The *Pseudomonas aeruginosa* T3SS can contribute to traversal of an *in situ* epithelial multilayer independently of the T3SS needle

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Running Title: T3SS needle-independent epithelial traversal by *P. aeruginosa*

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ABSTRACT

Multilayered epithelia lining our tissue surfaces normally resist traversal by opportunistic bacteria. Previously, we developed a strategy to experimentally perturbate this resistance *in situ* in the corneas of mouse eyes and used it to show that traversal of a multilayered epithelium by *Pseudomonas aeruginosa* requires ExsA, the transcriptional activator of its type 3 secretion system (T3SS). Here, we developed a novel strategy for quantitively localizing individual traversing bacteria within the *in situ* multilayered corneal epithelium and explored contributions of T3SS components. The results showed that T3SS translocon and T3SS effector mutants had reduced epithelial traversal efficiency. Surprisingly, a Δ*pscC* mutant unable to assemble the T3SS needle traversed as efficiently as wild-type *P. aeruginosa*, while a Δ*exsD* mutant 'constitutively on' for T3SS expression was traversal defective. Dispensability of the T3SS needle for effector-mediated traversal was confirmed using a mutant lacking the T3SS operon except the effector genes (Δ*pscU-L* mutant). That mutant reacquired the ability to traverse if complemented with rhamnose-inducible *exsA*, but not if the effector genes were also deleted (Δ*pscU-L*Δ*exoSTY*). Western immunoblot confirmed ExoS in culture supernatants of rhamnose-induced *exsA*-complemented Δ*pscU-L* mutants lacking all T3SS needle protein genes. Together, these results show that epithelial traversal by *P. aeruginosa* can involve T3SS effectors and translocon proteins independently of the T3SS needle previously thought essential for T3SS function. This advances our understanding of *P. aeruginosa* pathogenesis and has relevance to development of therapeutics targeting the T3SS system.

IMPORTANCE

While the capacity to cross an epithelial barrier can be a critical step in bacterial pathogenesis, our understanding of mechanisms involved is derived largely from cell culture experimentation. The latter is due to practical limitations of *in vivo*/*in situ* models and challenge of visualizing individual bacteria in the context of host tissue. Here, factors used by *P. aeruginosa* to traverse an epithelial multilayer *in situ* were studied by: 1) leveraging the transparent properties and superficial location of the cornea, 2) using our established method for enabling bacterial traversal susceptibility, and 3) developing a novel strategy for accurate and quantitative localization of individual traversing bacteria *in situ*. Outcomes showed that T3SS translocon and T3SS effector proteins synergistically contribute to epithelial traversal efficiency independently of the T3SS needle. These findings challenge the assumption that the T3SS needle is essential for T3SS effectors or translocon proteins to contribute to bacterial pathogenesis.

70 **INTRODUCTION**

71

72 *Pseudomonas aeruginosa* is a common cause of opportunistic infections, able to cause disease in
73 many body sites including the airways, GI tract, skin, urinary tract, eye, brain, heart and blood (1,
74 2). Susceptibility can be associated with wounds, burns, immunocompromise, chemotherapy,
75 surgery, predisposing diseases (e.g. cystic fibrosis), and use of indwelling medical devices (3–5).
76 In the eye, *P. aeruginosa* is a leading cause of corneal infection (microbial keratitis), estimated to
77 cause vision loss in approximately 2 million people worldwide each year (6–8). A major risk factor
78 for keratitis is contact lens wear (8–10) and *P. aeruginosa* is the most frequently isolated pathogen
79 from lens-associated infections (6, 10). Recently, an outbreak of multidrug resistant *P. aeruginosa*
80 infections was reportedly transmitted *via* simple artificial tears eyedrops, causing infections of the
81 eye and beyond. These infections resulted in multiple instances of vision loss and several deaths,
82 in some instances without contact lens wear or prior eye disease (11–13).

83 Type three secretion systems (T3SSs) are among various tools Gram-negative bacteria use to
84 export factors across their otherwise impermeable cell membranes. A T3SS is additionally able to
85 inject effector toxins across host cell membranes into the cytoplasm of a target host cell to alter its
86 biology. *P. aeruginosa* encodes a single T3SS that has been shown to make significant
87 contributions to virulence during acute infections of the cornea (14–17) and other body sites (18–
88 22). The *P. aeruginosa* T3SS is encoded by 42 known genes encoding proteins of a multimeric
89 needle core, a translocon pore, and one or more effector toxins (23). ExsA is the only known
90 transcription factor for these genes. Effectors of the *P. aeruginosa* T3SS, like those of other Gram-
91 negative bacteria, can have a multitude of effects on host cells, including modifying their biology
92 to support an intracellular lifestyle (24–27).

93 All environment-exposed body surfaces are covered by multilayers of epithelial cells, that
94 when healthy resist traversal by *P. aeruginosa* and other opportunistic bacteria. This includes the
95 cornea of the eye, supremely capable of preventing bacterial colonization through a repertoire of
96 intrinsic defenses, some inherent within the corneal epithelium, others conferred by other factors
97 present at the ocular surface (28–34). The cornea's unique efficacy in this regard makes the corneal
98 surface our only environmentally exposed body surface devoid of a viable bacterial microbiome
99 (35, 36).

100 If *P. aeruginosa* does adhere to the cornea, which can occur after superficial injury, additional
101 barriers prevent it from traversing the multilayered epithelium to reach the underlying stroma,
102 access to which is required for the initiation of infectious pathology (i.e. keratitis) (28, 37–41).
103 Thus, infection susceptibility requires alterations to the epithelial barrier beyond superficial injury.
104 While that can be accomplished by full thickness injury (42, 43), *P. aeruginosa* corneal infection
105 is most commonly associated with contact lens wear, which predisposes the epithelium to bacterial
106 traversal more subtly by mechanisms not yet well understood (44, 45).

107 A plethora of studies have been done to explore how *P. aeruginosa* crosses a cultured epithelial
108 cell layer *in vitro*, using a variety of epithelial cell types. For example, airway epithelial cells or
109 MDCK cells were used to show roles for elastase, exotoxin A, type 4 pili, flagella, and the T3SS
110 (46–50). Our own *in vitro* studies using cultured corneal epithelial cells revealed roles for proteases
111 (41), type 4 pilus-associated twitching motility (51), and the T3SS effector ExoU; the latter
112 encoded by only a subset of *P. aeruginosa* strains (cytotoxic strains) and which enables traversal
113 by killing epithelial cells (52). Recently, a study using *in vitro* grown organoids of human airway
114 epithelial cells showed that both the T3SS and type VI secretion system (T6SS) can contribute to
115 *P. aeruginosa* "translocation" (traversal) of an epithelial barrier *via* goblet cell invasion (53).

116 Thus, our current understanding of how *P. aeruginosa* (and other bacteria) traverse susceptible
117 epithelial layers is based largely on *in vitro* cell culture study outcomes wherein bacteria and host
118 cells are studied in isolation from other factors normally found in their environment *in vivo*. Yet
119 our prior studies have shown that *in vivo* factors modify how bacteria interact with cells, including
120 basement membranes, mucosal fluids, other cell types, nerves, soluble factors, and environmental
121 conditions specific to the tissue site (28, 32, 37, 39, 41, 54, 55). Other limitations of the *in vitro*
122 literature on this topic are that most studies used epithelial cell monolayers that differ from *in vivo*
123 multilayers that contain layers with cells in multiple states of differentiation. Further, transformed
124 cells used by many studies are generally locked into one state of differentiation irrespective of
125 whether they can polarize correctly *in vitro* (e.g. MDCK cells, HeLa cells).

126 Factors that have hindered development of *in vivo*/*in situ* models for studying epithelial
127 traversal by *P. aeruginosa* and other bacteria include deliberately bypassing epithelial and other
128 tissue barriers to enable infection (e.g. by wounding or bacterial injection through them).
129 Moreover, introducing bacteria *in vivo/in situ* can trigger inflammation that can break down
130 subsequent barriers to bacterial dissemination, reducing or even eliminating the need for bacteria
131 to contribute. Another obstacle to an *in vivo/in situ* study if individual bacteria need to be localized
132 is the challenge of visualizing them in the context of host tissue.

133 Our published studies have shown that pretreatment of *in vitro* grown corneal epithelial cells
134 with mucosal fluid (tear fluid) increased their resistance to *P. aeruginosa* adhesion, invasion, and
135 cytotoxicity, and increased their traversal resistance when grown as multilayers (39, 56, 57). This
136 was accompanied by profound changes to the epithelial cell's transcriptome (57). Tear fluid
137 exposure also changes gene expression in *P. aeruginosa*, including multiple genes involved in
138 virulence and survival, reducing its ability to traverse cultured corneal epithelial cells - without

139 directly impacting bacterial viability (39, 57, 58). Other *in vivo* factors that are not present in
140 epithelial cell cultures can modify epithelial-microbe interactions. In the corneal epithelium they
141 include nerves and immune cells, both of which contribute to maintaining epithelial cell
142 homeostasis while also directly recognizing and responding to microbes (28, 31, 32, 59–61). Thus,
143 while cell culture studies have provided a good starting point for understanding epithelial barrier
144 function and mechanisms by which *P. aeruginosa* traverses epithelial cells, *in vivo/in situ* studies
145 are warranted.

146 Some of our published studies have attempted to address this knowledge gap. In one study, we
147 enabled traversal susceptibility *in vivo* in mice by scratching through the corneal epithelium, then
148 waiting for the time point at which the cell multilayer was reestablished but still remained
149 permissive to bacterial traversal, which occurred 6 hours after scratching (38). Bacterial location
150 and inoculation was quantified in fixed and stained tissue sections. The results showed that mutants
151 lacking ExsA, the transcriptional activator for the T3SS, were unable to traverse the epithelium
152 (38). Since wound healing might have played into the outcome, we later performed a second study
153 enabling susceptibility using more gentle superficial injury, by gently blotting the surface with a
154 KimWipe™, then EGTA-treatment prior to inoculation (37). Using this strategy, we found that
155 bacteria traversing the epithelium did not penetrate past the basal lamina, which when intact
156 functions as a non-specific size exclusion filter (37, 41). This avoids bacterial entry into the stroma
157 and therefore subsequent pathology/inflammation that could complicate studies of how bacteria
158 overcoming epithelial barrier function. In a later study, the blot/EGTA model was used to quantify
159 *P. aeruginosa* traversal of murine corneal epithelium by combining 3D confocal imaging to
160 identify fluorescent bacteria with confocal reflectance microscopy to image the non-fluorescent
161 cornea (62). Results again showed that ExsA was required for *P. aeruginosa* to traverse the corneal

162 epithelium when the mouse was immunocompetent (62).

