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 Extracellular DNA (eDNA) is present in significant quantities in both clinical and environmental
50 settings, and provides a vast reservoir of genetic material that can be sampled by bacteria that are
51 competent for natural transformation[5]. In many naturally competent bacterial species Type IV pili
52 (T4P) are required for natural transformation[4]. While the exact role of T4P in natural transformation
53 is unclear, the generally accepted model is that DNA binds to the pilus structure, which retracts and
54 pulls the DNA to the cell surface. It is unclear whether or not DNA is translocated across the outer
55 membrane through the PilQ secretin pore. The incoming DNA can then be accessed by the ComEA
56 DNA translocation machinery in the periplasm, which mediates DNA uptake possibly by a ratchet
57 mechanism[4,6]. In Gram-positive bacterial species that do not produce T4P, natural transformation
58 involves a number of proteins with homology to T4P proteins which are thought to form a pseudopilus
59 structure that spans the cell wall and is coupled to the DNA translocation complex at the cytoplasmic
60 membrane[4,7]. Once exogenous DNA has been taken up by the cell it can be stably incorporated into
61 the genome via recombination or transposition, or be maintained as an independent replicon if
62 plasmid DNA is taken up by an appropriate host[7]. Natural plasmid transformation has also been

63 described in *Escherichia coli*[8]. This appears to be a distinct mechanism which does not require
64 homologs of the T4P/competence pseudopilus or DNA translocation machinery[9].
65
66 *Pseudomonas aeruginosa* is a highly antibiotic resistant Gram-negative bacterium which is a part of
67 the 'ESKAPE' group of pathogens that pose a serious health risk worldwide. *P. aeruginosa* readily
68 acquires antibiotic resistance determinants, and demonstrates a high degree of genomic diversity and
69 malleability similar to that seen in naturally transformable bacteria[10,11]. Despite this, *P. aeruginosa*
70 has long been thought to be incapable of natural transformation[12]. *P. aeruginosa* is a model organism
71 for studying T4P[13]. Interestingly, *P. aeruginosa* produces copious quantities of eDNA under
72 conditions that promote T4P production such as in static broth cultures[14], biofilms[15] and during
73 twitching motility-mediated biofilm expansion[16,17]. We therefore hypothesised that *P. aeruginosa*
74 may be competent for natural transformation under conditions that promote both T4P expression and
75 eDNA production. Here we show that some clinical and laboratory strains of *P. aeruginosa* are in
76 fact capable of natural transformation under these conditions.
77

78 # Methods

79 **Strains, plasmids and growth conditions**

80 *P. aeruginosa* strains used in this study were PAO1 (ATCC 15692), PAK[18], PA14[19], PA103[20],
81 $PAO1_{GFP}$ which contains mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* encoding *gfp* and *aac1* (Gm^R) inserted
82 downstream of *glmS*[21], $PAO1_{CTX}$ which contains miniCTX2 encoding *tet* (Tc^R) inserted into the *attB*
83 site of the chromosome[22] and T4P mutants PAK*pilA*::*TcR*[23], and Tn*5*-B21 mutants of *pilQ*[24], *pilT*[25],
84 *pilV*[26], *fimV*[27]. The *P. aeruginosa* CF sputum clinical isolates were obtained from David Armstrong
85 at Monash Medical Centre (Melbourne, Australia), and the otitis externa *P. aeruginosa* clinical
86 isolates were obtained from Di Olden at Gribbles Pathology Melbourne (Australia). The pUCPSK
87 plasmid used is a non-conjugative *E. coli*-*P. aeruginosa* shuttle vector encoding *bla* which confers

88 carbenicillin resistance (Carb^R) in *P. aeruginosa*[28]. *E. coli* Dh5α (*recA, endA1, gyrA96, hsdR17, thi-*
89 *1, supE44, relA1, φ80, dlacZΔM15*) was used as a host strain for pUCPSK and was miniprepped from
90 *P. aeruginosa* and *E. coli* strains using a Qiagen miniprep kit according to manufacturer's
91 instructions.
92
93 *P. aeruginosa* was cultured on lysogeny broth (LB) solidified with agar at 1.5% (w/v) for routine
94 maintenance and at 1.5% or 1% (w/v) for colony biofilm assays and grown in cation-adjusted Mueller
95 Hinton Broth (CAMHB) at 37°C for all static broth and flow biofilm assays. Antibiotics were used
96 at the following concentrations (w/v) as required: ampicillin 50 µg/ml for *E. coli* and carbenicillin
97 250 µg/ml, gentamicin 100 µg/ml and tetracycline 100 µg/ml for *P. aeruginosa*.
98
99 **Bioinformatics and data and statistical analyses**
100 Homologs of proteins involved in natural transformation were identified in *P. aeruginosa* PAO1
101 using BLASTp[29]. The Pseudomonas.com resource[30] and the PAK genome[31] were used to identify *P.*
102 *aeruginosa* orthologs.
103 Data was graphed and analysed using Graph Pad Prism version 8.0. The number of replicates and any
104 statistical tests are described in Figure legends.
105
106 **Colony biofilm assay**
107 Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. A 10
108 µL plastic loop was used to generate a 1 cm patch of the overnight culture on a dry 1% LBA plate.
109 This was then incubated overnight at 37°C. The next day 10 µL of pUCPSK plasmid DNA (at a final
110 concentration of 5 µg) was spotted onto the established colony biofilm and allowed to dry into the
111 cells. The plate was then incubated with the agar downwards at 37°C for the indicated time. After
112 incubation the colony biofilm was harvested from the plate into 1 mL LB, vortexed to resuspend and

