Pseudomonas aeruginosa is capable of natural transformation in biofilms

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Abbreviations:

extracellular DNA; eDNA, type IV pili; T4P

Abstract

Natural transformation is a mechanism that enables competent bacteria to acquire naked, exogenous DNA from the environment. It is a key process that facilitates the dissemination of antibiotic resistance and virulence determinants throughout bacterial populations. *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen that produces large quantities of extracellular DNA (eDNA) that is required for biofilm formation. *P. aeruginosa* has a remarkable level of genome plasticity and diversity that suggests a high degree of horizontal gene transfer and recombination but is thought to be incapable of natural transformation. Here we show that *P. aeruginosa* possesses homologs of all proteins known to be involved in natural transformation in other bacterial species. We found that *P. aeruginosa* in biofilms is competent for natural transformation of both genomic and plasmid DNA. Furthermore, we demonstrate that type IV pili (T4P) facilitate but are not absolutely essential for natural transformation in *P. aeruginosa*.

38 ## Introduction

39 The continued increase in antimicrobial resistance (AMR) levels is considered to be a significant
40 global threat[1]. Horizontal gene transfer (HGT) is a key source of bacterial genome variation and
41 evolution and is largely responsible for the acquisition of antibiotic resistance genes by bacterial
42 pathogens[2]. Bacteria can acquire and heritably incorporate new genetic information via three HGT
43 mechanisms: conjugation, transduction and natural transformation. Conjugation is a cell-contact
44 dependent mechanism that transfers DNA directly from the cytoplasm of one bacterial cell into
45 another. Transduction involves encapsidation of DNA into a bacteriophage which then injects the
46 DNA into the recipient cell. The third HGT mechanism is natural transformation which involves the
47 import of naked DNA from the environment through a specialised DNA transport apparatus[3,4].

48

49 In many naturally competent bacterial species Type IV pili (T4P) are required for natural
50 transformation[4]. While the exact role of T4P in natural transformation is unclear, the generally
51 accepted model is that DNA binds to the pilus structure, which retracts and pulls the DNA to the cell
52 surface. It is unclear whether or not DNA is translocated across the outer membrane through the PilQ
53 secretin pore. The incoming DNA can then be accessed by the ComEA DNA translocation machinery
54 in the periplasm, which mediates DNA uptake possibly by a ratchet mechanism[4,5]. In Gram-positive
55 bacterial species that do not produce T4P, natural transformation involves a number of proteins with
56 homology to T4P proteins which are thought to form a pseudopilus structure that spans the cell wall
57 and is coupled to the DNA translocation complex at the cytoplasmic membrane[4,6]. Once exogenous
58 DNA has been taken up by the cell it can be stably incorporated into the genome via recombination
59 or transposition, or be maintained as a plasmid if plasmid DNA is taken up by an appropriate host[6].

60 Extracellular DNA (eDNA) is present in significant quantities in both clinical and environmental
61 settings, and provides a vast reservoir of genetic material that can be sampled by bacteria that are
62 competent for natural transformation[7].

63

64 *Pseudomonas aeruginosa* is a highly antibiotic resistant Gram-negative bacterium which is a part of
65 the 'ESKAPE' group of pathogens that pose a serious health risk worldwide. *P. aeruginosa* readily
66 acquires antibiotic resistance determinants, and demonstrates a high degree of genomic diversity and
67 malleability similar to that seen in naturally transformable bacteria[8,9]. Despite this, *P. aeruginosa* has
68 long been thought to be incapable of natural transformation[10]. *P. aeruginosa* is a model organism for
69 studying T4P[11]. Interestingly, *P. aeruginosa* produces copious quantities of eDNA under conditions
70 that promote T4P production such as in static broth cultures[12], biofilms[13] and during twitching
71 motility-mediated biofilm expansion[14,15]. We therefore hypothesized that *P. aeruginosa* may be
72 competent for natural transformation under conditions that promote both T4P expression and eDNA
73 production. Here we show that some strains of *P. aeruginosa* are in fact capable of natural
74 transformation under these conditions.

75

76 **Results**

77 Bioinformatic analyses of the sequenced *P. aeruginosa* strains PAO1, PA14 and PAK show that each
78 of these strains encode homologs of all genes known to be involved in natural transformation in other
79 bacterial species (Table 1). To determine if *P. aeruginosa* might be capable of natural transformation
80 in biofilms, we established biofilms with a 1:1 mixture of $PAO1_{GFP}$ (Gm^R) and PAO1 [pUCPSK]
81 ($Carb^R$) biofilms in 10 cm Tygon tubing under continuous flow conditions. Biofilm effluent was
82 collected each day for 4 days and bacteria tested for their ability to grow on LB agar plates containing
83 both gentamicin and carbenicillin. An average of 50-100 Gm^R/$Carb^R$ colonies (resistant to both
84 gentamicin and carbenicillin) were obtained from the effluent of mixed $PAO1_{GFP}$ and PAO1
85 [pUCPSK] biofilms on day 1 and confluent lawns of Gm^R/$Carb^R$ colonies obtained from day 2
86 onwards. The presence of mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the chromosomal attTn7 site in these
87 colonies was confirmed by PCR amplification. To confirm that these colonies also possessed

88 pUCPSK, plasmid DNA was extracted, transformed into *E. coli* and confirmed by sequencing.
89 Neither the $PAO1_{GFP}$ or PAO1 [pUCPSK] strains used to establish these mixed biofilms, or effluent
90 from control single strain biofilms were able to grow on the dual antibiotic selection plates. As PAO1
91 lacks a prophage capable of transduction and pUCPSK is a non-conjugative plasmid, these results
92 suggest that HGT of extracellular plasmid DNA and/or chromosomal DNA might occur via natural
93 transformation in *P. aeruginosa* biofilms.