163 A limitation of both of our prior studies was that neither method accounted for the complex
164 shape of the cornea, which is curved rather than flat. The problem this presents is that at any plane
165 of imaging the back and front surfaces of the corneal epithelium are not in line. This made it
166 difficult to accurately determine depth of penetration across the population and ascertain whether
167 T3SS mutants were penetration defective, or simply less efficient at traversing the epithelium.

168 Here, we report development of an advanced analytical imaging approach allowing for precise
169 metrics of location to be determined for all individual bacteria within the curved multilayered
170 epithelium *in situ*. Use of these methods showed that the T3SS impacts penetration/traversal
171 efficiency of *P. aeruginosa* rather than being absolutely required, with roles played by both the
172 T3SS translocon and T3SS effector proteins. Unexpectedly, results showed that the T3SS needle
173 was dispensable for this phenotype, and actually interfered when constitutively expressed.

174

175 **RESULTS**

176

177 **Transcription of the T3SS promotes *P. aeruginosa* traversal efficiency in the multilayered**
178 **corneal epithelium *in situ.*** Our prior study showed that the transcriptional activator of the T3SS
179 (ExsA) contributed to *P. aeruginosa* traversal of the mouse eye's corneal epithelium (62). Here,
180 we leveraged recent advances in imaging and image analysis to obtain more detail about how ExsA
181 impacts bacterial location, taking into account the complexities of the cornea's shape while more
182 accurately assessing location of individual members of the traversing population.

183 As in our previous study, we used confocal reflectance microscopy (CRM) to image the corneal
184 epithelium and GFP-expression to detect bacteria. Raw confocal images were then imported into

185 Imaris software v9.9 for processing, the CRM signal pseudo-colored red (Fig. 1A). As detailed in
186 the methods section, the reflectance signal from the stroma was manually excluded, and a
187 "Surface" representing only signal from the epithelium was generated (Fig. 1B). Apical and basal
188 boundaries of the epithelium were then identified (Fig. 1C). Individual bacteria were identified as
189 a "Spot", creating objects in the image that each represented one population member (Fig. 1D).
190 The distance (in microns) from the apical and basal boundary of the epithelium was measured for
191 each bacterial "Spot", and the depth of penetration of each bacterium was calculated as a
192 percentage to provide its relative position in between the upper and lower epithelial boundaries,
193 with 0% being surface adherent and 100% meaning that the bacterium reached the underlying
194 basal lamina (Fig. 1E). Another metric quantified was the proportion of the population able to
195 penetrate beyond the 50% (midpoint) normalized to the thickness of the epithelium in that region
196 of the tissue (as in Fig. 2B).

197 The first experiment was conducted using *P. aeruginosa* strain PAO1F wild-type compared to
198 an isogenic Δ*exsA* mutant: corneas were incubated with 600 µl of ~10^{11} CFU/ml bacteria for 6 h
199 (see Methods). The goal was to compare the outcome to our prior study that used more rudimentary
200 methods. For wild-type, the median traversal depth of individual bacteria was 47.29% of the
201 epithelial thickness, the upper quartile reaching at least 76.02% depth (Fig. 2A). Fig. 2B shows
202 that 47.27% of the bacterial population penetrated beyond 50% thickness of the epithelium. In
203 contrast, median penetration depth for Δ*exsA* mutant was only 7.31% depth, the upper quartile
204 being at only 12.86% depth (Fig. 2A). Only 2.67% penetrated beyond the midway point (50%
205 depth) (Fig. 2B). While these findings confirmed the importance of the T3SS in corneal epithelial
206 traversal by *P. aeruginosa* shown in our earlier publications, they provided more granularity on
207 how it impacts bacterial location/distribution. The pattern that emerged showed that the *exsA*

208 mutants lacking the entire T3SS were not completely defective in ability to penetrate beyond the
209 epithelial surface, with significant variability among the population. While wild-type penetrated
210 more deeply than T3SS mutants over the 6 h time span of these experiments, there was again
211 significant variability with only a fraction fully traversing to the level of the underlying basal
212 lamina. For this reason, the capacity to penetrate the corneal epithelium is referred to as traversal
213 efficiency for the remainder of this manuscript.
214
215 **Efficient traversal involves T3SS effectors and the T3SS translocon but does not require the**
216 **T3SS needle apparatus.** To explore which ExsA-regulated T3SS components are required for
217 traversal efficiency, we compared wild-type *P. aeruginosa* to mutants lacking the T3SS needle
218 apparatus (Δ*pscC*), the T3SS effectors (Δ*exoSTY*), or the T3SS translocon proteins (Δ*popBD*).
219 As shown in Fig. 2A, both the T3SS effector mutants (Δ*exoSTY*) and the T3SS translocon
220 mutants (Δ*popBD*) were defective in traversal efficiency compared to wild-type reaching median
221 traversal depths of 11.16% and 10.91% respectively, comparable to the Δ*exsA* mutant. Compared
222 to the Δ*exsA* mutant, a greater proportion of the Δ*popBD* or Δ*exoSTY* mutant populations traversed
223 to over 50% depth; 30.18% and 19.27% respectively (Fig. 2B). Surprisingly, the Δ*pscC* mutant
224 lacking the T3SS needle, was the most traversal efficient of all of the mutants examined, with
225 median traversal at 45.24% depth, similar to wild-type.
226 These outcomes were surprising given that the T3SS needle is considered important for T3SS
227 functions. Thus, we additionally tested a Δ*exsD* mutant that is constitutively active for the T3SS
228 and therefore consistently expresses T3SS components (including the needle). This differs from
229 wild-type which instead requires induction by host cell contact or low calcium to express the T3SS,
230 and it is effectively the opposite of a Δ*exsA* mutant which consistently lacks T3SS expression.

231 Results with the Δ*exsD* mutant showed that constitutive expression of the T3SS actually interferes
232 with traversal efficiency, showing a median traversal depth of only 12.66% (Fig. 2A), with only
233 21.42% of the population penetrating beyond 50% depth (Fig. 2B). Representative examples of
234 epithelium traversal by wild-type bacteria and T3SS mutants are shown in Supplemental Fig. S1.
235 Since traversal efficiency data within these different populations was not normally distributed,
236 the Kruskal-Wallis test with Dunn's multiple comparisons was used to compare each mutant to
237 the Δ*exsA* mutant. Due to the very large sample sizes (10,000-60,000 datapoints each group), all
238 comparisons were found to be highly statistically significant ($P < 0.0001$), even when median
239 values and distributions appeared visually comparable (Fig. 2A). This was also true when
240 comparing mutants to wild-type. For this reason, the magnitude and the direction of the differences
241 are important to consider in evaluating the biological significance of the outcomes. Taking those
242 parameters into consideration, the data showed that traversal efficiency was supported by
243 combined efforts of the T3SS effectors and the T3SS translocon. They further suggested that the
244 T3SS needle was not required, and that constitutively expressing it and other T3SS factors in the
245 entire population actually detracted from traversal efficiency. Since this result was unexpected, we
246 used Sanger sequencing to re-confirm that the Δ*pscC* mutant used contained a clean deletion of
247 *pscC* (data not shown). In other controls, the T3SS-GFP reporter pJNE05 (P_{exoS} expression) was
248 used to confirm that P_{exoS} was activated upon EGTA-exposure similarly to wild-type in each of the
249 mutants shown to be defective in traversal in Fig. 2: Δ*popBD*, Δ*exoSTY* and Δ*exsD* (Supplemental
250 Fig. S2). Since P_{exoS} expression is a reliable surrogate for *exsA* expression, this outcome confirmed
251 that the mutants were not defective for T3SS induction, at least under the conditions used.
252
253 **Complementation of ExsA in the Δ*pscU-L* T3SS operon mutant restored T3SS effector-**

254 **dependent traversal efficiency.** The above results using T3SS needle mutants and constitutive
255 expression of the T3SS showed that the capacity to make the T3SS needle did not correlate with
256 efficient traversal despite involvement of the T3SS effectors. Here, we directly tested the
257 hypothesis that the effectors can function without the needle.

258 First, we generated a mutant lacking the 36 genes in the main T3SS operons, from Δ*pscU* to
259 Δ*pscL* (designated Δ*pscU-L*). This mutant still encodes the known T3SS effectors in PAO1 (ExoS,
260 ExoT and ExoY) and their chaperones under endogenous promoters. Results showed the Δ*pscU-*
261 *L* mutant had low traversal efficiency (Fig. 3A, B) thereby phenocopying the Δ*exsA* mutant. This
262 was to be expected since the Δ*pscU-L* mutant lacks the gene encoding ExsA in addition to genes
263 encoding all of the T3SS machinery proteins, and while it does encode the T3SS effectors, these
264 are not transcribed, translated or released without ExsA.

265 Next, we tested if expressing the T3SS effectors in this mutant lacking T3SS machinery related
266 proteins could rescue traversal efficiency. This was done by complementing the mutant with ExsA
267 using a chromosome-integrating vector to induce expression of *exsA* under a rhamnose-sensitive
268 promoter. This vector was first tested in the background of a Δ*exsA* mutant using a GFP reporter
269 pJNE05 for P_{exoS} expression as a surrogate for *exsA* induction (as above, Supplemental Fig. S2).
270 Rhamnose addition induced GFP expression and it significantly increased bacterial traversal
271 compared to the no rhamnose control for the Δ*exsA*:P_{rha}*exsA* mutant (Supplemental Fig. S3A, B).
272 We next showed that Rhamnose-induction of *exsA* expression in the Δ*pscU-L* mutant background
273 increased median traversal to 11.09% depth versus the rhamnose-induced vector control at 7.34%
274 depth (Fig. 3A), and it also increased the percentage of the population that traversed to at least
275 50% depth to 18.35% vs. 0.00 % (Fig. 3B). Showing that partial restoration of traversal efficiency
276 by Δ*pscU-L*:P_{rha}*exsA* depended on the T3SS effectors, it was abrogated when the three exotoxin

277 genes were also deleted (Median: 5.76% depth, population over halfway 0.00%) (Fig. 3A, B). An
278 *in vitro* control with pJNE05 confirmed activation of P_{exoS} in the Δ*pscU-L* mutant after *exsA*
279 complementation with rhamnose addition relative to a vector-complemented control: Mean +/- SD
280 P_{exoS} expression (FITC/OD_{600}) for Δ*pscU-L*:P_{rha}*exsA* + rhamnose was 3397 ± 1033 relative
281 fluorescence units versus 1567 ± 1235 for Δ*pscU-L*:P_{rha}Vector + rhamnose (P = 0.0061, Student's
282 t-Test).