113 then incubated at 37°C for 30 min to fully resuspend the cells. The cell suspension was then spread
114 plated between two 150 mm LBA plates with appropriate antibiotic selection and incubated for 24 hr
115 at 37°C.
116
117 **Static broth assay**
118 Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. 40µL
119 of overnight culture was subcultured into 2 ml fresh CAMHB with DNA added at the indicated
120 concentration. The media, cells and DNA were then mixed and incubated at 37°C statically for 24 hr.
121 Note for the shaking broth assay the same setup was used however the culture was incubated with
122 shaking at 250 rpm. In both cases after incubation the cell suspension was then spread plated between
123 two 150 mm LBA plates with appropriate antibiotic selection and incubated for 24 hr at 37°C.
124 To image the aggregates present in static broth cultures *P. aeruginosa* was grown as described for the
125 static broth assay in a glass-bottomed Ibidi® µ-Dish and visualised using DeltaVision Elite inverted
126 research microscope with a × 100 1.4 numerical aperture UPlanFLN objective, InsightSSI
127 illumination, SoftWorX acquisition software, fitted with a WeatherStation environmental chamber
128 (Applied Precision, GE Healthcare, Issaquah, WA, USA) and a scientific CMOS 15-bit camera
129 (pco.edge, PCO AG, Kelheim, Germany).
130
131 **Isolation of DNA for use in continuous flow biofilm assays**
132 Chromosomal DNA (gDNA) was purified from PAO1Tn7::*gfp-aac1* cells using the Epicentre®
133 Masterpure DNA purification kit. Extracellular DNA (eDNA) was purified from a confluent lawn of
134 PAO1Tn7::*gfp-aac1* cultured overnight on MacConkey agar containing 5% (v/v) glycerol. Bacteria
135 were suspended in sterile phosphate buffered saline (PBS), centrifuged and the supernatant filtered
136 through 0.2 µm PES membrane. eDNA present in the supernatant was further purified by removal of

137 proteins and ethanol precipitation as reported previously[32]. Sterility of all DNA samples was
138 confirmed prior to use by plating onto LB agar.
139
140 **Continuous flow biofilm assays**
141 10 cm lengths of Tygon laboratory tubing (2mm ID) were inoculated with 1/100 dilution of an
142 overnight culture of *P. aeruginosa* in CAMHB and allowed to attach for 2 hours under static
143 conditions after which continuous flow was commenced at a rate of 80 µL/min at room temperature.
144 Influent media was CAMHB containing either no added DNA or DNA added at a final concentration
145 of 1 µg/mL for pUCPSK plasmid DNA, or 0.5 mg/mL for gDNA or eDNA. At harvest, the attached
146 biofilm was removed by sonication and biofilm-associated bacteria collected by centrifugation.
147 Transformants were selected by plating onto LB agar with appropriate antibiotic selection and
148 incubated for 24 hr at 37°C. To visualise natural transformation by PAO1 continuous flow biofilms,
149 an IBIDI® µ-slide I (with flow kit) was inoculated and cultured as described for the Tygon tubing
150 biofilms. Biofilms were imaged using an Olympus IX71 inverted research microscope with a × 100
151 1.4 numerical aperture UPlanFLN objective, FViewII monochromatic camera and AnalySIS
152 Research acquisition software (Olympus Australia, Notting Hill, VIC, Australia) fitted with an
153 environmental chamber (Solent Scientific, Segensworth, UK) for fluorescent imaging.
154
155 **Confirmation of natural transformation events from continuous flow biofilms**
156 The presence of mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the chromosomal attTn7 site was confirmed by PCR
157 using primers $Tn7_{-up}$ (5'CGTATTCTTCGTCGGCGTGAC3') and $Tn7_{-down}$
158 (5'CGAAGCCGCCGACAAGGG3'). Expression of GFP was confirmed by epifluorescence
159 microscopy on an Olympus IX71. The presence of mini-CTX2 at the chromosomal *attB* site was
160 confirmed by PCR using primers P_{ser-up} (5'CGAGTGGTTTAAGGCAACGGTCTTGA3') and P_{ser-}
161 $_{down}$ (5'AGTTCGGCCTGGTGGAACAACTCG 3')[22]. To confirm the presence of pUCPSK in *P.*

162 *aeruginosa*, plasmid DNA was extracted from *P. aeruginosa*, transformed into *E. coli*, extracted and
163 confirmed by sequencing with $M13_{-FUP}$ (5'TGTAAAACGACGGCCAGT3').
164

165 **Results**

166 Bioinformatic analyses of the sequenced *P. aeruginosa* strains PAO1, PA14 and PAK show that each
167 of these strains encode homologs of all genes known to be involved in natural transformation in other
168 bacterial species (Table 1). To determine if *P. aeruginosa* might be capable of natural transformation
169 in biofilms, we established biofilms with a 1:1 mixture of $PAO1_{GFP}$ (Gm^R) and PAO1 [pUCPSK]
170 ($Carb^R$) under continuous flow conditions. Biofilm effluent was collected each day for 4 days and
171 bacteria tested for their ability to grow on LB agar plates containing both gentamicin and
172 carbenicillin. An average of 50-100 Gm^R/$Carb^R$ colonies (resistant to both gentamicin and
173 carbenicillin) were obtained from the effluent of mixed $PAO1_{GFP}$ and PAO1 [pUCPSK] biofilms on
174 day 1 and confluent lawns of Gm^R/$Carb^R$ colonies obtained from day 2 onwards. The presence of
175 mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the chromosomal *attTn7* site in these colonies was confirmed by PCR
176 amplification. To confirm that these colonies also possessed pUCPSK, plasmid DNA was extracted,
177 transformed into *E. coli* and confirmed by sequencing. Neither the $PAO1_{GFP}$ or PAO1 [pUCPSK]
178 strains used to establish these mixed biofilms, or effluent from control single strain biofilms were
179 able to grow on the dual antibiotic selection plates. As PAO1 lacks a prophage capable of transduction
180 and pUCPSK is a non-conjugative plasmid, these results suggest that HGT of plasmid DNA and/or
181 chromosomal DNA might occur via natural transformation in *P. aeruginosa* biofilms.
182