94

95 To determine if HGT of chromosomal DNA could occur via natural transformation, we established
96 biofilms with a 1:1 mixture of $PAO1_{GFP}$ (Gm^R) and $PAO1_{CTX}$ (Tc^R) in 10 cm Tygon tubing under
97 continuous flow conditions. Biofilm effluent was collected each day for 8 days and bacteria tested
98 for their ability to grow on LB agar plates containing both gentamicin and tetracycline. Whilst no
99 Gm^R/Tc^Rcolonies were obtained from days 1-4, from days 5-8 an average of 3 Gm^R/Tc^R colonies that
100 were resistant to both antibiotics and expressed GFP were recovered per day. The presence of both
101 mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* and mini-CTX2 in these colonies was confirmed by PCR, and GFP
102 expression observed with epifluorescence imaging. Importantly, neither the $PAO1_{GFP}$ or $PAO1_{CTX}$
103 strains used to inoculate the mixed biofilms or effluent from control single-strain biofilms were able
104 to grow on the dual antibiotic selection plates. As neither conjugation or transduction is likely to
105 account for these HGT events in PAO1 biofilms, these results suggest that PAO1 is able to acquire
106 and incorporate chromosomal DNA and plasmids encoding antibiotic resistance genes via natural
107 transformation in biofilms.

108

109 To confirm that *P. aeruginosa* is indeed capable of natural transformation and to rule out any
110 possibility of HGT through transduction or conjugation, we performed a series of experiments to
111 follow the uptake of purified, sterile exogenous DNA. We first grew wildtype strains PAK and PAO1
112 on LB agar overnight to form a colony biofilm on the surface of the agar. We then added 5 μg of

113 sterile DNA (or the equivalent volume of sterile water) of the plasmid pUCPSK, onto the surface of
114 the colony biofilm. After 2 hr incubation at 37°C the colony was resuspended and cells plated onto
115 media containing carbenicillin to select for transformants that had acquired the plasmid. Only colony
116 patches that had been exposed to plasmid DNA yielded $Carb^R$ colonies (Figure 1A), whereas colony
117 patches exposed to sterile water yielded none. The transformation efficiency for PAK and PAO1 was
118 24.7 ± 10.1 and 5.8 ± 1.9 transformants/µg plasmid DNA, respectively. To confirm that the
119 carbenicillin resistant colonies had acquired pUCPSK, plasmid DNA was extracted, re-transformed
120 into *E. coli* and confirmed by sequencing. These observations indicate that a proportion of cells within
121 colony biofilms of *P. aeruginosa* are competent for natural transformation and are able to take up and
122 maintain plasmid DNA.

123

124 Given that both *P. aeruginosa* strains PAK and PAO1 appeared to be naturally competent, we wanted
125 to determine if this was also the case for other commonly utilised lab strains (PA14 and PA103) and
126 clinical isolates. All *P. aeruginosa* strains were first confirmed to be carbenicillin sensitive prior to
127 use. Thirteen *P. aeruginosa* otitis externa and twelve cystic fibrosis (CF) lung sputum isolates were
128 assayed for the ability to uptake pUCPSK plasmid DNA in a colony biofilm. Of these, 7/12 otitis
129 externa and 6/10 CF isolates were able to uptake exogenous plasmid DNA (Figure 1B). No $Carb^R$
130 colonies were obtained in the no plasmid DNA controls for each strain. Interestingly, a range of
131 transformation efficiencies were observed in both clinical and lab strains. Of the lab strains, PA14
132 was the least capable of natural transformation with PAK the most efficient. These results
133 demonstrate that many lab and clinical isolate strains of *P. aeruginosa* are capable of natural
134 transformation within colony biofilms, albeit with different efficiencies.

135

136 *P. aeruginosa* also expresses T4P when cultured in static nutrient broth[11]. Under these conditions *P.*
137 *aeruginosa* forms biofilms and suspended microcolony aggregates that contain eDNA[12]. To

138 determine if natural transformation also occurred in broth cultures, 10 µg of sterile pUCPSK plasmid
139 DNA (or the equivalent volume of sterile water) was added to a subculture of *P. aeruginosa* wildtype
140 PAK or PAO1 and incubated statically at 37°C for 24 hrs to allow biofilms and aggregates to form.
141 Cells were then recovered and plated onto media containing carbenicillin to select for transformants.
142 $Carb^R$ colonies were obtained for both PAO1 and PAK under these conditions, whereas no $Carb^R$
143 colonies were identified in the water control. As was observed with colony biofilm transformations
144 (Fig 1A), PAK was more efficient for natural transformation of pUCPSK than PAO1 in static broth
145 cultures (Fig 2A).