283 In Fig. 3A all comparisons between groups were significant (P < 0.0001 for most comparisons,
284 and P = 0.025 comparing Δ*pscU-L* [no rhamnose] *vs.* Δ*pscU-L*Δ*exoSTY*:P_{rha}*exsA* [+ rhamnose],
285 Kruskal-Wallis test with Dunn's multiple comparisons). The latter comparison compared two
286 mutants both unable to express T3SS effectors or any of the T3SS machinery-related proteins, only
287 one able to express the transcriptional activator ExsA (Fig. 3A, B). Thus, ExsA expression in the
288 absence of those other components had the least impact on traversal efficiency. Taken together,
289 these results show that ExsA-driven expression of *exoSTY* can increase traversal efficiency of a
290 mutant lacking all the T3SS machinery and thus one or more effectors are necessary for traversal.
291 However, it remains possible that ExsA regulates the expression of a factor(s) outside of the main
292 T3SS operon that is/are needed for effector-mediated traversal.
293

294 **The T3SS translocon contributes to traversal efficiency beyond roles of the T3SS effectors,**
295 **also independently of the T3SS needle.** To explore if the T3SS translocon contributes to traversal
296 efficiency beyond the impact of the T3SS effectors, we studied deletion of *popB* in the background
297 of a Δ*exoSTY* mutant (already reduced in traversal efficiency compared to wild-type). This led to
298 a small but statistically significant further reduction in median traversal depth: Δ*exoSTY* median
299 at 10.91% depth vs. Δ*popB*Δ*exoSTY* median at 8.10% depth (Fig. 4A, Δ*exoSTY* data reproduced

300 from Fig. 2) (P < 0.0001, Kruskal-Wallis test with Dunn's multiple comparisons). A much bigger
301 reduction was seen in the percent of population traversing beyond the 50% point: Δ*exoSTY* 19.27%
302 vs. Δ*popB*Δ*exoSTY* 1.62%.

303 We next tried the opposite experiment, deleting a T3SS effector gene (*exoS*) in a T3SS
304 translocon mutant (*popB*). This also showed separable contributions for the T3SS effectors and the
305 T3SS translocon, the median depth for a Δ*popB* mutant at 12.30% depth vs. median for
306 Δ*popB*Δ*exoS* mutants at 9.42% depth (Fig. 4A) (P < 0.0001, Kruskal-Wallis test with Dunn's
307 multiple comparisons). Again, the difference became more obvious when assessing the percent of
308 the population penetrating beyond 50% depth; Δ*popB* 17.76% vs. Δ*popB*Δ*exoS* 5.86% (Fig. 4B).
309 Taken together, these results showed that T3SS translocon proteins and T3SS effector proteins can
310 have additive impacts on traversal efficiency and specifically implicate ExoS and PopB. However,
311 difference in traversal beyond 50% depth shown between Δ*popB*Δ*exoS* 5.86% and Δ*popB*Δ*exoSTY*
312 1.62% implicate a role(s) for ExoT and/or ExoY.

313 Having already shown that the T3SS needle was dispensable for effector mediated traversal,
314 we next asked if it was needed for the translocon's contribution. Thus, we mutated the translocon
315 pore proteins PopB and PopD (Δ*popBD* mutant) in the background of the T3SS needle mutant
316 (Δ*pscC*) already shown to be traversal competent (Fig. 2). This reduced traversal efficiency of the
317 needle mutant: Δ*pscC* mutant median 45.24% depth, population over halfway 43.84% vs.
318 Δ*pscC*Δ*popBD* mutant median at 36.11% depth, population over halfway at 35.00% (Fig. 4A, B,
319 Δ*pscC* data reproduced from Fig. 2) (P < 0.0001, Kruskal-Wallis test with Dunn's multiple
320 comparisons). This showed that the T3SS translocon can contribute to traversal efficiency in the
321 absence of the T3SS needle.

322 All traversal results and strain characteristics are summarized in Table 1. Together, these

323 outcomes implicate two separate contributors to T3SS-mediated traversal efficiency: the T3SS
324 effectors (including ExoS), and the T3SS translocon pore (including PopB), each functioning
325 independently of the T3SS needle which can instead play an inhibitory role. Interestingly, the
326 magnitude of their individual contributions appear similar in magnitude, the Δ*exoSTY* and Δ*popB*
327 mutants showing no significant difference in impact (Fig. 4).
328
329 **Exotoxin S protein can be released by a T3SS operon mutant.** Results in Figs. 2-4 show that
330 the T3SS effectors can contribute to traversal efficiency without the T3SS needle genes. Our
331 current understanding of T3SS transcription and expression is that in the presence of Ca^{2+} or
332 absence of host cell contact, low levels of T3SS expression occur due to repression of ExsA by
333 ExsD (63). Upon host cell contact or Ca^{2+} deprivation, the T3SS regulator ExsE is secreted, leading
334 to ExsA de-repression and T3SS transcription. *In vitro*, Δ*pscC* needle mutants do not secrete ExsE
335 and do not activate high levels of T3SS expression (64). To test if an alternate mechanism of T3SS
336 effector toxin expression might be operating in our *ex vivo* traversal model or T3SS mutants, the
337 Δ*exsA* or Δ*pscU-L* mutant carrying rhamnose-inducible *exsA* were tested for ExoS protein
338 expression *in vitro* using LB broth without EGTA calcium chelation to specifically test rhamnose
339 induction compared to controls (vector only or no rhamnose). After growth in broth culture to
340 'mid-log' phase, bacterial pellet and supernatant samples were collected and probed for ExoS *via*
341 affinity-purified polyclonal antibody using SDS-PAGE and Western immunoblot as described
342 previously (64). ExoS protein was detected in the pellet and supernatant of Δ*exsA*:P_{rha}*exsA* with
343 rhamnose induction (as expected) and in Δ*pscU-L*:P_{rha}*exsA* with inclusion of rhamnose but not in
344 its vector control (Fig. 5). These data confirmed that ExsA-driven ExoS expression and release
345 can occur for mutants lacking needle protein genes.

346

347 **DISCUSSION**

348

349 Previously, we showed that the transcriptional activator of the T3SS (ExsA) is required for *P.*
350 *aeruginosa* to traverse susceptible multilayered epithelium in the context of live tissue, shown
351 using the eyes of immunocompetent mice (38, 62). Here, we explored the role of T3SS
352 components.

353 The cornea has several advantages over other tissues for this type of investigation being readily
354 accessible for manipulation or imaging, optically clear when healthy allowing imaging without 2-
355 photon microscopy or tissue clearing protocols and is relatively separated from other body sites
356 reducing complexity associated with cross-talk that can occur during infection. Its epithelial
357 surface also lacks a microbiome (35, 36), allowing a pathogen to be studied alone or in combination
358 with other bacteria in a controlled fashion. Use of subtle epithelial injury to enable susceptibility
359 to bacterial traversal, as done in this study using tissue paper blotting then EGTA treatment,
360 preserves the underlying basal lamina barrier, prohibiting bacterial access to the vulnerable stroma
361 and allowing study of epithelial traversal by bacteria without complexities introduced by later steps
362 of infection development. Thus, this mouse eye model for studying epithelial traversal shares some
363 advantages of *in vitro* cell/tissue culture models in reducing complexity but benefits from *in situ*
364 context.

365 A challenge of *in situ* tissue imaging for quantitively localizing bacteria is variations in tissue
366 shape that can make it difficult to ascertain position relative to upper and lower boundaries across
367 an entire tissue sample. Here, we developed methods that account for the cornea's curved surface,
368 variations in thickness and topography in different regions and resulting from cell exfoliation, and

369 any changes to these parameters after exposure to bacteria. This allowed for more precise
370 quantitation of bacterial location between the epithelial surface and underlying basal lamina,
371 separately accounting for all individual bacteria present in the epithelial layer.

372 To calibrate outcomes to our previously published methods, we first compared wild-type *P.*
373 *aeruginosa* to mutants lacking the entire T3SS *(exsA* mutants) which confirmed the critical role
374 for the T3SS in corneal epithelium traversal. However, the more accurate localization method
375 showed traversal was not an absolute quality, with both wild-type *P. aeruginosa* and *exsA* mutants
376 showing a distribution/spread for individual bacteria in their penetration rates. Thus, we referred
377 to "traversal efficiency" rather than "traversal" to describe subsequent outcomes.

378 When roles of specific T3SS components were examined, some results aligned with *in vitro*
379 cell culture study findings (65). For example, mutants lacking genes for known T3SS exotoxins
380 had reduced traversal efficacy compared to wild-type. However, our study showed that mutants in
381 T3SS translocon pore proteins also showed reduced traversal efficiency, with mutants lacking both
382 translocon and exotoxin proteins showing an even more profound defect similar to *exsA* mutants
383 unable to express any T3SS components. This implicated both the T3SS effectors and the T3SS
384 translocon proteins, their roles being additive.

385 Not all outcomes aligned with *in vitro* study findings. Mutants lacking the T3SS needle were
386 just as traversal efficient as wild-type *in situ.* This was a surprising and confusing result because
387 the T3SS needle is generally thought critical for T3SS function, including for secretion or
388 expression of the very same T3SS components we found required for traversal efficiency. Thus,
389 we used a mutant lacking the entire T3SS operon except the genes encoding the effector exotoxins
390 (Δ*pscU-L* mutant) (66) and induced effector expression by complemented it with rhamnose
391 inducible *exsA* (the transcriptional activator of all T3SS-related genes). This promoted traversal

392 efficiency in the mutant despite it lacking T3SS needle and other machinery related proteins.
393 Confirming it was T3SS effector gene expression driving the phenotype in the T3SS operon mutant
394 not than other genes outside the T3SS operon, traversal was no longer promoted by *exsA*
395 complementation when the effector genes were also mutated (i.e. Δ*pscU-L exoSTY* mutant). Since
396 Δ*pscU-L* mutants lack T3SS translocon genes additionally needed for full traversal efficiency, it
397 was not surprising that *exsA* complementation did not fully restore the operon mutant to wild-type
398 traversal efficiency. Indeed, efficiency approximated translocon (*popBD*) mutants that similarly
399 encode T3SS effectors but not translocon proteins, but that differ in encoding T3SS needle
400 proteins. Together these results confirmed that the T3SS needle protein genes are not needed for
401 T3SS effector-mediated traversal efficiency in this *in situ* model.