183 To determine if HGT of chromosomal DNA did indeed occur via natural transformation, and the
184 above result was not just due to HGT of plasmid DNA, we established biofilms with a 1:1 mixture of
185 $PAO1_{GFP}$ (Gm^R) and $PAO1_{CTX}$ (Tc^R) under continuous flow conditions. Biofilm effluent was
186 collected each day for 8 days and bacteria tested for their ability to grow on LB agar plates containing

187 both gentamicin and tetracycline. Whilst no Gm^R/Tc^Rcolonies were obtained from days 1-4, from
188 days 5-8 an average of 3 Gm^R/Tc^R colonies that were resistant to both antibiotics and expressed GFP
189 were recovered per day. The presence of both mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* and mini-CTX2 in these
190 colonies was confirmed by PCR. Importantly, neither the $PAO1_{GFP}$ or $PAO1_{CTX}$ strains used to
191 inoculate the mixed biofilms or effluent from control single-strain biofilms were able to grow on the
192 dual antibiotic selection plates. As neither conjugation or transduction is likely to account for these
193 HGT events in PAO1 biofilms, these results suggest that *P. aeruginosa* is able to acquire and
194 incorporate chromosomal DNA and plasmids encoding antibiotic resistance genes via natural
195 transformation in biofilms.

196

197 To confirm that *P. aeruginosa* is indeed capable of natural transformation and to rule out any
198 possibility of HGT through transduction or conjugation, we performed a series of experiments to
199 follow the uptake of purified, sterile exogenous DNA. We firstly examined plasmid DNA uptake
200 under continuous flow biofilm conditions. PAO1 flow biofilms were cultured in the presence or
201 absence of purified pUCPSK plasmid DNA (at a final concentration of 1 μg/mL) and the amount of
202 natural transformation within the biofilm biomass and effluent assessed at days 3, 4 and 5. $Carb^R$
203 colonies were obtained from both the biofilm biomass and biofilm effluent (Fig. 4a). No $Carb^R$
204 colonies were obtained in the no DNA control.

205

206 We also examined if natural transformation by uptake of exogenous chromosomal DNA occurs in
207 biofilms cultured under continuous flow. *P. aeruginosa* PAO1 flow biofilms were cultured in the
208 presence and absence of sterile gDNA or eDNA obtained from $PAO1_{GFP}$ (Gm^R) (at a final
209 concentration of 0.5 mg/mL) in the media influent. After 5 days the number of Gm^R colonies
210 recovered from the biofilm biomass were counted. This revealed extremely variable rates of natural
211 transformation of gDNA by cells within the biofilm biomass across multiple experiments (Fig. 1b).

212 This is not unexpected as the rate is likely to be dependent upon the time at which the natural
213 transformation event occurred. If this event occurred early in the assay, we would expect many
214 transformants recovered due to proliferation of the transformed cells. However, if transformation
215 occurred later we would expect far fewer transformants as these did not have as long to proliferate.
216 For the eDNA experiments, while some Gm^R transformants were obtained (Fig. 1c), the rate of natural
217 transformation was overall much lower than for gDNA (Fig. 1b). This may be due to the integrity of
218 the DNA as we observed via agarose gel electrophoresis that the eDNA used in these experiments
219 was quite degraded compared with the gDNA, presumably through the action of nucleases present in
220 the extracellular milieu. No Gm^R colonies were obtained for continuous flow biofilms in the absence
221 of gDNA or eDNA indicating that the gentamicin resistant cells recovered from these assays was due
222 to the presence of the exogenous chromosomal DNA. To further rule out the possibility of
223 spontaneous resistance, the presence of the mini-Tn7-Gm^R-$P_{A1/04/03}$-*egfp* at the chromosomal attTn7
224 site in the biofilm-derived Gm^R colonies was confirmed by PCR. The presence of the *gfp* gene in the
225 Gm^R colonies was also confirmed by visualisation of GFP expression using epifluorescence
226 microscopy (Fig. 2b). No GFP expression was observed in the PAO1 inoculum strain (Fig. 2a). As it
227 was not possible to directly visualise biofilms cultured in Tygon tubing, PAO1 continuous flow
228 biofilms were cultured in transparent flow cells over 5 days in the presence and absence of gDNA
229 obtained from $PAO1_{GFP}$. Epifluorescence microscopy revealed microcolonies of GFP-expressing
230 bacteria within the biofilm (Fig. 2c-f). No GFP expression was observed in the no DNA control
231 biofilms.

232

233 To further investigate natural transformation by *P. aeruginosa* we set out to determine if this process
234 with also occurring in colony biofilms on agar plates. We first grew wildtype strains PAK and PAO1
235 on LB agar overnight to form a colony biofilm on the surface of the agar. We then added 5 μg of
236 sterile DNA (or the equivalent volume of sterile water) of the plasmid pUCPSK, onto the surface of
237 the colony biofilm. After 2 hr incubation at 37°C the colony was resuspended and cells plated onto

238 media containing carbenicillin to select for transformants that had acquired the plasmid. Only colony
239 patches that had been exposed to plasmid DNA yielded $Carb^R$ colonies (Fig. 3a), whereas colony
240 patches exposed to sterile water yielded none. The transformation efficiency for PAK and PAO1 was
241 24.7 ± 10.1 and 5.8 ± 1.9 transformants/μg plasmid DNA, respectively. To confirm that the
242 carbenicillin resistant colonies had acquired pUCPSK, plasmid DNA was extracted, re-transformed
243 into *E. coli* and confirmed by sequencing. These observations indicate that a proportion of cells within
244 colony biofilms of *P. aeruginosa* are competent for natural transformation and are able to take up and
245 maintain plasmid DNA.