146

147 To determine if transformation efficiency was dependent on the amount of DNA added, we performed
148 static broth transformation assays with increasing amounts of plasmid DNA. Whilst we observed an
149 increase in the number of transformants with increasing amounts of plasmid DNA added (Figure 2C),
150 the transformation efficiency (transformants/µg DNA) was relatively unchanged over the range of
151 DNA quantities used for transformation (0.1-30 µg) (Figure 2D).

152

153 *P. aeruginosa* produces more T4P when cultured in static broth cultures than under shaking
154 conditions[11]. We investigated the effects on natural transformation efficiency of culturing under static
155 or shaking conditions and found that although some natural transformation was still observed under
156 shaking conditions, more transformants were obtained with static culture conditions (Figure 2B),
157 consistent with a role of T4P in natural transformation. To directly examine the role of T4P in natural
158 transformation of *P. aeruginosa,* we added plasmid DNA to static broths of PAK mutants defective
159 in the production of the pilin subunit (*pilA*), in T4P assembly (*pilV*, *pilQ, fimV*) and in T4P retraction
160 (*pilT*). Interestingly, all T4P mutants were capable of some natural transformation of pUCPSK,
161 however a significant reduction in transformation efficiency compared to wildtype PAK was
162 observed (Figure 2E). No $Carb^R$ colonies were identified in the no DNA controls. There was no

163 apparent difference in the transformation efficiency of mutants which either didn't have any surface-
164 assembled T4P (*pilA, pilV, pilQ, fimV*) or were unable to retract T4P (*pilT*) (Figure 2E). These suggest
165 that in *P. aeruginosa*, T4P facilitate transport of DNA to the cell surface but are not essential for
166 natural transformation of *P. aeruginosa*. Furthermore, these observations indicate that during natural
167 transformation in *P. aeruginosa* the DNA is not being translocated through the PilQ secretin pore.

168

169 To further examine the hypothesis that *P. aeruginosa* cells within biofilms are competent for natural
170 transformation, we examined plasmid DNA uptake under flow biofilm conditions. PAO1 flow
171 biofilms were cultured in the presence or absence of purified pUCPSK plasmid DNA and the amount
172 of natural transformation within the biofilm biomass and in the effluent assessed at days 3, 4 and 5.
173 Natural transformation was observed in both the biofilm biomass and biofilm effluent (Figure 2F).
174 No $Carb^R$ colonies were obtained in the no DNA control.

175

176 We have shown that *P. aeruginosa* is capable of natural transformation by uptake of exogenous
177 plasmid DNA in colony biofilms, in static and shaking broth cultures and in flow biofilms (Figures
178 1, 2). We were also interested in determining whether *P. aeruginosa* was also able to uptake
179 chromosomal DNA from the environment and integrate this into the chromosome. To examine this,
180 chromosomal DNA from $PAO1_{GFP}$ (Gm^R) was purified from either a whole cell lysate (gDNA) or the
181 total (sterile) eDNA from confluent agar plate culture and applied to static broth cultures of PAK or
182 PAO1 for 24 hr. Cells were recovered and cultured on agar containing gentamicin to select for
183 transformants. These assays revealed that natural transformation of gDNA occurred at a low
184 frequency for both PAK and PAO1 in static broth cultures (Figure 3A). No natural transformation
185 with sterile eDNA was observed in static broth cultures for either PAK or PAO1 (Figure 3A). This
186 may be due to the integrity of the DNA as we observed via agarose gel electrophoresis that the eDNA
187 used in these experiments was quite degraded compared with the gDNA, presumably through the

188 action of nucleases present in the extracellular milieu. No gentamicin resistant colonies were obtained
189 in the no DNA controls.
190
191 We also examined if natural transformation by uptake of exogenous chromosomal DNA occurs in
192 biofilms cultured under continuous flow. *P. aeruginosa* PAO1 flow biofilms were cultured in 10 cm
193 Tygon tubing in the presence and absence of sterile gDNA or eDNA obtained from $PAO1_{GFP}$ (Gm^R)
194 in the media influent. After 5 days the number of Gm^R colonies recovered from the biofilm biomass
195 were counted. This revealed extremely variable rates of natural transformation of gDNA by cells
196 within the biofilm biomass across multiple experiments (Figure 3B). This is not unexpected as the
197 rate is likely to be dependent upon the time at which the natural transformation event occurred. If
198 this event occurred early in the assay, we would expect many transformants recovered due to
199 proliferation of the transformed cells. However, if transformation occurred later we would expect far
200 fewer transformants as these did not have as long to proliferate. For the eDNA experiments, while
201 some Gm^R transformants were obtained (Figure 3C), the rate of natural transformation was overall
202 much lower than for gDNA (Figure 3B). No Gm^R colonies were obtained for continuous flow biofilms
203 in the absence of gDNA or eDNA indicating that the gentamicin resistant cells recovered from these
204 assays was due to the presence of the exogenous chromosomal DNA. To further rule out the
205 possibility of spontaneous resistance, the presence of the mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the
206 chromosomal attTn7 site in the biofilm-derived Gm^R colonies was confirmed by PCR. The presence
207 of the *gfp* gene in the Gm^R colonies was also confirmed by visualisation of GFP expression using
208 epifluorescence microscopy (Figure 4B). No GFP expression was observed in the PAO1 inoculum
209 strain (Figure 4A). As it was not possible to directly visualise biofilms cultured in Tygon tubing,
210 PAO1 continuous flow biofilms were cultured in transparent flow cells over 5 days in the presence
211 and absence of gDNA obtained from $PAO1_{GFP}$. Epifluorescence microscopy revealed microcolonies
212 of GFP-expressing bacteria within the biofilm (Fig 4C-F). No GFP expression was observed in the
213 no DNA control biofilms.

