm^2) of three experiments performed in triplicate was quantified using image analysis of stained
477 plate region and normalized to 1.
478
479 **Figure 3. Detergents, and not antibiotics, promote twitching motility equivalent to the bile**
480 **salts response. (A)** Subsurface twitching motility assays of *P. aeruginosa* (PAO1) were
481 performed three times in triplicate on LB agar (-) and LB agar supplemented with the membrane-
482 disruptive detergents: bile salts (BSs), Triton X-100 (TX100), Triton X-114 (TX114), and
483 sodium dodecyl sulfate (SDS). **(B)** Twitching motility assays of *P. aeruginosa* (PAO1) were also
484 performed on LB agar supplemented with ampicillin (Amp), ciprofloxacin (Cipro), gentamicin
485 (Gent), and polymyxin B (PB). After 48 hours of incubation, the agar was removed, and the
486 twitching area was quantified using image analysis. Asterisks (*) specify that the twitching area
487 values at the indicated additives are statistically different ($P<0.05$) from those of the no-additive
488 LB (-) control, and twitching values that are not statistically different ($P>0.05$) are represented by
489 (ns).
490
491 **Figure 4. Hydrophilic glass surfaces increase twitching motility of *P. aeruginosa* in LB**
492 **media.** Subsurface twitching motility assays of wildtype (WT) and a *pilA* mutant strain ($pilA^-$) of
493 *P. aeruginosa* (PAO1) were performed in LB agar on polystyrene and glass microscope slides
494 placed within a polystyrene Petri dish. The twitching motility of WT and $pilA^-$ *P. aeruginosa*
495 was also analyzed in LB supplemented with 0.5% bile salts (BSs) on polystyrene and glass
496 microscope slides. To test the effect of hydrophobic surfaces, the twitching motility of WT and
497 $pilA^-$ *P. aeruginosa* was additionally analyzed on polystyrene and glass microscope slides coated

498 with a polydimethylsiloxane (PDMS) solution. The microscope slides not coated in PDMS, were
499 coated with 70% ethanol as a control. All of these conditions were tested via three biological
500 replicates in triplicate. After 18 hours of incubation, the agar was removed, and the twitching
501 area was quantified using image analysis. Asterisks (*) specify that the twitching area values on
502 the indicated surfaces are statistically different ($P<0.05$) from those of the polystyrene control,
503 and twitching values that are not statistically different ($P>0.05$) are represented by (ns).
504
505 **Figure 5. Tissue-treated plates (hydrophilic) promote twitching motility over untreated**
506 **plates (hydrophobic) with LB. (A)** Subsurface twitching motility assays of wildtype (WT) and
507 a *pilA* mutant strain (pilA$^-$) of *P. aeruginosa* (PAO1) were performed three times in triplicate in
508 LB agar on polystyrene and plasma-treated polystyrene 6-well plates with or without the addition
509 of 0.5% bile salts (BSs). **(B)** Twitching motility assays of wildtype (WT) and a *pilA* deletion
510 strain (pilA$^-$) of *A. nosocomialis* (M2) were also performed three times in triplicate in LB agar on
511 polystyrene and plasma-treated polystyrene 6-well plates. After 18 hours of incubation, the agar
512 was removed, and the twitching area was quantified using image analysis. Asterisks (*) specify
513 that the twitching area values on the indicated surfaces are statistically different ($P<0.05$) from
514 those of the polystyrene control.
515

516 **Supplemental Materials**

517

518 **Table S1. Composition of LB and MacConkey media.** Ingredients and their concentration are

519 listed for both media used.

520

521 **Figure S1. Twitching motility analysis of *Pseudomonas aeruginosa* PA14 in the presence of**

522 **bile salts.** Subsurface twitching motility assays inoculated with *P. aeruginosa* PA14 were

523 performed in triplicate on LB agar supplemented with or without 0.5% bile salts (BSs). After 48

524 hours of incubation, the agar was removed, and cells adhered to the plate were stained with 1%

525 crystal violet. Asterisks (*) specify that the twitching area values with bile salts for the indicated

526 strains are statistically different ($P<0.05$) from those without bile salts.

527

528 **Table S2. Properties of detergents.** Characteristics of detergents and their maximum non-

529 inhibitory concentration (MaxNIC) in P. aeruginosa.