246

247 Given that both *P. aeruginosa* strains PAK and PAO1 appeared to be naturally competent, we wanted
248 to determine if this was also the case for two other commonly utilised lab strains (PA14 and PA103)
249 and clinical isolates. All *P. aeruginosa* strains were first confirmed to be carbenicillin sensitive prior
250 to use. Thirteen *P. aeruginosa* otitis externa and twelve cystic fibrosis (CF) lung sputum isolates were
251 assayed for the ability to uptake pUCPSK plasmid DNA in a colony biofilm. Of these, 7/12 otitis
252 externa and 6/10 CF isolates were able to uptake exogenous plasmid DNA (Fig. 3b). No $Carb^R$
253 colonies were obtained in the no plasmid DNA controls for each strain. Interestingly, transformation
254 ability varied amongst both clinical and lab strains. Of the lab strains, PA14 was the least capable of
255 natural transformation with PAK the most efficient. These results demonstrate that many lab and
256 clinical isolate strains of *P. aeruginosa* are capable of natural transformation within colony biofilms,
257 albeit with different efficiencies.

258

259 *P. aeruginosa* also expresses T4P when cultured in static nutrient broth[13]. Under these conditions *P.*
260 *aeruginosa* forms biofilms and suspended microcolony aggregates that contain eDNA[14,33]. To
261 determine if natural transformation also occurred in static broth cultures, 10 μg of sterile pUCPSK
262 plasmid DNA (or the equivalent volume of sterile water) was added to a subculture of *P. aeruginosa*

263 wildtype PAK or PAO1 and incubated statically at 37°C for 24 hrs. Cells were then recovered and
264 plated onto media containing carbenicillin to select for transformants. $Carb^R$ colonies were obtained
265 for both PAO1 and PAK under these conditions, whereas no $Carb^R$ colonies were identified in the
266 water control. As was observed with colony biofilm transformations (Fig. 3a), PAK was more
267 efficient for natural transformation of pUCPSK than PAO1 in static broth cultures, with efficiencies
268 of 24.6 ± 5.5 and 1.0 ± 0.3 transformants/μg plasmid DNA, respectively (Fig. 4a).

269

270 To determine if transformation efficiency was dependent on the amount of DNA added, we performed
271 static broth transformation assays with increasing amounts of plasmid DNA. Whilst we observed an
272 increase in the number of transformants with increasing amounts of sterile plasmid DNA added (Fig.
273 S1), the transformation efficiency (transformants/μg DNA) was relatively unchanged over the range
274 of DNA quantities used for transformation (0-30 μg) (Fig. 4b).

275

276 We were also interested in determining whether *P. aeruginosa* was also able to uptake exogenous
277 chromosomal DNA and integrate this into the chromosome in static broth cultures. To examine this,
278 chromosomal DNA from $PAO1_{GFP}$ (Gm^R) was purified from either a sterile whole cell lysate (gDNA)
279 or the total sterile eDNA from confluent agar plate culture and 15 μg added to static broth cultures of
280 PAK or PAO1 for 24 hr. Cells were recovered and cultured on agar containing gentamicin to select
281 for transformants. These assays revealed that natural transformation of gDNA occurred at a low
282 frequency for both PAK and PAO1 in static broth cultures (Fig. S2). No natural transformation with
283 sterile eDNA was observed in static broth cultures for either PAK or PAO1 (Fig. S2). As mentioned
284 above for eDNA in continuous flow biofilms, this may be due to the degraded nature of the eDNA
285 compared to the gDNA as visualised by agarose gel electrophoresis. No gentamicin resistant colonies
286 were obtained in the no DNA controls.

287

288 *P. aeruginosa* produces more T4P when cultured in static broth cultures than under shaking
289 conditions[13]. We investigated the effects on natural transformation efficiency of culturing under static
290 or shaking conditions and found that although some natural transformation was still observed under
291 shaking conditions, more transformants were obtained with static culture conditions (Fig. 5a),
292 consistent with a role of T4P in natural transformation. To directly examine the role of T4P in natural
293 transformation of *P. aeruginosa,* we added 10 µg sterile pUCPSK plasmid DNA to static broths of
294 PAK mutants defective in the production of the pilin subunit (*pilA*), in T4P assembly (*pilV*, *pilQ,*
295 *fimV*) and in T4P retraction (*pilT*). Interestingly, all T4P mutants were capable of some natural
296 transformation of pUCPSK, however a significant reduction in transformation efficiency compared
297 to wildtype PAK was observed (Fig. 5b). No $Carb^R$ colonies were identified in the no DNA controls.
298 There was no apparent difference in the transformation efficiency of mutants which either didn't have
299 any surface-assembled T4P (*pilA, pilV, pilQ, fimV*) or were unable to retract T4P (*pilT*) (Fig. 5b).
300
301 Given that wildtype *P. aeruginosa* forms aggregates in a static broth[14,33] we considered the possibility
302 that natural transformation was occurring within these aggregates in our static broth transformation
303 assay. To determine if the reduction in transformation efficiency by T4P mutants (Fig. 5b) could be
304 accounted for by the inability to form aggregates, we used microscopy to image wildtype PAK and
305 T4P mutants under static broth conditions. This revealed that mutants unable to make surface-
306 assembled T4P (*pilA, pilV, pilQ, fimV*) were also unable to form aggregates (Fig. 5c). A *pilT* mutant,
307 which has a T4P retraction defect, was however still able to form aggregates (Fig. 5c). While the total
308 number of aggregates was less than that observed for wildtype (Fig. 5c), this decrease does not
309 account for the observed decrease in natural transformation efficiency (Fig. 5b). This suggests that it
310 is the lack of functional T4P effects natural transformation and not other factors associated with
311 aggregate formation.
312

313 Overall these data suggest that in *P. aeruginosa*, T4P facilitate transport of DNA to the cell surface
314 but are not essential for natural transformation, at least of plasmid DNA. Furthermore, these
315 observations indicate that during natural transformation in *P. aeruginosa* the PilQ secretin pore is not
316 absolutely required for translocation of plasmid DNA across the outer membrane.