214

215 ## Discussion

216 Here we have demonstrated, in contrast to current dogma, that *P. aeruginosa* is capable of natural
217 transformation of both plasmid and chromosomal DNA under conditions that promote the expression
218 of T4P and eDNA production, such as in biofilms. We found that whilst T4P appear to be involved
219 in facilitating DNA uptake, T4P are not absolutely required for natural transformation in *P.*
220 *aeruginosa.* Furthermore, our data suggests that the PilQ secretin pore is not absolutely required for
221 translocation of DNA across the outer membrane in this organism. This is in contrast to the other
222 Gram-negative bacteria in which it appears that T4P and the secretin pore are required for natural
223 transformation.

224

225 The finding that *P. aeruginosa* is capable of natural transformation is a paradigm shift in our
226 understanding of how this pathogen acquires genetic diversity. Indeed, recombination has recently
227 been identified as a major means of genetic diversity in *P. aeruginosa* cystic fibrosis (CF) lung
228 isolates although the source of DNA and the mechanism of HGT was not determined[16]. Natural
229 transformation may be an important mechanism for the acquisition of antibiotic resistance and
230 virulence genes in this ESKAPE pathogen and a significant contributor to the rapid increase in
231 number of multidrug resistant *P. aeruginosa* strains that are an emerging problem worldwide.

232

233 ## Methods

234 **Strains, plasmids and growth conditions**

235 *P. aeruginosa* strains used in this study were PAO1 (ATCC 15692), PAK[17], PA14[18], PA103[19],
236 $PAO1_{GFP}$ which contains mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* encoding *gfp* and *aac1* (Gm^R) inserted
237 downstream of *glmS*[20], $PAO1_{CTX}$ which contains miniCTX2 encoding *tet* (Tc^R) inserted into the *attB*

238 site of the chromosome[21] and T4P mutants PAK*pilA*::*TcR*[22], and Tn*5*-B21 mutants of *pilQ*[23], *pilT*[24],
239 *pilV*[25], *fimV*[26]. The *P. aeruginosa* CF sputum clinical isolates were obtained from David Armstrong
240 at Monash Medical Centre (Melbourne, Australia), and the otitis externa *P. aeruginosa* clinical
241 isolates were obtained from Di Olden at Gribbles Pathology Melbourne (Australia). The pUCPSK
242 plasmid used is a non-conjugative *E. coli-P. aeruginosa* shuttle vector encoding *bla* which confers
243 carbenicillin resistance ($Carb^R$) in *P. aeruginosa*[27]. *E. coli* Dh5α (*recA, endA1, gyrA96, hsdR17, thi-*
244 *1, supE44, relA1, φ80, dlacZΔM15*) was used as a host strain for pUCPSK and was miniprepped from
245 *P. aeruginosa* and *E. coli* strains using a Qiagen miniprep kit according to manufacturer's
246 instructions.

247

248 *P. aeruginosa* was cultured on lysogeny broth (LB) solidified with agar at 1.5% (w/v) for routine
249 maintenance and at 1.5% or 1% (w/v) for colony biofilm assays and grown in cation-adjusted Mueller
250 Hinton Broth (CAMHB) at 37°C for all static broth and flow biofilm assays. Antibiotics were used
251 at the following concentrations (w/v) as required: ampicillin 50 µg/ml for *E. coli* and carbenicillin
252 250 µg/ml, gentamicin 100 µg/ml and tetracycline 100 µg/ml for *P. aeruginosa*.

253

254 **Bioinformatics and data and statistical analyses**

255 Homologs of proteins involved in natural transformation were identified in *P. aeruginosa* PAO1
256 using BLASTp[28]. The Pseudomonas.com resource[29] and the PAK genome[30] were used to identify *P.*
257 *aeruginosa* orthologs.

258 Data was graphed and analyzed using Graph Pad Prism version 8.0. The number of replicates and
259 any statistical tests are described in figure legends.