530

531 **Table S3. Antimicrobial susceptibility testing of *P. aeruginosa* strain PAO1.**

532

Table 1.

Strain		**Description**	**Reference**
Acinetobacter nosocomialis	M2	Metro Health Systems clinical isolate	(Niu et al., 2008)
	M2	*pilA* deletion	(Harding et al., 2013)
Acinetobacter baumannii	Ab0057	Walter Reed Army Medical Center clinical isolate	(Hamidian et al., 2017)
	AYE	Valenciennes Hospital clinical isolate	(Adams et al., 2008)
Pseudomonas aeruginosa	PAO1	Wildtype	(Stover et al., 2000)
	PW8622	*pilA* transposon insertion (*pilA*-H02::ISphoA/hah)	(Held. et al., 2012)

Fig. 1

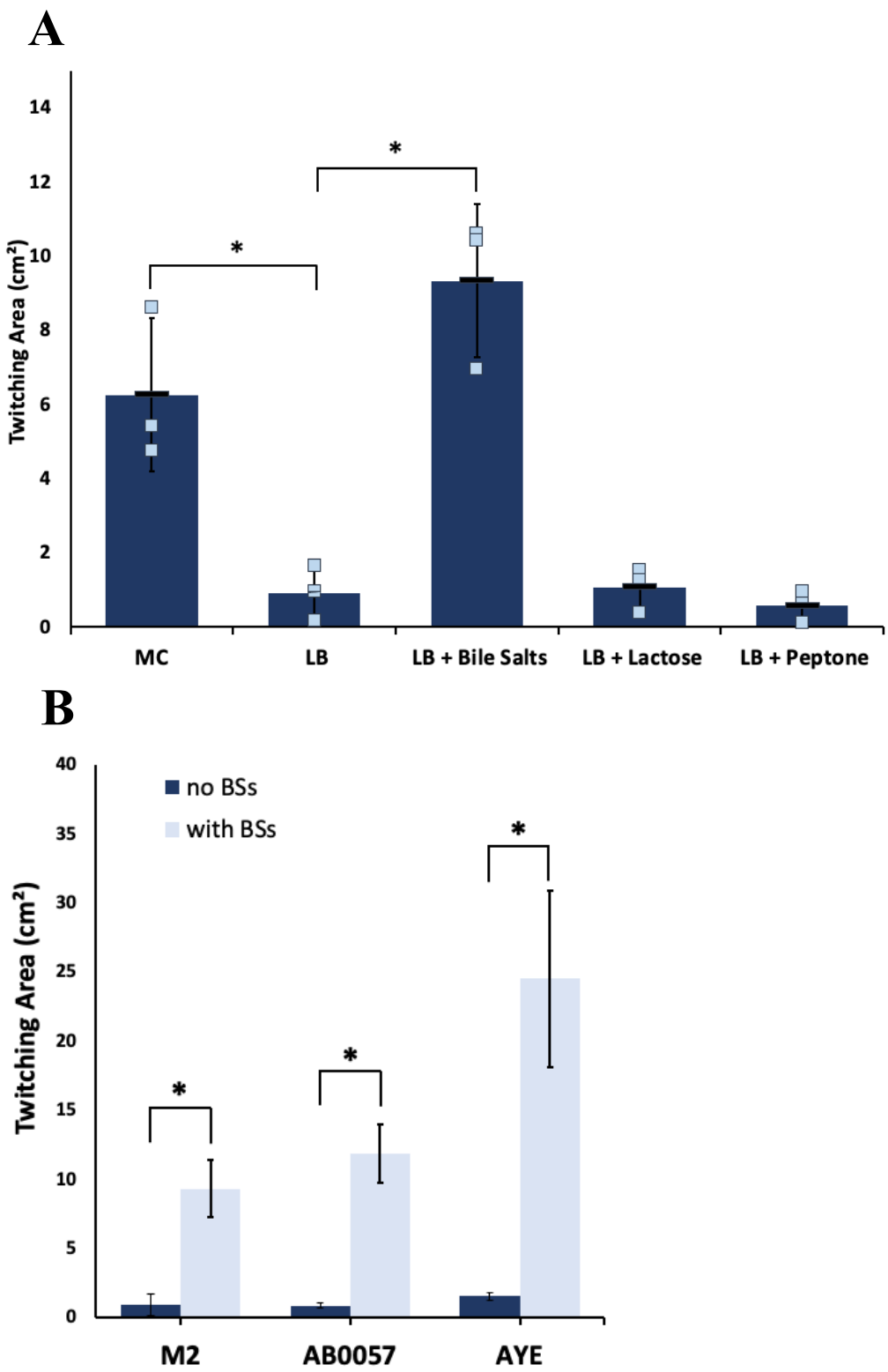
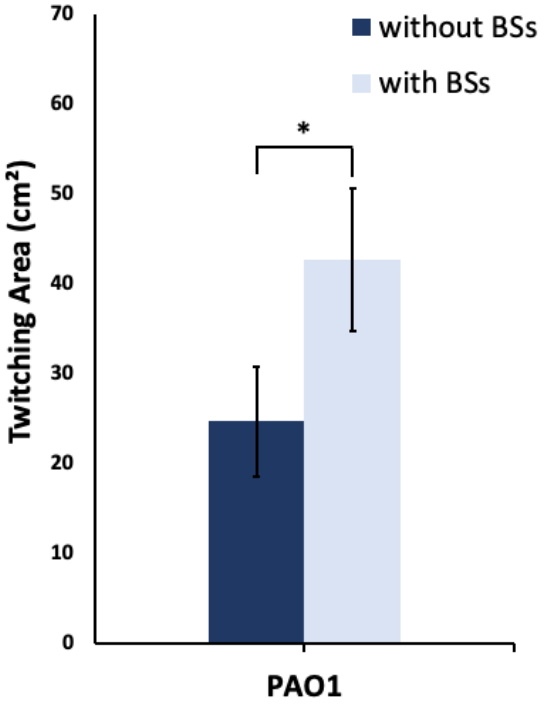