317

318 **Discussion**

319 Here we have demonstrated, in contrast to current dogma, that *P. aeruginosa* is capable of natural
320 transformation of both plasmid and chromosomal DNA under conditions that promote the expression
321 of T4P and eDNA production, such as in static broth cultures and biofilms. We found that whilst T4P
322 appear to be involved in facilitating plasmid DNA uptake, T4P are not absolutely required for natural
323 transformation of plasmid DNA in *P. aeruginosa.* Furthermore, our data suggests that the PilQ
324 secretin pore is not absolutely required for translocation of plasmid DNA across the outer membrane
325 in this organism.

326

327 It is thought that separate uptake mechanisms for plasmid or chromosomal DNA are present in *E.*
328 *coli*[8]. While natural transformation of chromosomal DNA and integration into the genome has not
329 been demonstrated in *E. coli*, T4P/competence pseudopilus and DNA translocation machinery
330 homologs have been shown to be responsible for uptake of chromosomal DNA for use as a nutrient
331 source[34]. Natural plasmid transformation in *E. coli* does not utilise the competence pseudopilus
332 uptake system although the precise mechanism for this process has not been determined[9]. At this
333 stage we cannot rule out the possibility that there are two mechanisms in *P. aeruginosa* involved in
334 the natural transformation of plasmid DNA or chromosomal DNA.

335

336 The finding that *P. aeruginosa* is capable of natural transformation is a paradigm shift in our
337 understanding of how this pathogen acquires genetic diversity. Indeed, recombination has been

338 identified as a major means of genetic diversity in *P. aeruginosa* cystic fibrosis (CF) lung isolates
339 although the source of DNA and the mechanism of HGT was not determined[35]. Natural
340 transformation may be an important mechanism for the acquisition of antibiotic resistance and
341 virulence genes in this ESKAPE pathogen and a significant contributor to the rapid increase in the
342 number of multidrug resistant *P. aeruginosa* strains that are an emerging problem worldwide.

343

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351

352 ## Author information

353 **Contributions**

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

357

358 ## Ethics declarations

359 The authors declare no conflict of interest.

360

361

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437 opportunistic human pathogen Pseudomonas aeruginosa. *Genome Biol. Evol.* **7,** 18–34 (2014).
438

439 **Tables and Figures**

440 **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

441 *Using *P. aeruginosa* PAO1 gene nomenclature

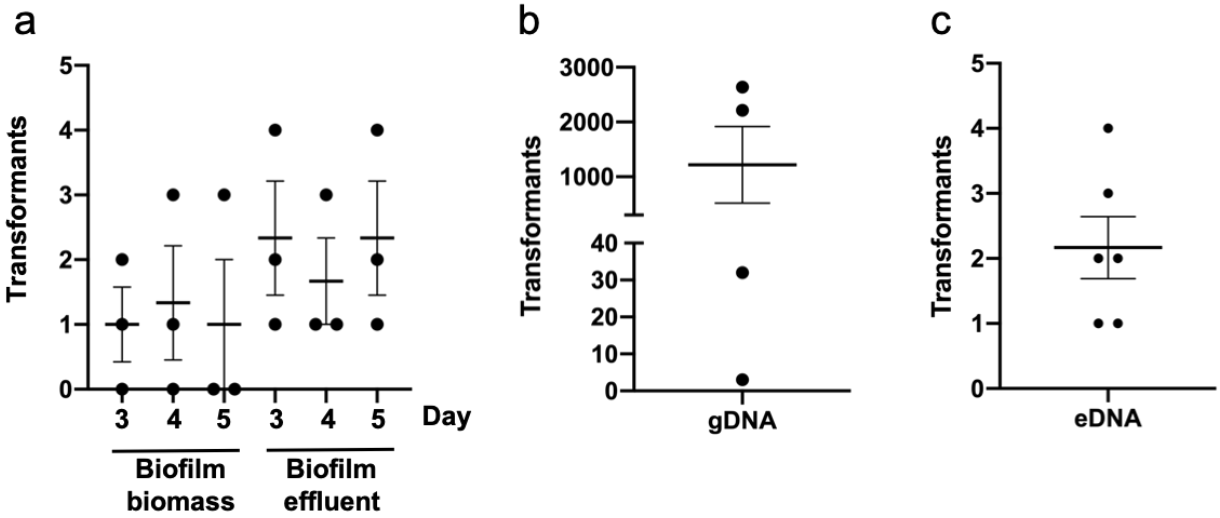

Figure 1. *P. aeruginosa* is capable of natural transformation in continuous flow biofilms. Carbenicillin or gentamicin resistant transformants obtained from continuous flow biofilms of PAO1 in Tygon tubing. For (a) pUCPSK was added to the media influent (at a final concentration of 1 μg/mL) with either the biofilm biomass or effluent harvested and plated on carbenicillin selective agar plates on the indicated day. For (b-c) sterile gDNA or eDNA obtained from $PAO1_{GFP}$ (Gm^R) (at a final concentration of 0.5 mg/mL) was added to the media influent and the biofilm biomass harvested and plated on gentamicin selective agar plates after 5 days. The mean of each set of technical triplicates was calculated to give an n≥3 which is presented as mean ± S.E.M.