260

261 **Colony biofilm assay**

262 Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. A 10
263 µL plastic loop was used to generate a 1 cm patch of the overnight culture on a dry 1% LBA plate.
264 This was then incubated overnight at 37°C. The next day 10 µL of DNA at the indicated concentration
265 was spotted onto the established colony biofilm and allowed to dry into the cells. The plate was then
266 incubated with the agar downwards at 37°C for the indicated time. After incubation the colony biofilm
267 was harvested from the plate into 1 mL LB, vortexed to resuspend and then incubated at 37°C for 30
268 min to fully resuspend the cells. The cell suspension was then spread plated between two 150 mm
269 LBA plates with appropriate antibiotic selection and incubated for 24 hr at 37°C.
270
271 **Static broth assay**
272 Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. 40µL
273 of overnight culture was subcultured into 2 ml fresh CAMHB with DNA added at the indicated
274 concentration. The media, cells and DNA were then mixed and incubated at 37°C statically for 24 hr.
275 Note for the shaking broth assay the same setup was used however the culture was incubated with
276 shaking at 250 rpm. In both cases after incubation the cell suspension was then spread plated between
277 two 150 mm LBA plates with appropriate antibiotic selection and incubated for 24 hr at 37°C.
278
279 **Isolation of DNA for use in continuous flow biofilm assays**
280 Chromosomal DNA (gDNA) was purified from PAO1Tn7::*gfp-aac1* cells using the Epicentre®
281 Masterpure DNA purification kit. Extracellular DNA (eDNA) was purified from a confluent lawn of
282 PAO1Tn7::*gfp-aac1* cultured overnight on MacConkey agar containing 5% (v/v) glycerol. Bacteria
283 were suspended in sterile phosphate buffered saline (PBS), centrifuged and the supernatant filtered
284 through 0.2 µm PES membrane. eDNA present in the supernatant was further purified by removal of
285 proteins and ethanol precipitation as reported previously[31]. Sterility of all DNA samples was
286 confirmed prior to use by plating onto LB agar.

287

288 **Continuous flow biofilm assays**

289 10 cm lengths of Tygon laboratory tubing (2mm ID) were inoculated with 1/100 dilution of an
290 overnight culture of *P. aeruginosa* in CAMHB and allowed to attach for 2 hours under static
291 conditions after which continuous flow was commenced at a rate of 80 µL/min at room temperature.
292 Influent media was CAMHB containing either no added DNA or DNA added at a final concentration
293 of 1 µg/mL for pUCPSK plasmid DNA, or 0.5 mg/mL for gDNA or eDNA. At harvest, the attached
294 biofilm was removed by sonication and biofilm-associated bacteria collected by centrifugation.
295 Transformants were selected by plating onto LB agar with appropriate antibiotic selection and
296 incubated for 24 hr at 37°C. To visualise natural transformation by PAO1 continuous flow biofilms
297 (Figure 4) of gDNA an IBIDI® µ-slide I (with flow kit) was inoculated and cultured as described for
298 the Tygon tubing biofilms. Biofilms were imaged using an Olympus IX71 inverted research
299 microscope fitted with phase contrast objectives and filtered halogen lamps for fluorescent imaging.

300

301 **Confirmation of natural transformation events from continuous flow biofilms**

302 The presence of mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the chromosomal attTn7 site was confirmed by PCR
303 using primers $Tn7_{-up}$ (5'CGTATTCTTCGTCGGCGTGAC3') and $Tn7_{-down}$
304 (5'CGAAGCCGCCGACAAGGG3'). Expression of GFP was confirmed by epifluorescence
305 microscopy on an Olympus IX71. The presence of mini-CTX2 at the chromosomal *attB* site was
306 confirmed by PCR using primers P_{ser-up} (5'CGAGTGGTTTAAGGCAACGGTCTTGA3') and P_{ser-}
307 $_{down}$ (5'AGTTCGGCCTGGTGGAACAACTCG 3')[21]. To confirm the presence of pUCPSK in *P.*
308 *aeruginosa*, plasmid DNA was extracted from *P. aeruginosa*, transformed into *E. coli*, extracted and
309 confirmed by sequencing with $M13_{-FUP}$ (5'TGTAAAACGACGGCCAGT3').

310

311

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Author information

Contributions

L.M.N, L.T, M.K, S.R.O, D.L and C.B.W performed experiments. L.M.N, L.T and C.B.W analyzed data. C.B.W. provided project administration and funding. L.M.N. and C.B.W. wrote the manuscript.

Ethics declarations

The authors declare no conflict of interest.