A
Twitching Area (cm²)
14
12
10
8
6
4
2
0
*
*
MC
LB
LB + Bile Salts
LB + Lactose
LB + Peptone
B
Twitching Area (cm²)
40
35
30
25
20
15
10
5
0
no BSs
with BSs
*
*
*
M2
AB0057
AYE

Fig. 2

A


without BSs
with BSs
*
Twitching Area (cm²)
70
60
50
40
30
20
10
0
PAO1

B

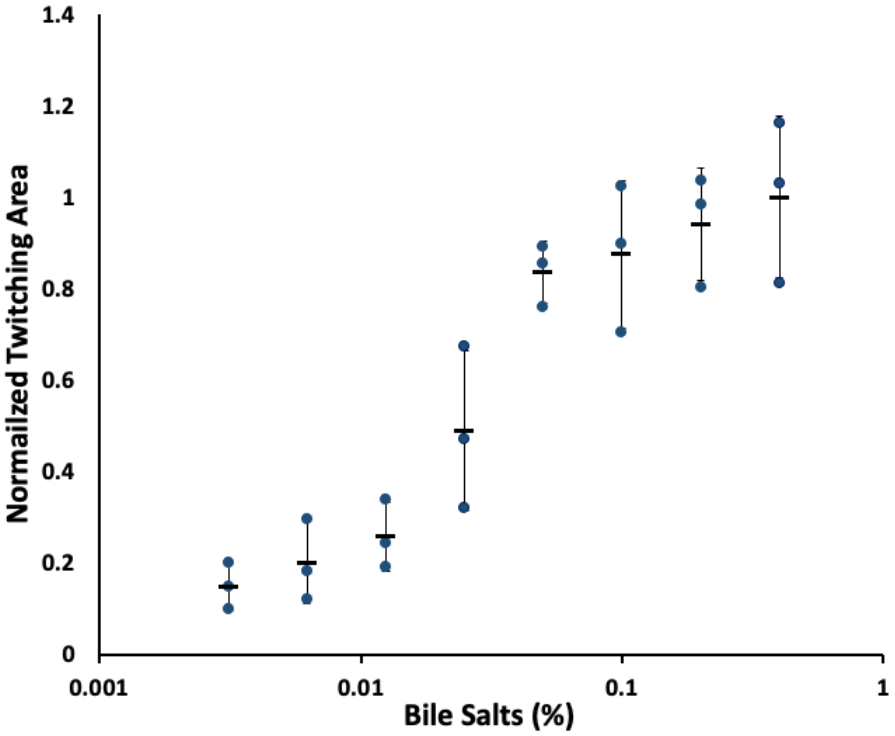

Normailzed Twitching Area
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
Bile Salts (%)