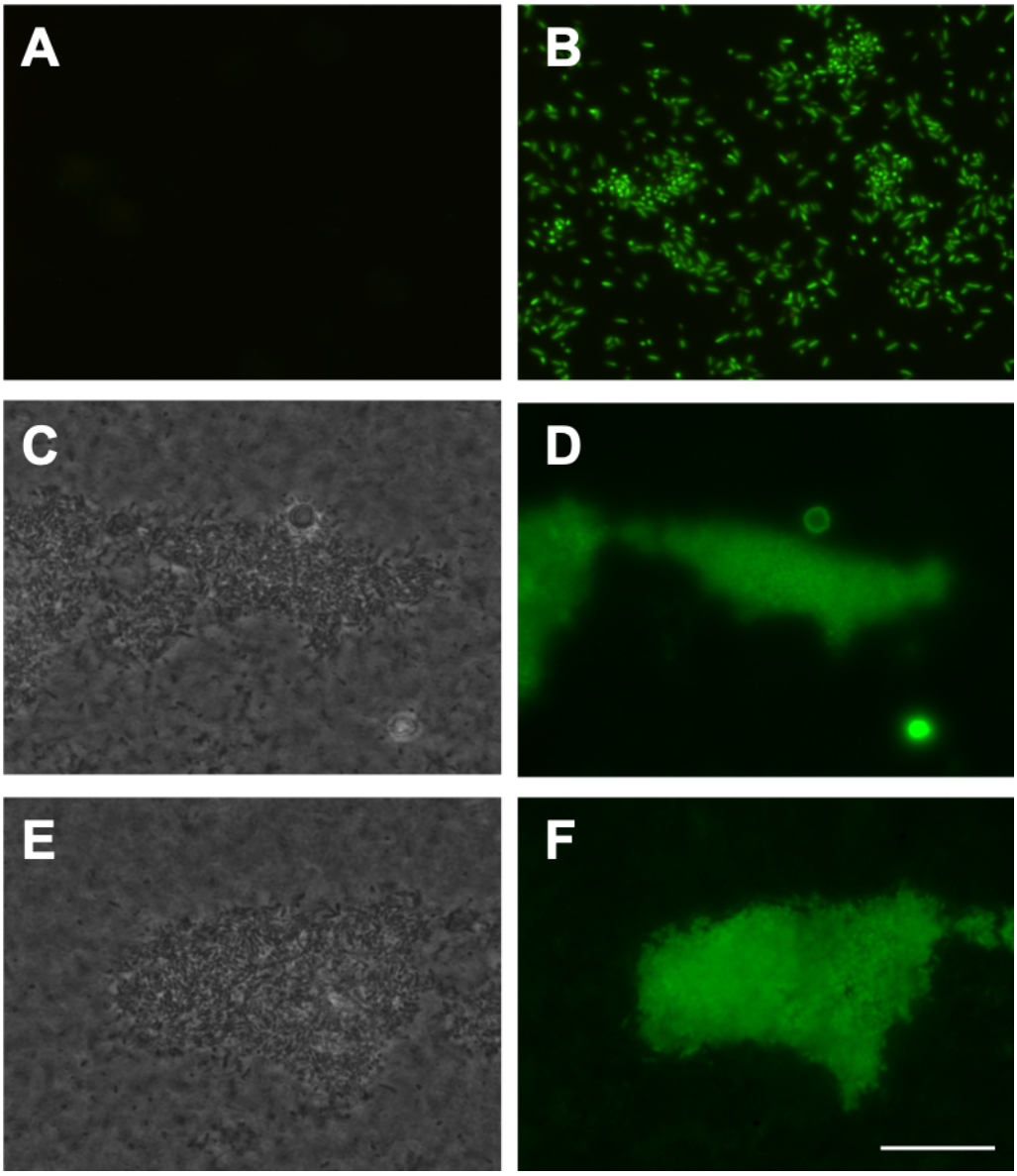

Figure 2. *P. aeruginosa* is capable of natural transformation of exogenous chromosomal DNA. The inoculum PAO1 strain did not express GFP (a). Sterile $PAO1_{GFP}$ gDNA (at a final concentration of 0.5 mg/mL) was added to the media influent of continuous flow biofilms of PAO1 in Tygon tubing (b) or flow 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). The biofilm biomass from PAO1 flow cells was visualized by phase contrast (c, e) or epifluorescence microscopy (d, f) which showed the presence of representative biofilm microcolonies expressing GFP. Scale bar 100 μm.