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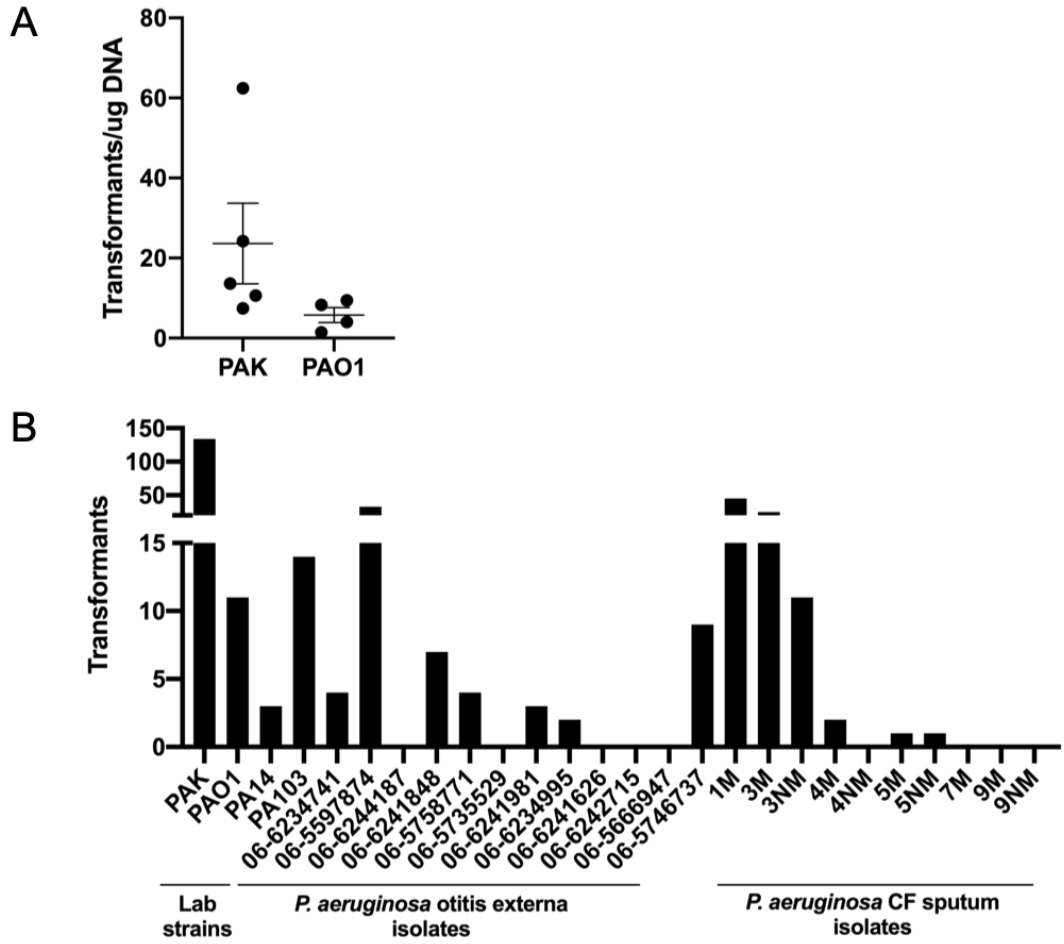
395

396 **Tables and Figures**

397 **Table 1.** Homologs of proteins involved in natural transformation in a range of bacteria.

Competence Protein	***Bacillus subtilis***	***Streptococcus pneumoniae***	***Haemophilus influenzae***	***Thermus thermophilus***	***Pseudomonas stutzeri***	***Neisseria gonorrhoeae***	***Pseudomonas aeruginosa****
T4P/Competence Pseudopilus							
Traffic NTPase(s)	ComGA	ComGA	PilB	PilF	PilT, PilU	PilF, PilT	PilB, PilT, PilU
Polytopic membrane protein	ComGB	ComGB	PilC	PilC	PilC	PilG	PilC
Pilins or pseudopilins	ComGC, -GD, -GE, -GG	CglC, CglD	PilA	PilA1, -A2, -A3, -A4	PilA1	PilE, ComP	PilA, -V, -W, -X, -E, FimT, FimU
Prepilin peptidase	ComC	CilC	PilD	PilD		PilD	PilD
Secretin/pilot	na	na	ComE	PilQ		PilQ/PilP	PilQ/PilP
DNA translocation machinery							
DNA receptor	ComEA	ComEA		ComEA		ComE	PA3140
Membrane channel	ComEC	ComEC	Rec-2	ComEC	ComA	ComA	PA2984
ATP-binding protein	ComFA	ComFA			ExbB		PA2983
Other							
			DprA (Smf)				PA0021
			TfoX (Sxy)				PA4703
			CRP				Vfr
			CyaA				CyaA, CyaB
			ComM				PA5290
			ComF				PA0489

398 *Using *P. aeruginosa* PAO1 gene nomenclature



399

400 **Figure 1. Lab and clinical strains of *P. aeruginosa* are capable of natural transformation of**
401 **plasmid DNA within colony biofilms.** pUCPSK DNA was applied to colony biofilms of (A) *P.*
402 *aeruginosa* PAK or PAO1, or (B) *P. aeruginosa* lab or clinical strains and incubated for 2 hr. Cells
403 were then harvested and the number of carbenicillin resistant transformants determined by spread
404 plating on selective media. For (A) the mean of each set of technical triplicates was calculated to
405 give an n≥4 which is presented as mean ± SEM. ($P > .05$; Mann-Whitney *U*-test). For (B) the
406 values presented are from n=1. For the CF sputum isolates the designation M refers to mucoid
407 phenotype, NM is non-mucoid.