Fig. 3

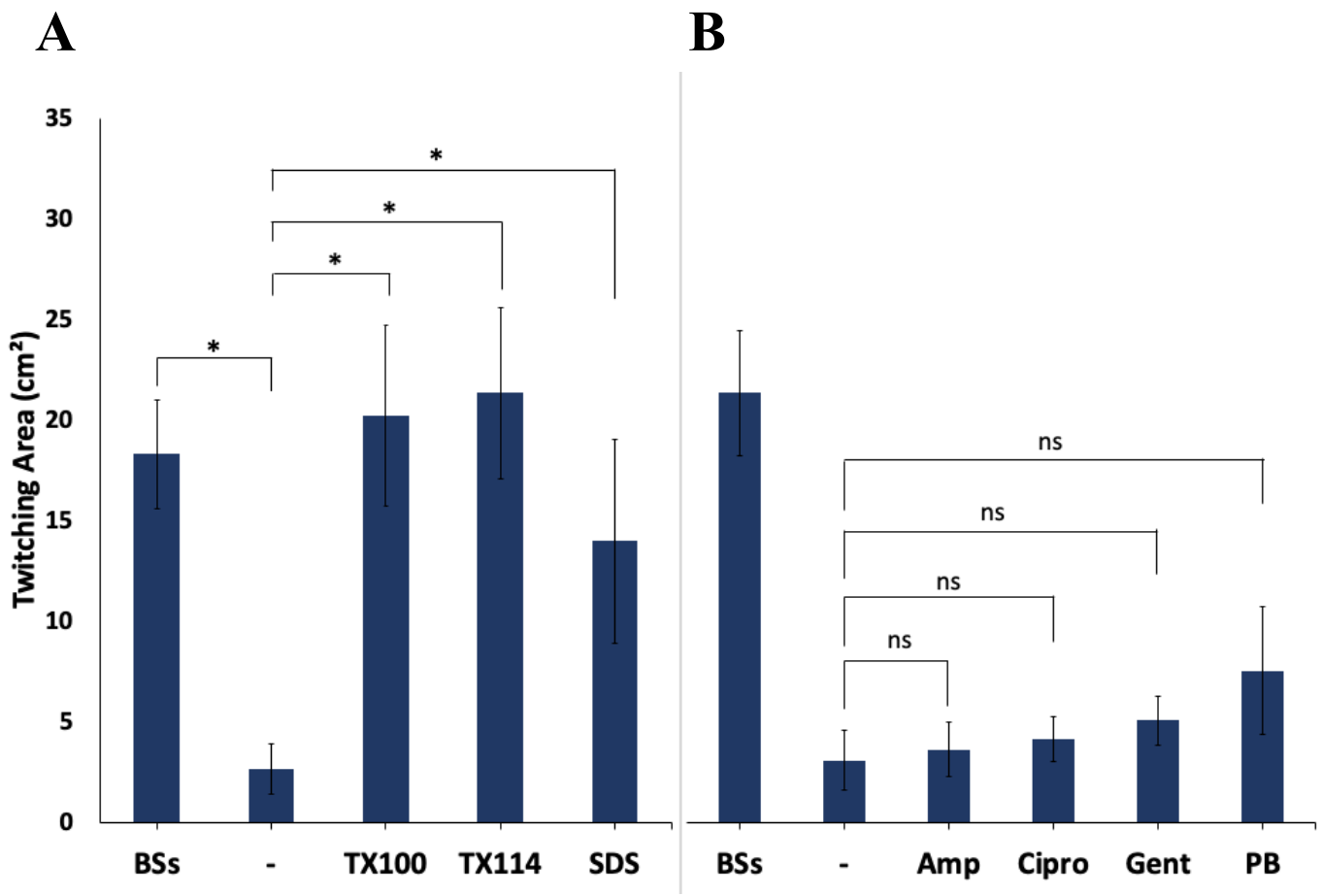

A
B
Twitching Area (cm²)
35
30
25
20
15
10
5
0
*
*
*
*
ns
ns
ns
ns
BSs
-
TX100
TX114
SDS
BSs
-
Amp
Cipro
Gent
PB