402 Since T3SS needle proteins are thought necessary for exporting T3SS effectors out of bacteria
403 and for injecting them into host cells this data raised two related questions. Can T3SS effectors
404 become extracellular without the needle, and if so, how do they impact traversal without being
405 injected into host cells?

406 Western immunoblotting confirmed the presence of ExoS in both supernatant and pellet of *in*
407 *vitro* grown Δ*pscU-L*:P_{rha}-*exsA* after rhamnose induction, showing that ExoS can become
408 extracellular without needle protein genes. While this might be an unusual feature of this mutant,
409 that would not explain results with other mutants that also show the needle is dispensable for T3SS
410 mediated traversal efficiency. A potential explanation is that bacterial lytic cell death releases
411 effectors, e.g. explosive cell lysis in *P. aeruginosa* (67), and a feature of other toxin release by
412 other bacteria (68, 69) and quite possible given the dense bacterial inoculum and routine 'natural'
413 bacterial cell death as a result of nutrient deprivation or from formation of biofilms (67). In the
414 context of our *in situ* assay, the presence of host-derived antimicrobial peptides (29, 33, 70) could

415 further contribute to bacterial cell death. Alternatively, T3SS proteins are packaged into OMVs
416 (Outer Membrane Vesicles) that are released into the extracellular environment, as shown for
417 *Salmonella enterica* T3SS-1 effectors and translocation proteins (71). Indeed, some T3SS proteins
418 were efficiently secreted *via* OMVs by mutants lacking a functional T3SS needle, and effector
419 toxins were shown delivered *via* OMVs into the cytoplasm of epithelial cells where they enhanced
420 pathogenesis of T3SS-deficient mutants (72). Pathogenic *E. coli* (O157:H7) can also efficiently
421 package T3SS translocation and effector proteins into OMVs in the absence of essential T3SS
422 needle proteins (73). Our own work showed that exposing *P. aeruginosa* to ocular surface tear
423 fluid or to purified lysozyme (a tear fluid ingredient) generates OMVs that are cytotoxic to the
424 corneal epithelium of mouse eyes *in vivo*. We also showed that priming the corneal surface with
425 these OMVs reduces defense against bacterial adhesion, and that tear fluid/lysozyme triggered
426 OMVs contain a ~48 kDa protein similar in size to ExoS (~49 kDa) and ExoT (~53 kDa) (74).
427 Another potential pathway for delivering T3SS effectors into a host cell without the T3SS needle
428 could be from an intracellular location. In this regard, it is interesting that the results specifically
429 implicated PopB and ExoS in traversal efficiency as our work has shown critical roles for both in
430 the intracellular lifestyle of *P. aeruginosa* (24–27, 75). Notwithstanding, even T3SS mutants are
431 internalized by epithelial cells and can persist inside intracellular vacuoles that can subsequently
432 fail to contain them (24–26, 75–78). In other words, T3SS factors can facilitate but are not essential
433 for bacterial entry into a host cell's cytoplasm. Inside epithelial or other cells, bacteria (including
434 *P. aeruginosa*) can use factors beyond T3SS factors to exert impacts on the host cell, and the host
435 cell can detect intracellular bacteria and in response alter its biology and/or induce programmed
436 cell death. Others have shown that extracellular ExoS itself can be internalized by eukaryotic cells,
437 and activates host cell TNF-α responses by triggering surface TLRs (79). While mechanisms might

438 overlap with some of the above, ExoS has also been shown to affect host cell function from an
439 extracellular location (80).

440 Given these many possibilities, a separate study will be needed to determine how T3SS effector
441 mediated traversal occurs without the T3SS needle. Importantly, much of our understanding of
442 gene expression in *P. aeruginosa* has been done using *in vitro* methods, often without host cells
443 present. Given its many environmental sensors and regulators of gene expression, regulation of the
444 *P. aeruginosa* T3SS *in situ/in vivo* could differ vastly from what has been shown *in vitro*. It is also
445 likely to be context dependent/complex due to microenvironments, and lack of homogeneity and
446 synchrony. Indeed, the result showing that *exsD* mutants (which differ from wild-type in
447 constitutively expressing the T3SS) are equally as traversal defective as mutants lacking the entire
448 T3SS are particularly poignant and highlight the importance of regulation. Further research on this
449 topic would benefit from more *in situ* experimentation to set the stage for well-designed *in vitro*
450 studies that can then tease apart mechanisms for the phenomena shown relevant to pathogenesis
451 *in situ/in vivo*.

452 The T3SS translocon proteins (PopB/PopD) were found to play roles additional to the
453 contribution of the effectors. This could relate to their ability to form pores in host cell membranes,
454 which can damage a cell or otherwise alter host cell function. Indeed, PopB-PopD complexes in
455 isolation have been shown to function as a pore-forming toxin by allowing K^+ efflux, resulting in
456 histone H3 modification and host cell subversion (81). A potential mechanism for the additive
457 impacts of translocon and effector proteins could be pore formation providing an entry mechanism
458 for effector delivery into the host cell. Whether the T3SS needle is also dispensable for translocon-
459 mediated traversal efficiency remains an open question that will require further investigation,
460 although it is suggested by the fact that needle mutants remain fully efficient.

461 Another factor to consider in interpreting the data is that T3SS needle proteins of *P. aeruginosa*
462 and other Gram-negative bacteria are recognized by mouse (and human) cells causing
463 inflammasome activation (82, 83), which can help the cornea and other tissues recognize, respond
464 and clear *P. aeruginosa* during experimental challenge *in situ* (35, 84). In this way, expressing
465 T3SS needle proteins could be detrimental to bacteria *in situ* (even if not *in vitro*) and using needle-
466 independent mechanism for traversal could be advantageous. Other *in situ* factors (absent *in vitro*)
467 may also impact bacterial viability or growth during traversal and thus data interpretation.

468 Providing additional insights into pathogenesis, traversing bacteria tended to be bimodally
469 distributed across the epithelium. Typically, there was a cluster in the apical region from 0-15%
470 traversed, few across the midway point, then a group from 50-80% traversed. This distribution
471 may reflect bistability of T3SS expression in *P. aeruginosa* populations (26, 85), and/or a host
472 defense barrier, possibly related to specialized junctions in the suprabasal region of the corneal
473 epithelium that have been shown to be a barrier to leukocytes (86). Also worth noting, the very
474 large sample sizes (10,000-60,000 datapoints for each group) provided such a powerful analysis,
475 that most comparisons yielded statistically significant differences even when they were small.
476 Thus, we considered the magnitude of the differences in central tendency (median) and data spread
477 (upper and lower quartiles) to draw conclusions about biological significance.

478 In conclusion, this study used novel and powerful image analysis tools and an *in situ* model
479 with pared-down complexity to focus on an early step in pathogenesis, traversal of a superficial
480 multilayered epithelium. The goal was to determine which T3SS components contribute. Results
481 showed roles for T3SS effectors, T3SS translocon proteins, and regulation of T3SS expression.
482 Differing from *in vitro* culture studies and challenging our general understanding about how the
483 *P. aeruginosa* T3SS supports infection pathogenesis, the T3SS needle was dispensable. Follow up

484 experiments confirmed that the T3SS effector ExoS could become extracellular in the absence of
485 the T3SS needle. How T3SS effectors and translocon proteins influence host cells to promote
486 traversal without the T3SS needle will require further work. Published knowledge about *P.*
487 *aeruginosa* and other Gram-negative bacteria suggest multiple possibilities (67, 72, 73, 87).

488 Outcomes of this study may or may not be applicable to how *P. aeruginosa* traverses epithelial
489 barriers other than the mouse corneal epithelium, but if so they might generally inform efforts to
490 develop therapies targeting the T3SS (88, 89). Importantly, the results highlight the need to move
491 beyond *in vitro* and cell culture studies when studying pathogenesis. At the same time, they support
492 complementary *in vitro* and cell culture experimentation that can further reduce complexity when
493 teasing apart details, and to study regulation in a more controlled system.

494 The model and imaging methods we have developed enable a single early stage of infection
495 pathogenesis to be studied *in situ* in the absence of various confounders generally present in
496 infection models (e.g. inflammation), and they enable individual bacteria to be visualized across
497 the entire tissue with their location quantified. These methods could prove useful for studying
498 pathogenic strategies of other microbes also able to traverse epithelial layers during infection,
499 including those not considered to be eye pathogens.

500

501 **MATERIALS AND METHODS**

502

503 **Bacteria.** *Pseudomonas aeruginosa* strain PAO1F was used for all experiments. Mutants in T3SS
504 components were either obtained from in-house strain collections or generated using two-step
505 allelic exchange (90). For this, pEXG2 vectors containing overlapping upstream and downstream
506 regions of the genes of interest (excluding the ORF) were cloned and transformed to *E. coli* SM10

507 (λ*pir*), which were then used as donor strains to deliver the vector to recipient *P. aeruginosa*
508 strains. Successful merodiploid colonies underwent sucrose counter-selection and PCR
509 verification of mutagenesis. For integration of rhamnose-inducible ExsA, Dr. Arne Rietsch
510 provided strains generated by Tn7 vector-mediated insertion and Flp-mediated excision of
511 antibiotic resistance markers (91). All bacteria, mutants and plasmids used are shown in Table 2.
512 In control experiments not shown, T3SS mutants did not show any growth defects relative to PAO1
513 in trypticase soy broth culture at 37 ºC *in vitro*.

514 For all experiments, bacteria were streaked from glycerol stocks stored at -80 ºC, plated onto
515 trypticase soy agar (TSA) plates with appropriate antibiotics (see Table 2) and grown at 37 ºC
516 overnight. For the rhamnose-induction traversal experiments, bacteria were grown on TSA also
517 supplemented with rhamnose (2 %). For T3SS-induction growth curves with plasmid pJNE05,
518 bacteria were grown in trypticase soy broth (TSB) supplemented with gentamicin (200 μg/ml),
519 monosodium glutamate (100 mM), glycerol (1%) and EGTA (2 mM) or alternatively with
520 rhamnose (2 %) instead of EGTA as specified.