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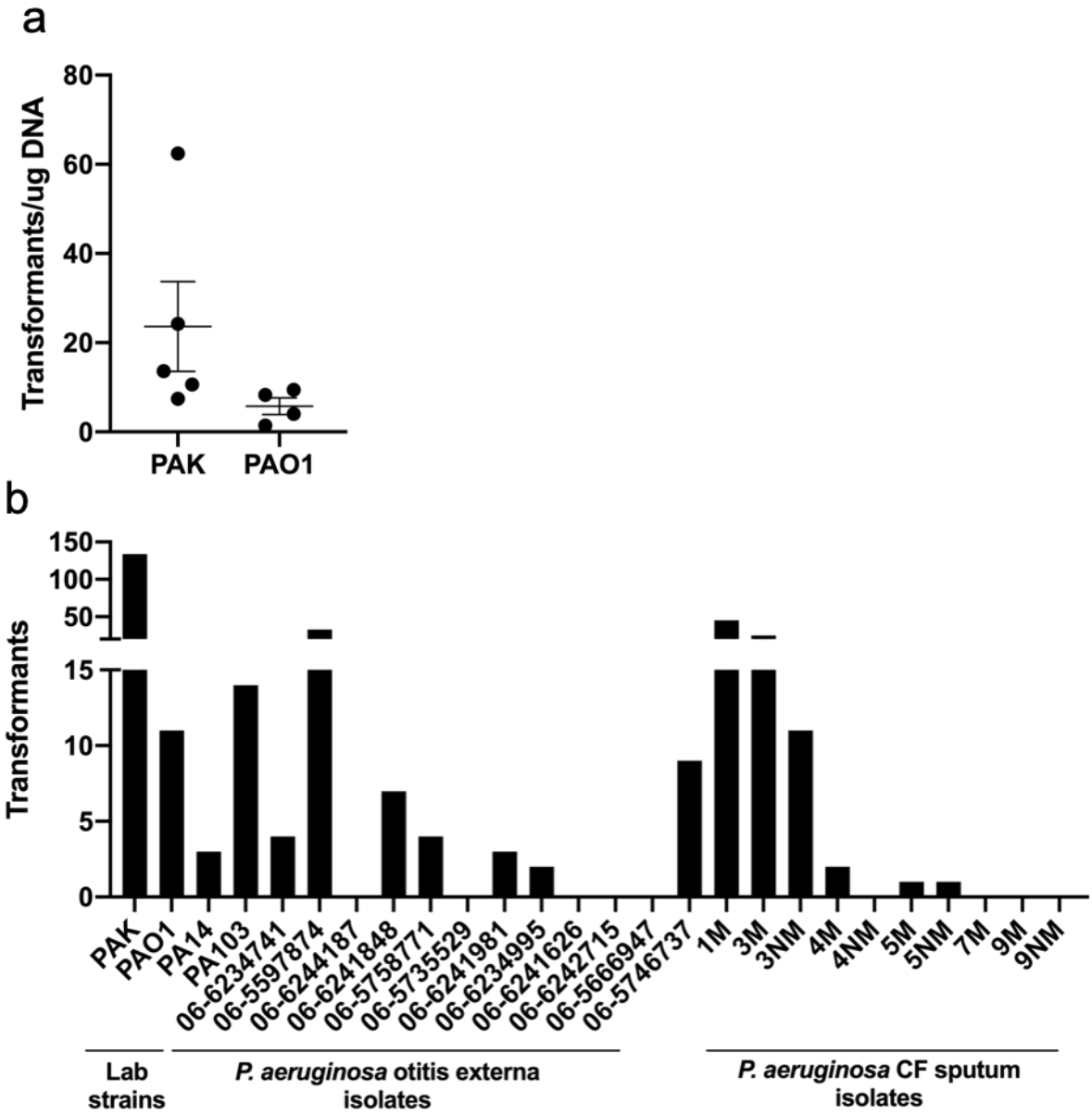


463

464 **Figure 3. Lab and clinical strains of *P. aeruginosa* are capable of natural transformation of**

465 **plasmid DNA within colony biofilms.** pUCPSK (5 µg) DNA was applied to colony biofilms of (a)

466 *P. aeruginosa* PAK or PAO1, or (b) *P. aeruginosa* lab or clinical strains and incubated for 2 hr.

467 Cells were then harvested and the number of carbenicillin resistant transformants determined by

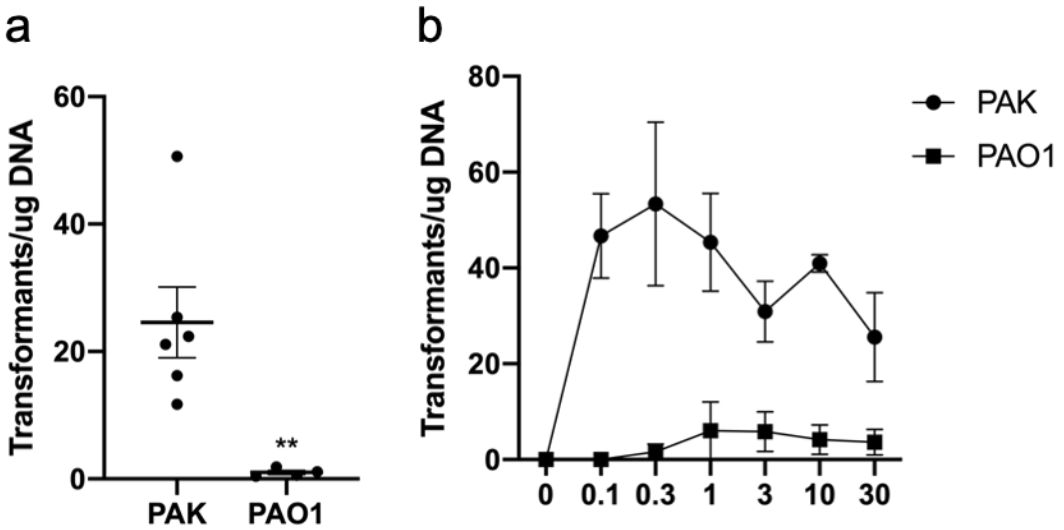
468 spread plating on selective media. For (a) the mean of each set of technical triplicates was

469 calculated to give an n≥4 which is presented as mean ± S.E.M. For (b) the values presented are

470 from n=1. For the CF sputum isolates the designation M refers to mucoid phenotype, NM is non-

471 mucoid.