Fig. 4

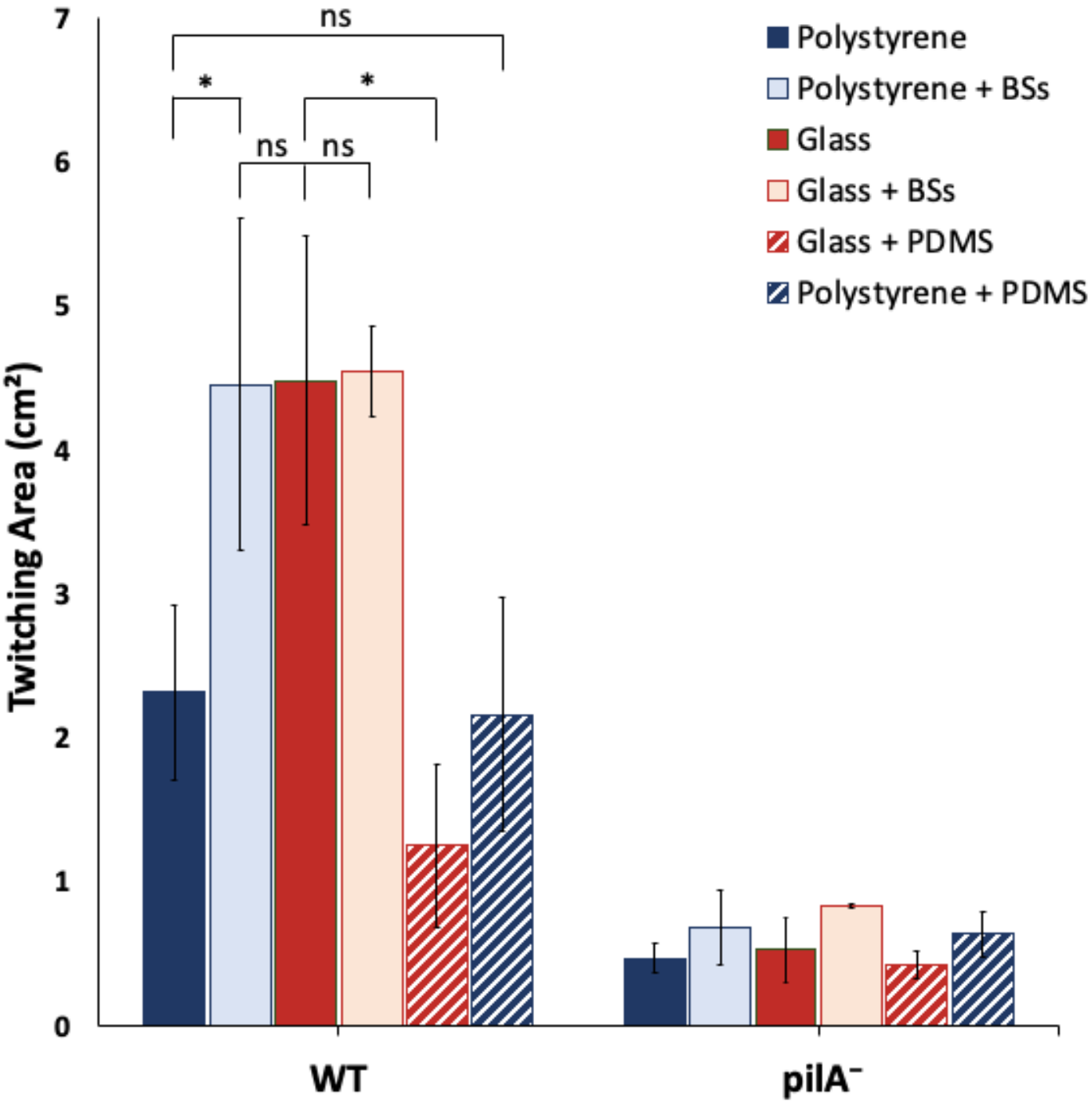
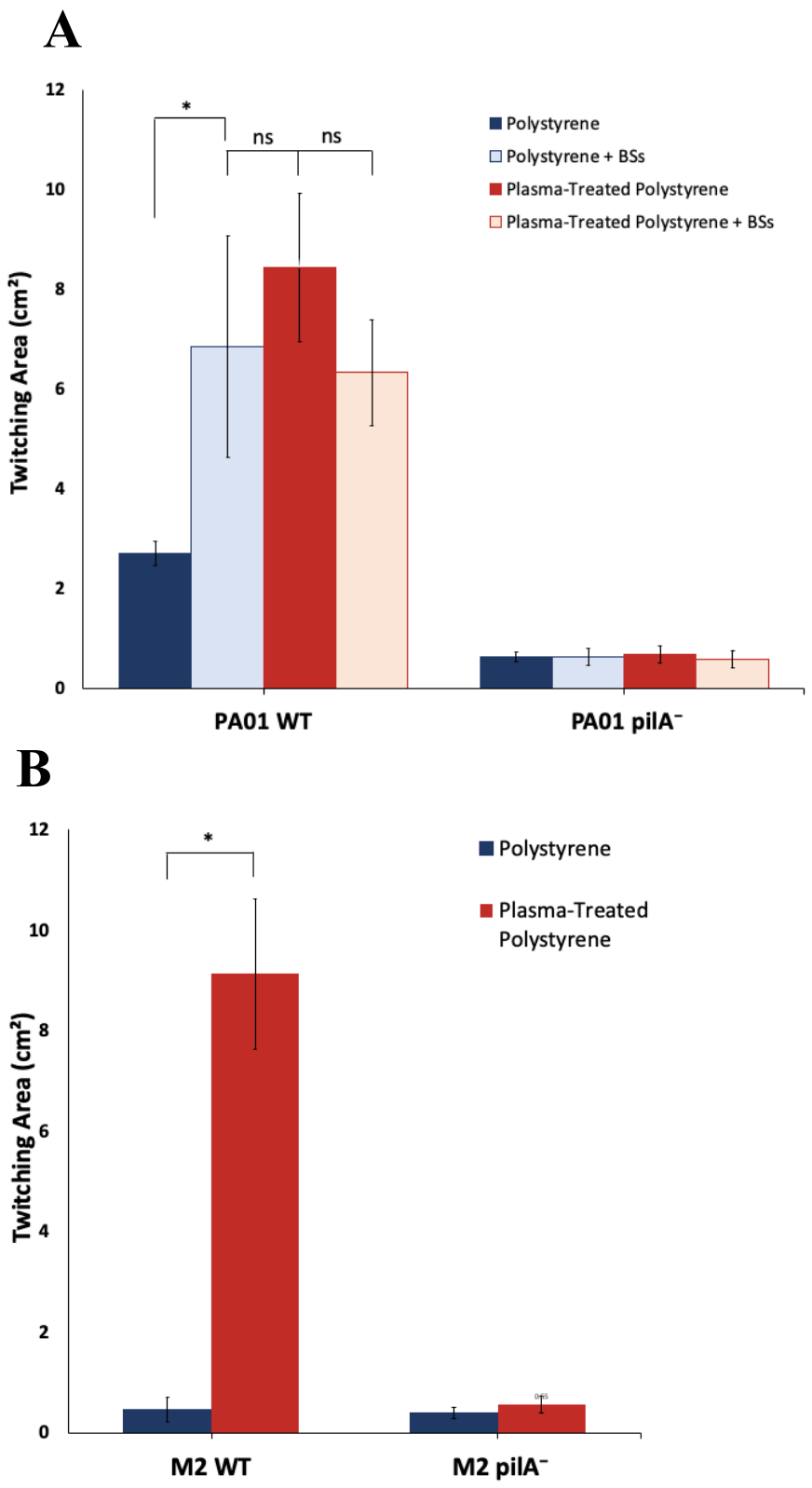

ns
*
*
ns
ns
Polystyrene
Polystyrene + BSs
Glass
Glass + BSs
Glass + PDMS
Polystyrene + PDMS
Twitching Area (cm²)
0
1
2
3
4
5
6
7
WT
pilA⁻

Fig. 5


A
12
10
8
6
4
2
0
Twitching Area (cm²)
*
ns
ns
Polystyrene
Polystyrene + BSs
Plasma-Treated Polystyrene
Plasma-Treated Polystyrene + BSs
PA01 WT
PA01 pilA⁻
B
12
10
8
6
4
2
0
Twitching Area (cm²)
*
Polystyrene
Plasma-Treated Polystyrene
M2 WT
M2 pilA⁻