521 For the *ex vivo* murine model of traversal, one bacterial "lawn-covered" TSA plate was used
522 per bacterial strain per condition. After overnight growth at 37 ºC, bacteria were collected with a
523 sterile loop and carefully suspended into serum-free DMEM (Dulbecco's Modified Eagle medium;
524 Gibco). DMEM was supplemented with 2% rhamnose when indicated. OD_{600} was measured, with
525 A_{600} 1.0 ~ 4 x 10^8 CFU/ml used for calculating density. Suspensions of 1.0 x 10^{11} CFU/ml were
526 used for *ex vivo* incubations, and concentrations confirmed by plating serial dilutions for viable
527 counts.

528

529 ***Ex vivo* murine model of traversal.** All procedures were approved by the Animal Care and Use

530 Committee of the University of California, Berkeley, an AAALAC accredited institution. For all
531 experiments, male or female C57BL6/J or mT/mG mice with tdTomato-labeled cell membranes
532 were used, 6 to 12 weeks old, and 3-6 eyes per experimental condition. Contralateral eyes from
533 the same mouse were used as controls when possible. All experiments were performed *ex vivo*, as
534 mice were euthanized by isoflurane inhalation prior to any further manipulations. After euthanasia,
535 each eye was lightly blotted with a Kimwipe (Kimtech), enucleated, then placed in 0.2µm filter-
536 sterilized 1X Phosphate-Buffered Saline (PBS) in a 48-well dish for subsequent steps. Eyes were
537 rinsed three times in PBS, then incubated in a 0.1M solution of EGTA at room temperature for 1
538 h. Eyes were rinsed three more times in PBS, then completely submerged in a 600 µl suspension
539 of bacteria, prepared as described above, and incubated at 37 ºC, 5 % CO_2 for 6 h, consistent with
540 our previous studies (62, 92). Following this incubation, eyes were rinsed with PBS three times to
541 remove excess non-adherent bacteria before fixation in 2 % paraformaldehyde (PFA) overnight at
542 4 ºC. Fixed eyes were then whole mounted on a glass cover slip with Loctite superglue with the
543 central cornea facing up before submersion in PBS and imaging.
544
545 **Imaging.** Whole-mounted fixed corneas were imaged using an Olympus Fluoview FV1000
546 upright laser scanning confocal microscope with a water immersion 20X objective (NA = 1.0). For
547 each eye, three to five non-intersecting fields of view on the central cornea were selected for
548 imaging with 3x optical zoom (60x total magnification) at 1024 x 1024 resolution and 0.8µm step
549 size. Fluorescent bacteria were imaged using FITC (488/509) and/or RFP (555/580) channels, and
550 corneal cells were imaged using an 640nm excitation channel for confocal reflectance microscopy
551 (CRM) (92, 93).
552

553 **Image analysis.** Images of corneas were processed on Imaris Software (v 9.9) as follows. First, a
554 "surface object" was manually drawn using the reflectance channel to select the total signal
555 underneath the corneal basement membrane. This object represented the corneal stroma and was
556 used to mask the remaining reflectance signal. This created a new channel with signal from only
557 the epithelium, which was used to automatically generate a new surface object.

558 From the created Epithelium surface object, a distance transformation was performed, and the
559 basement membrane and apical surface were identified as two new surface objects ~1µm away
560 from the Epithelium. Variability in the created objects due to differing corneal reflectance intensity
561 was minimized through use of automatic thresholds, and manual surveillance of the entire process
562 ensured consistency in appearances. Next, bacteria were identified through the 'Spots' tool, using
563 an XY size of 1.25 µm in the appropriate channel. Parameters for Spots creation were assessed
564 using an automatic threshold, adjusted manually to reduce false positive results (e.g., Spots where
565 there were no bacteria).

566 For each field of view, the following were measured: total volume and maximum thickness of
567 epithelium, total number of bacteria, and distance of each bacterium from apical surface and
568 basement membrane. Percent depth of traversal for each bacterium was calculated using the
569 formula: % Depth = Distance to Apical Surface / (Distance to Apical + Distance to Basement
570 Membrane) * 100 %. An inverted cumulative histogram was also generated from the % depth for
571 all bacteria in a condition with 5µm bins. The percent of the total population remaining deeper was
572 graphed, so 100 % of a population remained at 0 % traversal depth and 0 % remained at 100 %
573 depth.

574

575 **Western immunoblot.** Bacteria were inoculated in liquid cultures of LB media (containing 200

576 mM NaCl, supplemented with 10 mM $MgCl_2$) and grown with shaking overnight at 37 ºC.
577 Overnight cultures were diluted 1:300 into fresh media, grown for 2 h, then 1 ml of culture was
578 added to 1 ml pre-warmed media with 2 % rhamnose and 1 ml of culture was added to 1 ml pre-
579 warmed media as a control. The bacteria were grown for another hour before the OD_{600} was
580 measured for each tube, which were then placed on ice. 1 ml of each sample was pelleted, and the
581 entire supernatant was added to a fresh tube containing 10 % trichloroacetic acid for precipitation.
582 After 10 min on ice, two washes with acetone were conducted before drying out the precipitate at
583 room temperature. The cell pellet and supernatant precipitate were both resuspended in the same
584 volume of 1x SDS Buffer (Laemmli Solution [Bio-Rad], 1 mM DTT) to create a concentration of
585 4 x 10^9 CFU/ml, or an OD_{600} of 10 – typically 60-80 µl. Samples were boiled at 90 ºC for 10 min
586 to denature proteins before brief centrifugation.
587 For SDS-PAGE, 50 µl of each sample and Precision Plus Protein Standards (Bio-Rad) were
588 then applied to a 4-20 % Protean TGX Stain Free Gel (Bio-Rad) before separation at 200V for 35
589 min. Gels were imaged on the Bio-Rad Gel Dock, then transferred to nitrocellulose membranes
590 using the Mixed MW program on the TransBlot Turbo Transfer System. Membranes were blocked
591 with EveryBlot buffer (Bio-Rad) before incubation overnight at 4 ºC with an affinity-purified
592 Rabbit polyclonal anti-ExoS primary antibody (from Dr. Arne Rietsch, 1:5000). After washing 3x
593 with TBS-T (0.1 % Tween-20 in 1x TBS Buffer) for 5 min each, membranes were then incubated
594 for 1 h at RT with Goat anti-Rabbit HRP-conjugated secondary antibody (ThermoFisher A16096,
595 1:1000). Membranes were again washed with TBS-T, then exposed to Clarity Western ECL
596 substrate (Bio-Rad) for 5 min before chemiluminescence and colorimetric imaging.
597
598 **Statistical analysis.** Data on bacterial depth from Imaris were exported into Microsoft Excel and

599 GraphPad Prism 9 or 10 for visualization and analysis. The percent depth for each individual
600 bacterium was considered a unique data point and were aggregated across all fields of view for
601 each eye as one biological replicate. Data from 3-6 biological replicates for each condition were
602 combined into a single column for graphing and shown as median ± interquartile range. For all *ex*
603 *vivo* experiments, data did not pass normal distribution tests. The Kruskal-Wallis test with Dunn's
604 multiple comparisons or Kolmogorov-Smirnov test were used to compare groups. For growth
605 curve comparisons, One-way ANOVA with Dunnett's multiple comparisons or a paired Student's
606 t-Test were used. $P < 0.05$ was considered significant.
607
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614
615 **Contributions.** EJ, DS, NGK, AR, DE and SF designed the experiments; EJ, DS, NGK and AR
616 performed the experiments; EJ, AR, AS, DS, NGK, DE and SF analyzed and interpreted the data;
617 EJ, DE, AR and SF wrote the manuscript; DE and SF supervised the study.

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897 ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type
898 III secretion system. *Mol Microbiol* 68:657-671.
899

900 Table 1. Properties and epithelial traversal results for *P. aeruginosa PAO1F and T3SS mutants*

901

Strain	Exotoxins	Translocon	Needle	Epithelial Traversal
PAO1F	+	+	+	++
Δ*exsD*	++	++	++	+
Δ*pscC*	+	+	-	++
Δ*popBD*	+	-	+	+
Δ*exoSTY*	-	+	+	+
Δ*pscC*Δ*popBD*	+	-	-	++
Δ*popB*Δ*exoSTY*	-	-	+	-
Δ*pscU-L:*P_{rha}*exsA*	+	-	-	+
Δ*pscU-L*Δ*exoSTY:*P_{rha}*exsA*	-	-	-	-

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916 Table 2. Strains, mutants, and plasmids used in this study

Strain or mutant	Description	Source
PAO1F	Wild-type *P. aeruginosa*; All mutants made within this background	Dr. Alain Filloux *via* Dr. Arne Rietsch (94)
Δ*exsA*	*exsA* mutant; Constitutively T3SS^{Off}	Dr. Arne Rietsch
Δ*exsD*	*exsD* mutant; Constitutively T3SS^{On}	Dr. Timothy Yahr (63)
Δ*pscC*	*pscC* mutant; Lacking needle	Dr. Arne Rietsch (95)
Δ*popBD*	*popBD* double mutant; Lacking translocon pore	Dr. Arne Rietsch (95)
Δ*exoSTY*	*exoSTY* triple mutant; Lacking known exotoxins	Dr. Arne Rietsch (95)
Δ*pscU-L*	Total T3SS-operon mutant (knockout of all genes *pscU* to *pscL*); called *ΔU-L*	Dr. Arne Rietsch (66)
Δ*exsA:Prha-A*	*exsA* mutant with chromosome-integrated rhamnose-inducible *exsA*	Dr. Abby Kroken (27)
Δ*pscU-L:Prha-A*	Δ*U-L* with rhamnose-inducible *exsA*	This study
Δ*pscU-L:Prha-V*	Δ*U-L* with rhamnose-inducible vector control	This study
Δ*pscU-L* Δ*exoSTY:Prha-A*	Δ*U-L* lacking all known exotoxins, with rhamnose-inducible *exsA*	This study
Δ*popB*Δ*exoSTY*	*popB* mutant lacking all known exotoxins	Dr. Victoria Hritonenko (75)
Δ*pscC*Δ*popBD*	*popBD* mutant lacking T3SS needle	This study
Δ*popB*Δ*exoS*	*popB* mutant lacking *exoS*	This study
E. coli SM10(λ*pir*)	Mutagenesis vector donor strain	New England Biolabs
Plasmid	**Description (Resistance conferred, µg/ml)**	**Source**
pSMC2	Constitutive GFP (Carbenicillin, 400)	(96)
pJNE05	T3SS-GFP (P*exoS*). Plasmid encoding *gfp* fused to the ExsA-dependent promoter of *exoS*. (Gentamicin, 200)	(97)
pEXG2-Δ*pscU-L*	Integrating suicide plasmid for mutagenesis	Dr. Arne Rietsch
pEXG2-Δ*exoS*	Integrating suicide plasmid for mutagenesis	Dr. Arne Rietsch
pUC18-mini-Tn7T-PrhaB-*exsA*	Delivery vector for integrating *exsA* under Rhamnose-sensitive promoter to *att*Tn7 site (Carbenicillin, 100)	This study
pUC18-mini-Tn7T-PrhaB-Vector	Delivery vector for integrating empty expression vector under rhamnose-sensitive promoter to *att*Tn7 site (Carbenicillin, 100)	This study
pTNS1	Helper plasmid for conjugation of Tn7 integration plasmid (Ampicillin, 100)	(91)
pRK2013	Helper plasmid for conjugation of Tn7 integration plasmid (Kanamycin, 35)	(91)
pFLP2	Sucrose-sensitive vector to excise antibiotic resistance marker from *att*Tn7 site	(91)