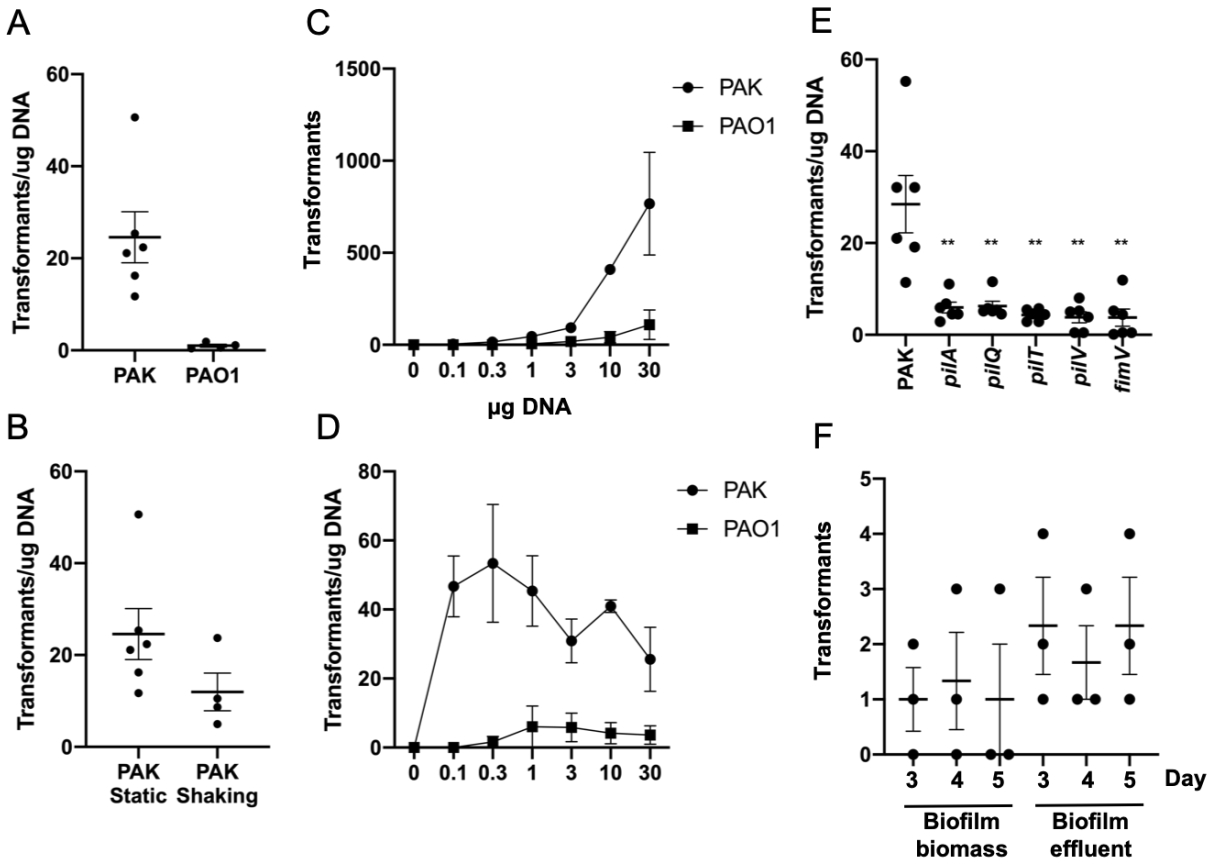
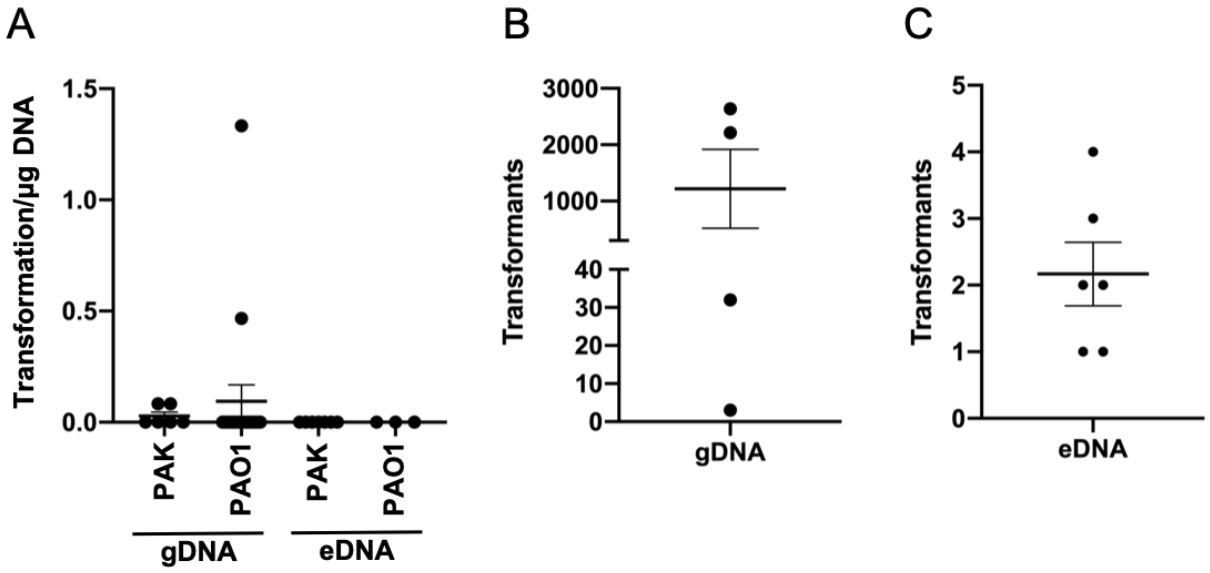


Figure 2. *P. aeruginosa* is capable of natural transformation of plasmid DNA in broth cultures and continuous flow biofilms. Carbenicillin resistant transformants obtained from static (A, C-E) or shaking (B) broth cultures incubated for 24 hr with pUCPSK or (F) from biofilm biomass or effluent of PAO1 continuous flow biofilms cultured with pUCPSK in the media influent harvested on the indicated day. The mean of each set of technical triplicates was calculated to give an n≥3 which is presented as mean ± SEM. For (A) and (E) ** $P < .005$; Mann-Whitney *U*-test compared to PAK. For (B) $P > .05$; Mann-Whitney *U*-test.