472



473

474 **Figure 4. *P. aeruginosa* is capable of natural transformation of plasmid DNA in static broth**

475 **cultures.** Carbenicillin resistant transformants obtained from static broth cultures incubated for 24

476 hr with (a) 10 µg pUCPSK or (b) 0-30 µg pUCPSK. The mean of each set of technical triplicates

477 was calculated to give an n≥3 which is presented as mean ± S.E.M. For (a) ** P <.005*;* Mann-

478 Whitney *U*-test compared to PAK.

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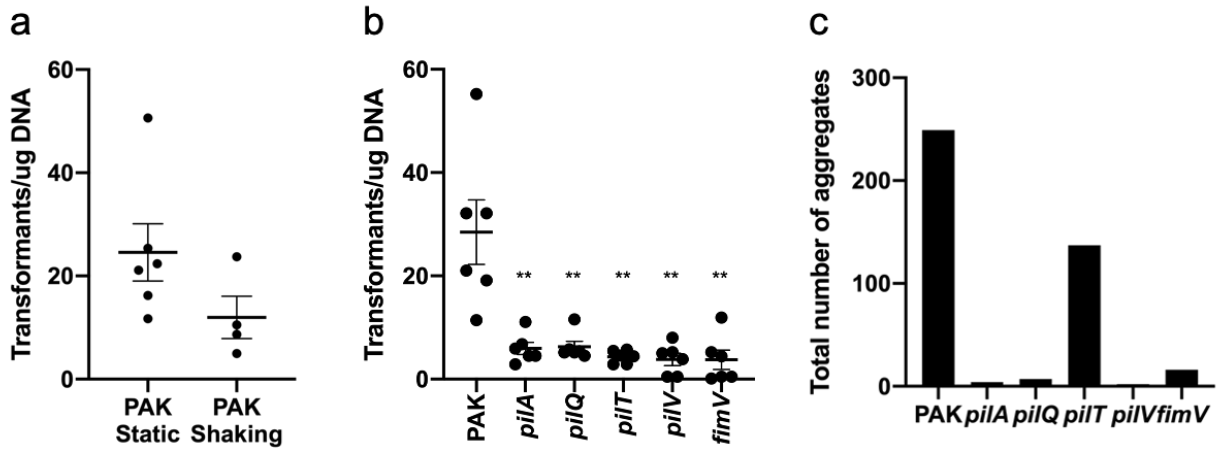


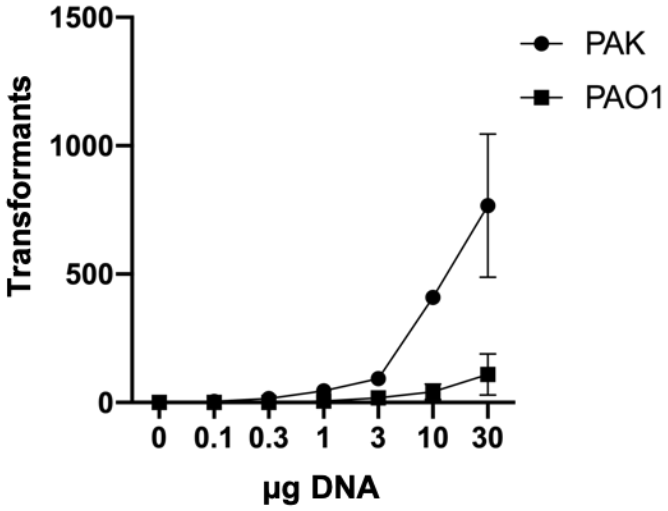
481

482 **Figure 5. T4P are not absolutely required for natural transformation of plasmid DNA.** Static
483 (a-c) or shaking (a) broth cultures were incubated for 24 hr with 10 µg pUCPSK and the number of
484 (a-b) carbenicillin resistant transformants or (c) aggregates observed determined. For (a-b) the mean
485 of each set of technical triplicates was calculated to give an n≥3 which is presented as mean ±
486 S.E.M. For (a) $P > .05$*;* Mann-Whitney *U*-test. For (b) ** $P < .005$*;* Mann-Whitney *U*-test compared
487 to PAK. For (c) the total number of aggregates from 60 random fields imaged on 3 separate days
488 (20 fields/day) is presented.

489

490 **Supplementary Figures**

491



492

493 **Supplementary Figure 1. Transformant numbers increase with plasmid DNA concentration**

494 **in static broth cultures.** Static broth cultures of PAK or PAO1 with 10 µg pUCPSK added were

495 incubated and the numbers of carbenicillin resistant transformants determined after 24 hr. The mean

496 of each set of technical triplicates was calculated to give an n≥3 which is presented as mean ±

497 S.E.M.

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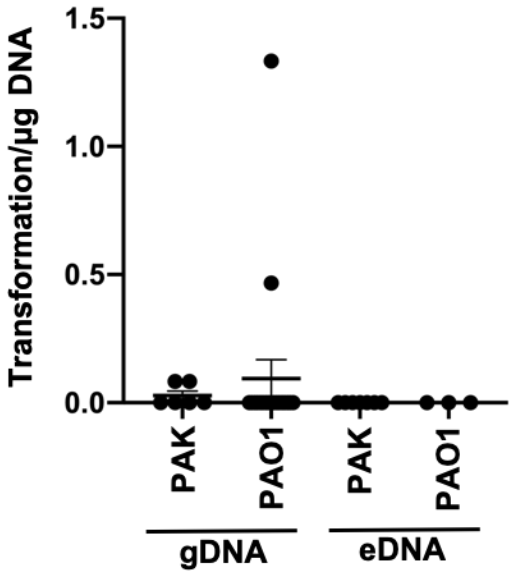

500

501 **Supplementary Figure 2. Natural transformation of gDNA and eDNA is inefficient in static**

502 **broth cultures of *P. aeruginosa*.** Static broth cultures of PAK or PAO1 with 15 µg gDNA or

503 eDNA were incubated and the numbers of gentamicin resistant transformants determined after 24

504 hr. The mean of each set of technical triplicates was calculated to give an n≥3 which is presented as

505 mean ± S.E.M.