917 **FIGURE LEGENDS**

918

919 **Figure 1. Method for quantitative imaging and analysis of bacterial traversal of the corneal**
920 **epithelium.** Whole murine eyeballs *ex vivo* were blotted with tissue paper, incubated in 0.1M
921 EGTA solution (1 h), then infected with ~1 x 10^{11} CFU/ml *P. aeruginosa* expressing GFP. Eyes
922 were fixed, whole mounted *en face*, and imaged by confocal microscopy. (A) Raw image imported
923 into Imaris 9.9 for 3D analysis, confocal reflectance signal shown in red and bacterial fluorescence
924 in green. (B) Boundary between stroma and epithelium manually delineated in sequential XZ slices
925 to exclude signal from stroma and create epithelium object. (C) Objects representing apical and
926 basal boundaries of epithelium. (D) Bacteria automatically identified by fluorescent signal quality.
927 (E) Bacteria colored by their depth of traversal, measured as a percentage of its position from the
928 apical to basal boundaries.

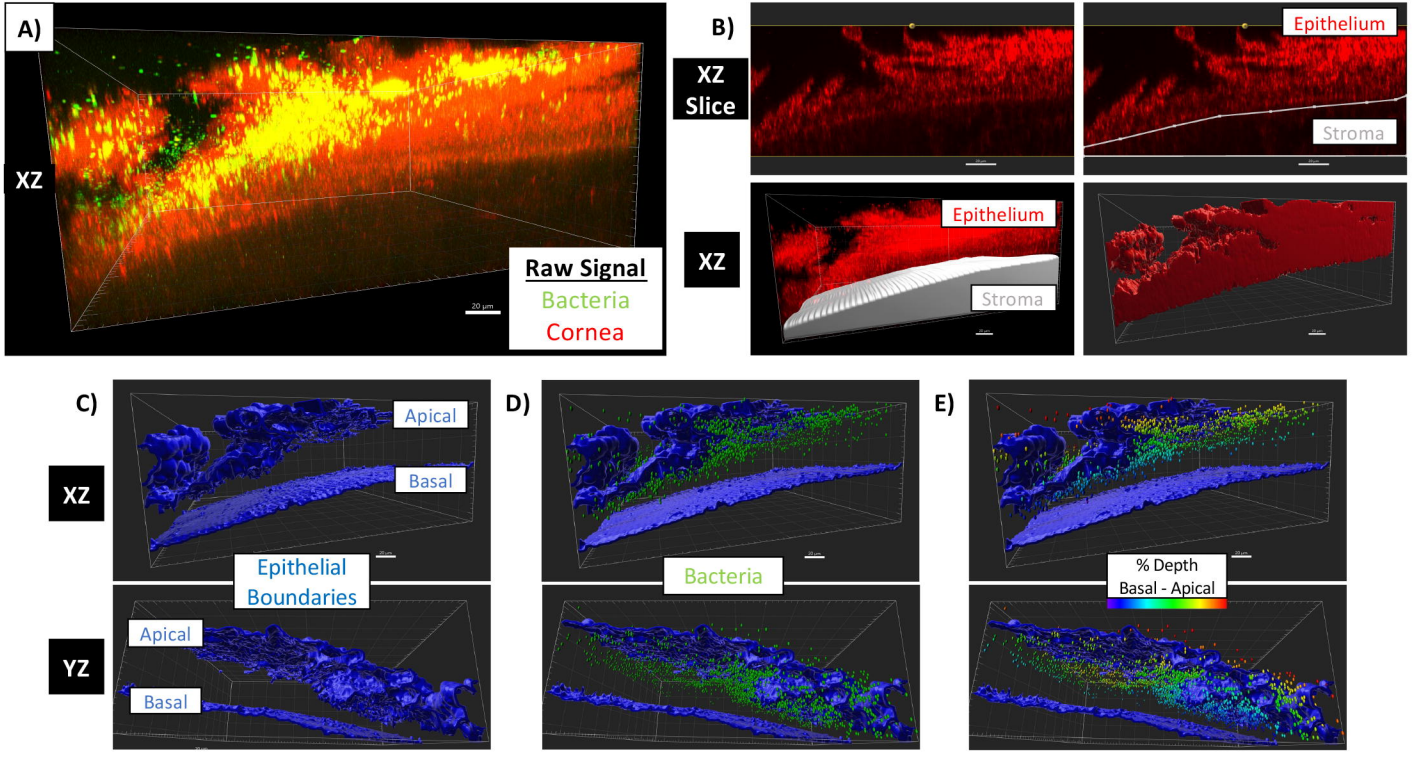
929

930 **Figure 2. Epithelial traversal by wild-type or T3SS mutants.** (A) Traversal depth after *ex vivo*
931 infection by PAO1F and T3SS mutants in genes encoding the transcriptional activator *exsA*,
932 transcriptional repressor *exsD*, known exotoxins *exoSTY*, needle component *pscC*, or translocon
933 pore *popBD*. Each data point represents the relative position (% depth) of one bacterium between
934 the apical and basal boundaries of the 3D corneal epithelium. All data points pooled from ≥ 4 eyes
935 per group. Error bars show the median +/- interquartile range. Note: All comparisons between the
936 T3SS mutants with the Δ*exsA* mutant were significant as were all comparisons with wild-type
937 PAO1F ($P < 0.0001$, Kruskal-Wallis test with Dunn's multiple comparisons). (B) Cumulative
938 histogram graphing traversal data (from Fig. 2A) of wild-type and T3SS mutants as a percent of
939 the total bacterial population found deeper in the epithelium. The vertical line indicates halfway

940 through the epithelium, highlighting the proportion of each strain that traversed more than 50% of
941 the epithelial layer.
942
943 **Figure 3. ExsA complementation in a T3SS operon-deficient background.** (A) Traversal depth
944 after *ex vivo* infection by a Δ*pscU-L* mutant which lacks all T3SS genes except *exoSTY* (and their
945 respective chaperones) and a Δ*pscU-L*Δ*exoSTY* mutant which also lacks effector toxins. Controls
946 include expression of an empty vector or *exsA* induced by overnight growth on and inclusion of
947 2% rhamnose during infection. Data were pooled from ≥ 3 eyes per strain. Error bars show median
948 +/- interquartile range. All comparisons between groups were significant at $P < 0.0001$ except for
949 Δ*pscU-L* vs. Δ*pscU-L*Δ*exoSTY*:P_{rha}-*exsA* + rhamnose, which had $P = 0.025$ (Kruskal-Wallis test
950 with Dunn's multiple comparisons). (B) Cumulative histogram graphing traversal data (from Fig.
951 3A) of T3SS operon-deficient mutants with ExsA complementation or controls as a percent of the
952 total bacterial population found deeper in the epithelium. The vertical line indicates halfway
953 through the epithelium, highlighting the proportion of each strain that traversed more than 50% of
954 the epithelial layer.
955
956 **Figure 4. Traversal by translocon and needle- or exotoxin-deficient mutants.** (A) Traversal
957 depth after *ex vivo* infection by T3SS translocon mutants in a background of Δ*pscC*, Δ*exoS*, or
958 Δ*exoSTY*. Data from Fig. 2A was used for Δ*pscC* and Δ*exoSTY* mutants. Data pooled from ≥3 eyes
959 per strain. Error bars show median +/- interquartile range. All comparisons between groups were
960 significant ($P < 0.0001$, Kruskal-Wallis test with Dunn's multiple comparisons) except for
961 Δ*exoSTY* vs. Δ*popB*. (B) Cumulative histogram graphing traversal data (from Fig. 4A) of the T3SS
962 mutants as a percent of the total bacterial population found deeper in the epithelium. The vertical

963 line indicates halfway through the epithelium, highlighting the proportion of each strain that
964 traversed more than 50% of the epithelial layer.
965
966 **Figure 5. Rhamnose-induction of ExsA-driven ExoS expression in both bacterial cells and**
967 **supernatant of a needle-deficient mutant Δ*pscU-L*.** Western immunoblot showing bacterial cell
968 pellets and supernatant fractions collected from Δ*exsA*:P_{rha}*exsA*, Δ*pscU-L*:P_{rha}Vector, Δ*pscU-*
969 *L*:P_{rha}*exsA* after growth to 'mid-log' phase in LB media, with induction of *exsA* by 2% rhamnose
970 inclusion for the conditions indicated. The location of bands corresponding with ExoS (~49 kDa)
971 detected by affinity-purified antibody and chemiluminescence are evident below the protein
972 standard annotated at 50 kDa.
973
974 **Supplemental Material**
975
976 **Supplemental Figure S1. Comparison of corneal epithelium traversal by *P. aeruginosa* and**
977 **its T3SS mutants.** Representative images of corneal epithelium traversal by wild-type PAO1F
978 compared to the Δ*pscC*, Δ*popBD* and Δ*exoSTY* mutants color-coded for traversal depth.
979
980 **Supplemental Figure S2. EGTA induction of *P. aeruginosa* T3SS gene expression was similar**
981 **between PAO1 and T3SS mutants.** OD_{600}-normalized GFP signal from pJNE05 (P*exoS*) carried
982 by PAO1F or its T3SS mutants in the translocon pore Δ*popBD*, known exotoxins Δ*exoSTY*, or the
983 transcriptional repressor Δ*exsD* after 24 h growth in T3SS-induction media [TSB plus gentamicin
984 (200 μg/ml), monosodium glutamate (100 mM), glycerol (1 %) and EGTA (2 mM)]. Similar P*exoS*
985 induction, i.e. T3SS induction, was observed across wild-type and mutants. N = 3 separate growth

986 curves per group. Mean +/- standard deviation, ns = not significant (One-way ANOVA with
987 Dunnett's Multiple Comparisons).
988
989 **Supplemental Figure S3. Rhamnose-induction of ExsA rescues *ex vivo* traversal.** (A) OD_{600}-
990 normalized GFP signal from pJNE05 (P*exoS*) carried by a Δ*exsA* mutant complemented with
991 rhamnose-inducible *exsA* with or without inclusion of rhamnose (Rha) (2 %) in the growth medium
992 [TSB with gentamicin (200 μg/ml), monosodium glutamate (100 mM) and glycerol (1%)] after 24
993 h of growth. N = 3 separate growth curves per group. Mean +/- standard deviation, ** $P < 0.01$
994 (Paired Student's t-Test). (B) Traversal depth after *ex vivo* infection of Δ*exsA* with expression of
995 *exsA* remaining off or induced by overnight growth with rhamnose (2 %). Data pooled from 3 eyes
996 per strain. Error bars show the median with interquartile range. **** $P < 0.0001$ (Kolmogorov-
997 Smirnov test).