416

417 **Figure 3. *P. aeruginosa* is capable of natural transformation of chromosomal DNA.** Sterile
418 gDNA or eDNA was added to (A) static broth cultures of PAK or PAO1 and incubated for 24 hr or
419 (B) in the media influent of continuous flow biofilms of PAO1 in Tygon tubing and incubated for 5
420 days. The mean of each set of technical triplicates was calculated to give an n≥3 which is presented
421 as mean ± SEM.

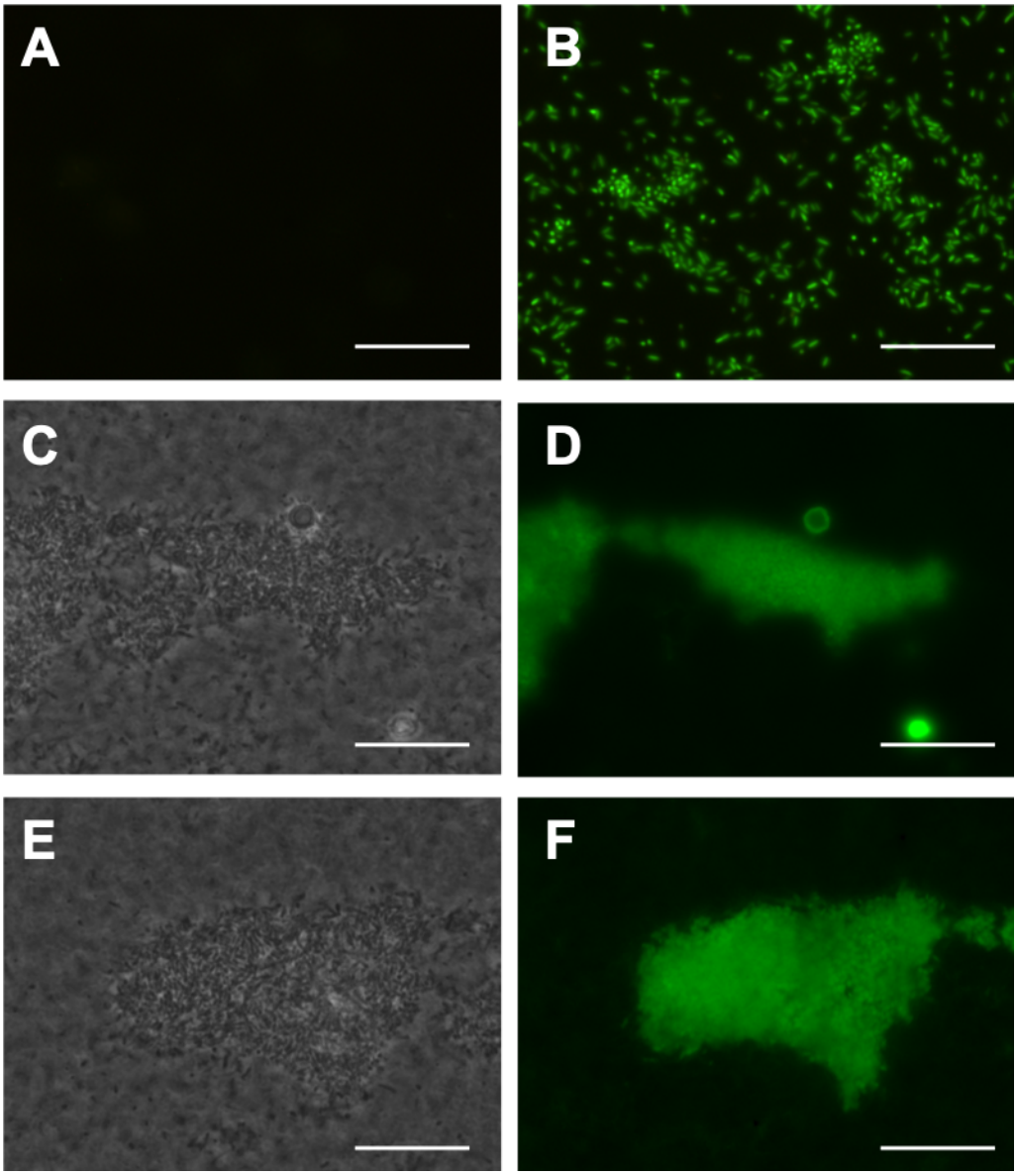


Figure 4. *P. aeruginosa* cultured in continuous flow biofilms can stably integrate and express antibiotic resistance and *gfp* gene cassettes by natural transformation of exogenous chromosomal DNA. Sterile $PAO1_{GFP}$ gDNA was added to the media influent of continuous flow biofilms of PAO1 in Tygon tubing (B) or flow cells (C-F) and incubated for 5 days. Gentamicin resistant (Gm^R) colonies obtained from Tygon tubing biofilms were resuspended in PBS and visualized by epifluorescence microscopy which showed all cells from Gm^R colonies expressed GFP (B) whereas the inoculum PAO1 strain did not express GFP (A). The biofilm biomass in flow cells was visualized by phase contrast (C, E) or epifluorescence microscopy (D, F) which showed the presence of biofilm microcolonies expressing GFP. Scale bar 100 µm.