A)
XZ
Raw Signal
Bacteria
Cornea
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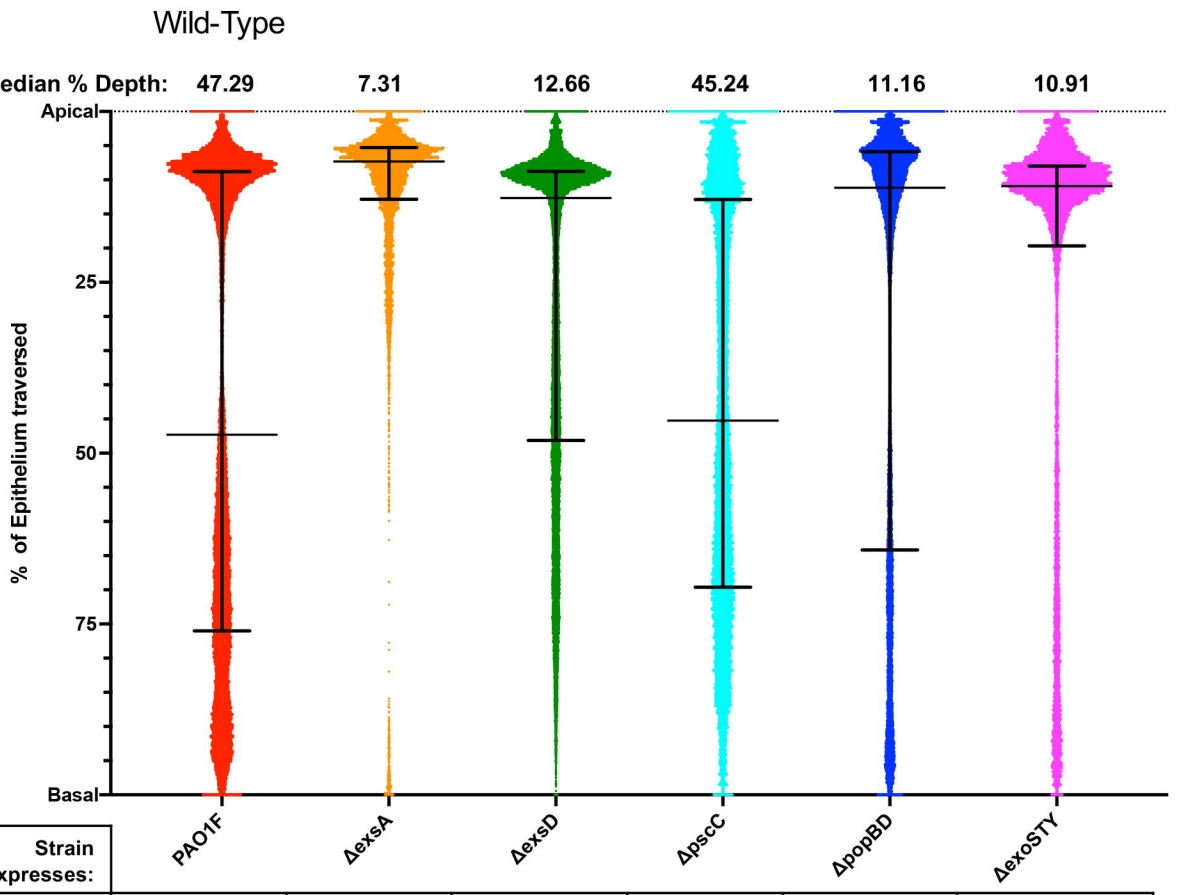
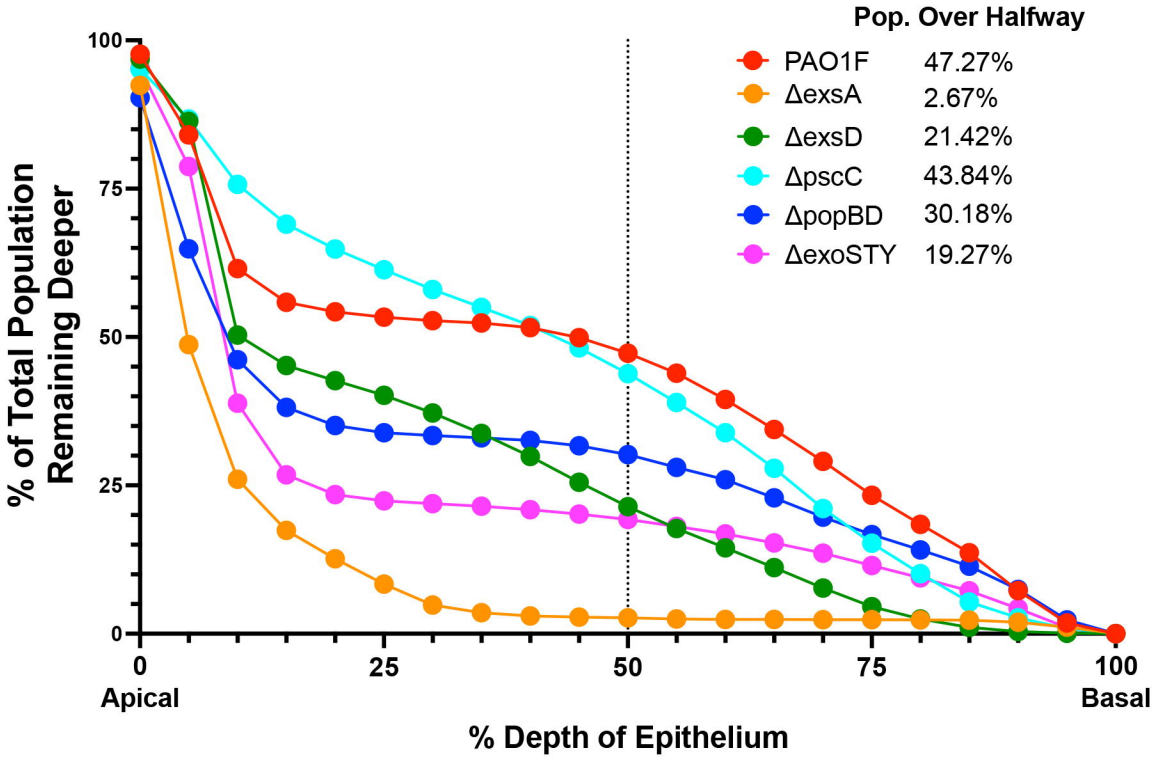
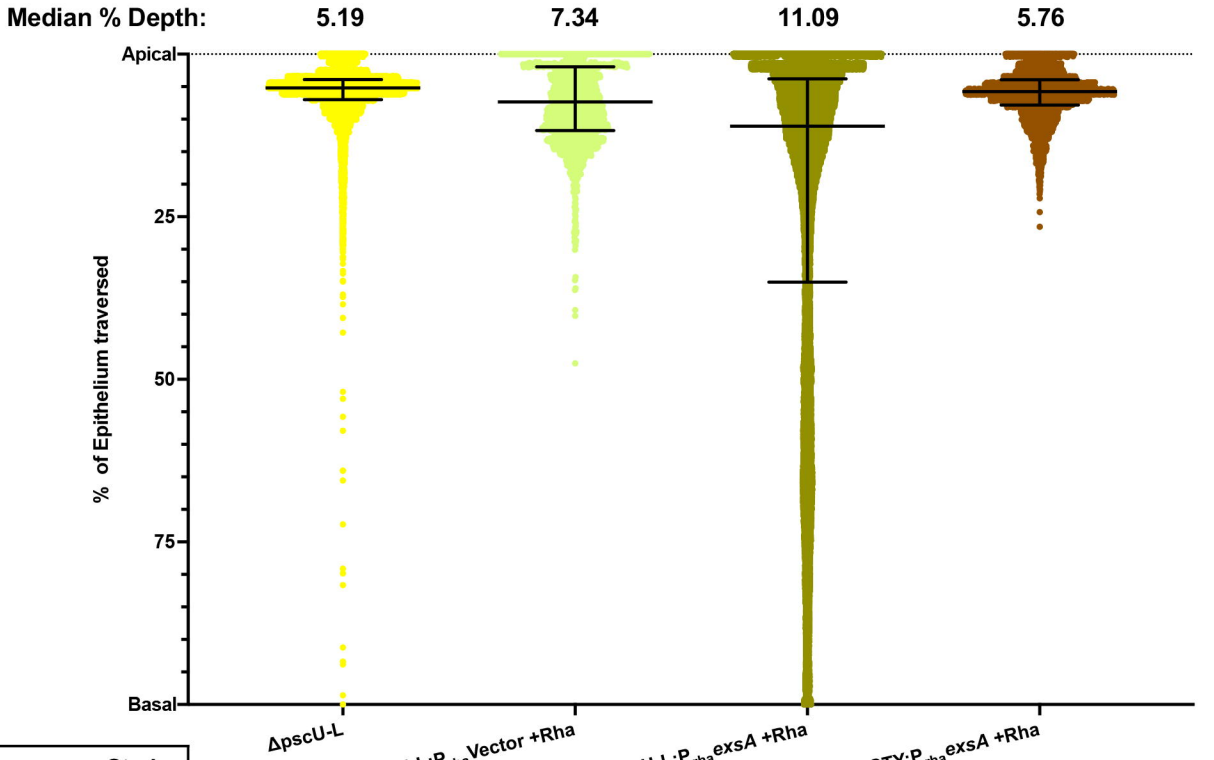


Strain Expresses:	PAO1F	ΔexsA	ΔexsD	ΔpscC	ΔpopBD	ΔexoSTY
ExsA	+	–	+	+	+	+
Needle	+	–	+	–	+	+
Translocon	+	–	+	+	–	+
Effectors	+	–	+	+	+	–

B)

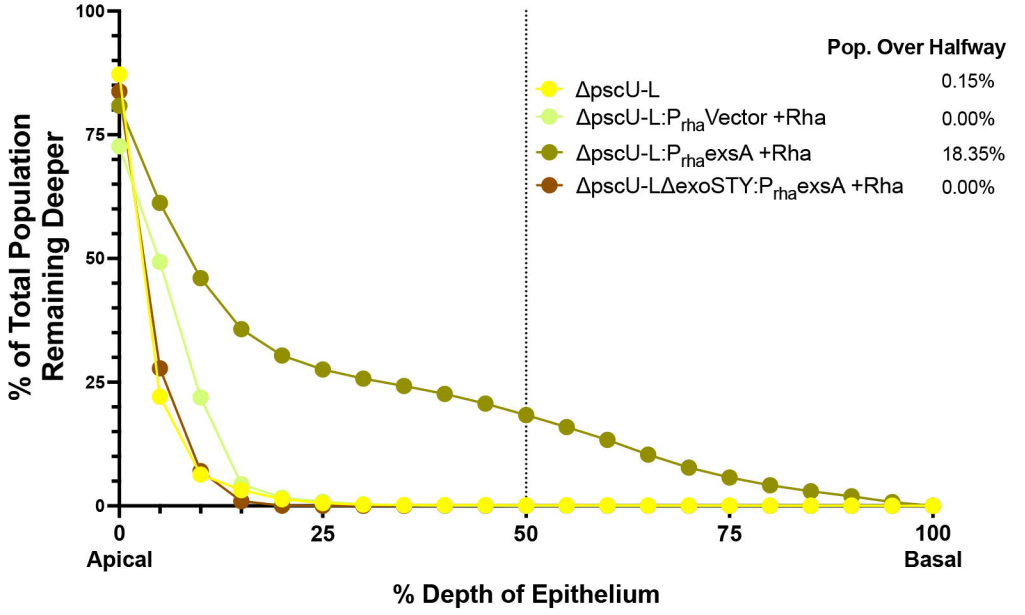


A)

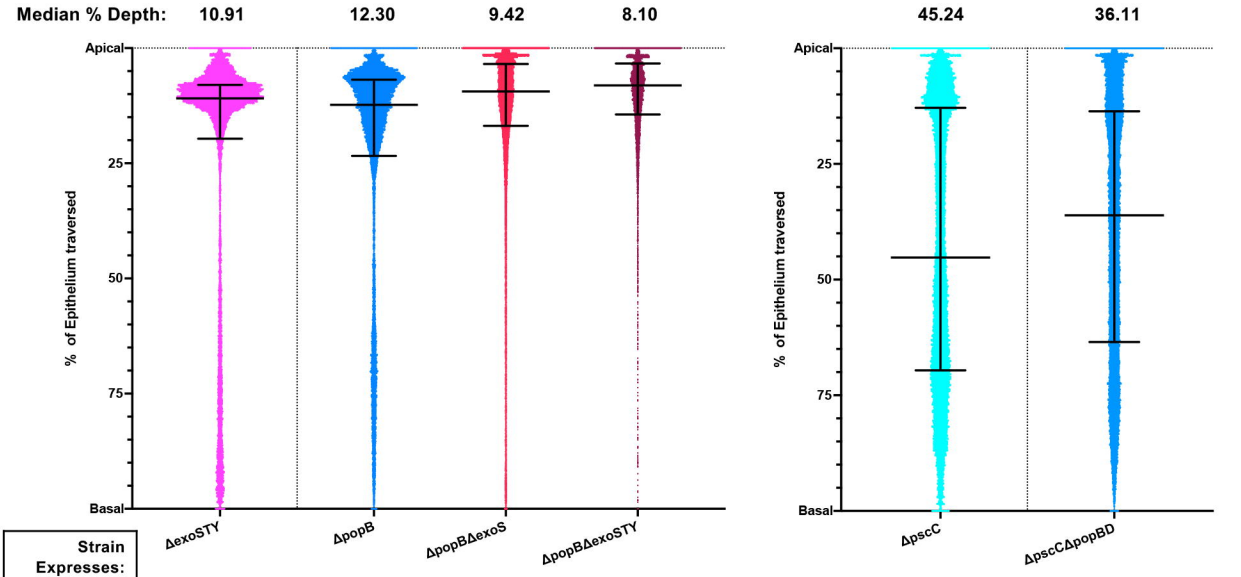


Strain Expresses:	ΔpscU-L	ΔpscU-L:P_{rha}Vector +Rha	ΔpscU-L:P_{rha}exsA +Rha	ΔpscU-LΔexoSTY:P_{rha}exsA +Rha
ExsA	–	–	+	+
Needle	–	–	–	–
Translocon	–	–	–	–
Effectors	–	–	+	–

B)

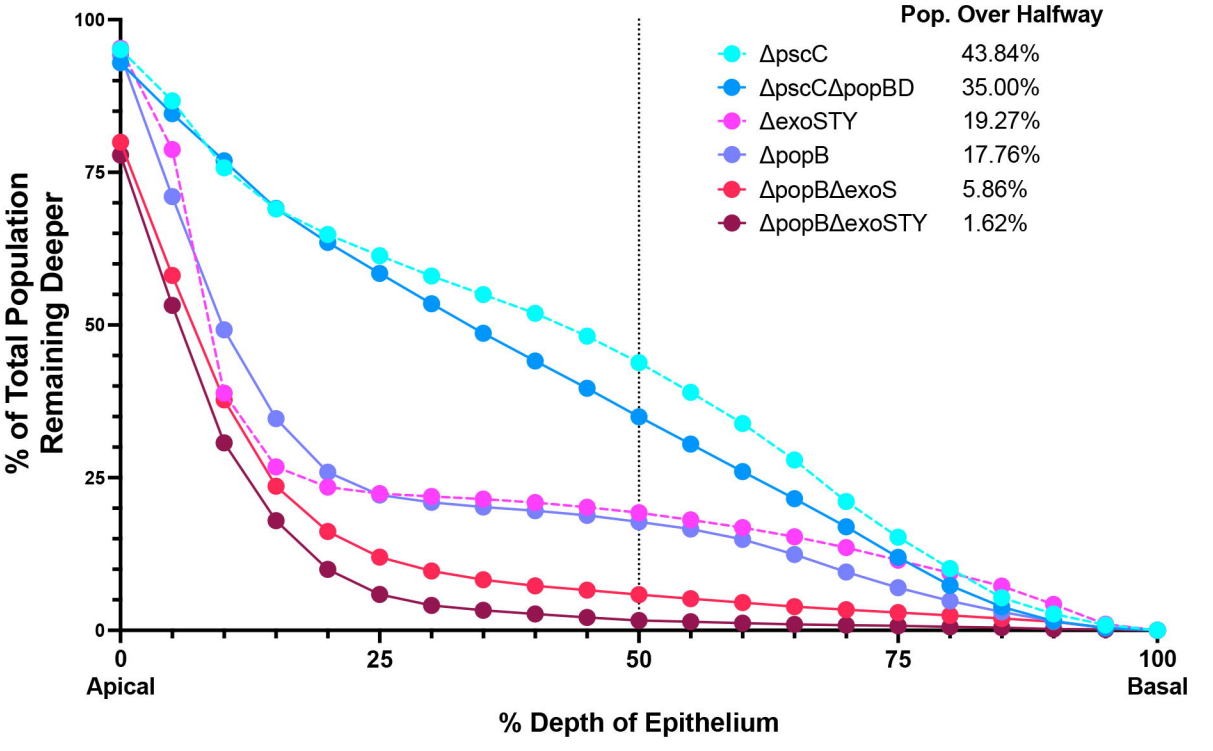


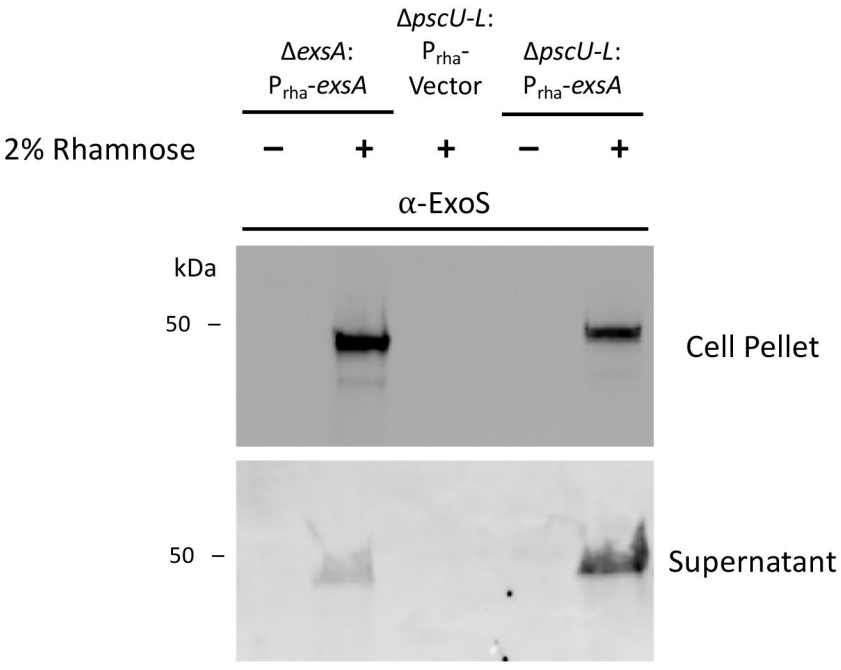
A)



Strain Expresses:	ΔexoSTY	ΔpopB	ΔpopBΔexoS	ΔpopBΔexoSTY	ΔpscC	ΔpscCΔpopBD
ExsA	+	+	+	+	+	+
Needle	+	+	+	+	–	–
Translocon	+	–	–	–	+	–
Effectors	–	+	only *exoTY*	–	+	+

B)




ΔexsA: Prha-exsA
ΔpscU-L: Prha-Vector
ΔpscU-L: Prha-exsA
2% Rhamnose
−
+
+
−
+
α-ExoS
kDa
50 –
Cell Pellet
50 –
Supernatant