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*.

Introduction

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

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

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

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

Results

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

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

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

To confirm that *P. aeruginosa* is indeed capable of natural transformation and to rule out any possibility of HGT through transduction or conjugation, we performed a series of experiments to follow the uptake of purified, sterile exogenous DNA. We first grew wildtype strains PAK and PAO1 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

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

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

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

Discussion

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

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

Methods

Strains, plasmids and growth conditions

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

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

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

Bioinformatics and data and statistical analyses

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

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

Colony biofilm assay

Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. A 10 µL plastic loop was used to generate a 1 cm patch of the overnight culture on a dry 1% LBA plate. This was then incubated overnight at 37°C. The next day 10 µL of DNA at the indicated concentration was spotted onto the established colony biofilm and allowed to dry into the cells. The plate was then incubated with the agar downwards at 37°C for the indicated time. After incubation the colony biofilm was harvested from the plate into 1 mL LB, vortexed to resuspend and then incubated at 37°C for 30 min to fully resuspend the cells. The cell suspension was then spread plated between two 150 mm LBA plates with appropriate antibiotic selection and incubated for 24 hr at 37°C.

Static broth assay

Overnight cultures of *P. aeruginosa* were grown in 2 ml CAMHB at 37°C, shaking at 250 rpm. 40µL of overnight culture was subcultured into 2 ml fresh CAMHB with DNA added at the indicated concentration. The media, cells and DNA were then mixed and incubated at 37°C statically for 24 hr. Note for the shaking broth assay the same setup was used however the culture was incubated with shaking at 250 rpm. In both cases after incubation the cell suspension was then spread plated between two 150 mm LBA plates with appropriate antibiotic selection and incubated for 24 hr at 37°C.

Isolation of DNA for use in continuous flow biofilm assays

Chromosomal DNA (gDNA) was purified from PAO1Tn7::*gfp-aac1* cells using the Epicentre® Masterpure DNA purification kit. Extracellular DNA (eDNA) was purified from a confluent lawn of PAO1Tn7::*gfp-aac1* cultured overnight on MacConkey agar containing 5% (v/v) glycerol. Bacteria were suspended in sterile phosphate buffered saline (PBS), centrifuged and the supernatant filtered through 0.2 µm PES membrane. eDNA present in the supernatant was further purified by removal of proteins and ethanol precipitation as reported previously[31]. Sterility of all DNA samples was 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 $P_{ser\text{-}up}$ (5'CGAGTGGTTTAAGGCAACGGTCTTGA3') and $P_{ser\text{-}down}$
307 (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').

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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.

References

1. O'Neill, J. *Tackling drug-resistant infections globally: Final report and recommendations*. (UK Government and Wellcome Trust, 2016).
2. Pallen, M. J. & Wren, B. W. Bacterial pathogenomics. *Nature* **449,** 835–842 (2007).
3. Davison, J. Genetic exchange between bacteria in the environment. *Plasmid* **42,** 73–91 (1999).
4. Dubnau, D. & Blokesch, M. Mechanisms of DNA uptake by naturally competent bacteria. *Annu. Rev. Genet.* (2019). doi:10.1146/annurev-genet-112618-043641
5. Ellison, C. K. *et al.* Retraction of DNA-bound type IV competence pili initiates DNA uptake

during natural transformation in Vibrio cholerae. *Nat. Microbiol.* **3,** 773–780 (2018).

6. Chen, I. & Dubnau, D. DNA uptake during bacterial transformation. *Nat. Rev. Microbiol.* **2,** 241–249 (2004).

7. Ibáñez de Aldecoa, A. L., Zafra, O. & González-Pastor, J. E. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. *Front. Microbiol.* **8,** 1390 (2017).

8. Kung, V. L., Ozer, E. A. & Hauser, A. R. The accessory genome of Pseudomonas aeruginosa. *Microbiol. Mol. Biol. Rev.* **74,** 621–641 (2010).

9. Shen, K. *et al.* Extensive genomic plasticity in Pseudomonas aeruginosa revealed by identification and distribution studies of novel genes among clinical isolates. *Infect. Immun.* **74,** 5272–5283 (2006).

10. Carlson, C. A., Pierson, L. S., Rosen, J. J. & Ingraham, J. L. Pseudomonas stutzeri and related species undergo natural transformation. *J. Bacteriol.* **153,** 93–99 (1983).

11. Whitchurch, C. B. in *Pseudomonas Volume 4 Molecular Biology of Emerging Issues* (ed. J. L. Ramos and R. C. Levesque) Springer USA: 139-188 (2006).

12. Allesen-Holm, M. *et al.* A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* **59,** 1114–1128 (2006).

13. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. Extracellular DNA required for bacterial biofilm formation. *Science* **295,** 1487 (2002).

14. Gloag, E. S. *et al.* Self-organization of bacterial biofilms is facilitated by extracellular DNA. *Proc. Natl. Acad. Sci. USA* **110,** 11541–11546 (2013).

15. Turnbull, L. *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7,** 11220 (2016).

16. Dettman, J. R., Rodrigue, N. & Kassen, R. Genome-wide patterns of recombination in the opportunistic human pathogen Pseudomonas aeruginosa. *Genome Biol. Evol.* **7,** 18–34 (2014).

17. Takeya, K. & Amako, K. A rod-shaped Pseudomonas phage. *Virology* **28,** 163–165 (1966).

18. Rahme, L. G. *et al.* Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268,** 1899–1902 (1995).
19. Liu, P. V. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. *J. Infect. Dis.* **128,** 506–513 (1973).
20. Klausen, M. *et al.* Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48,** 1511–1524 (2003).
21. Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43,** 59–72 (2000).
22. Watson, A. A., Mattick, J. S. & Alm, R. A. Functional expression of heterologous type 4 fimbriae in Pseudomonas aeruginosa. *Gene* **175,** 143–150 (1996).
23. Martin, P. R., Hobbs, M., Free, P. D., Jeske, Y. & Mattick, J. S. Characterization of pilQ, a new gene required for the biogenesis of type 4 fimbriae in Pseudomonas aeruginosa. *Mol. Microbiol.* **9,** 857–868 (1993).
24. Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V. & Mattick, J. S. Characterisation of a Pseudomonas aeruginosa twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* **101,** 33–44 (1991).
25. Alm, R. A. & Mattick, J. S. Identification of a gene, pilV, required for type 4 fimbrial biogenesis in Pseudomonas aeruginosa, whose product possesses a pre-pilin-like leader sequence. *Mol. Microbiol.* **16,** 485–496 (1995).
26. Semmler, A. B., Whitchurch, C. B., Leech, A. J. & Mattick, J. S. Identification of a novel gene,. *Microbiology (Reading, Engl.)* **146 (Pt 6),** 1321–32 (2000).
27. Watson, A. A., Alm, R. A. & Mattick, J. S. Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172,** 163–164 (1996).
28. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410 (1990).

29. Winsor, G. L. *et al.* Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res.* **44,** D646-53 (2016).

30. Cain, A. K. *et al.* Complete Genome Sequence of Pseudomonas aeruginosa Reference Strain PAK. *Microbiol. Resour. Announc.* **8,** (2019).

31. Miller, S. A., Dykes, D. D. & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16,** 1215 (1988).

Tables and Figures

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

*Using *P. aeruginosa* PAO1 gene nomenclature

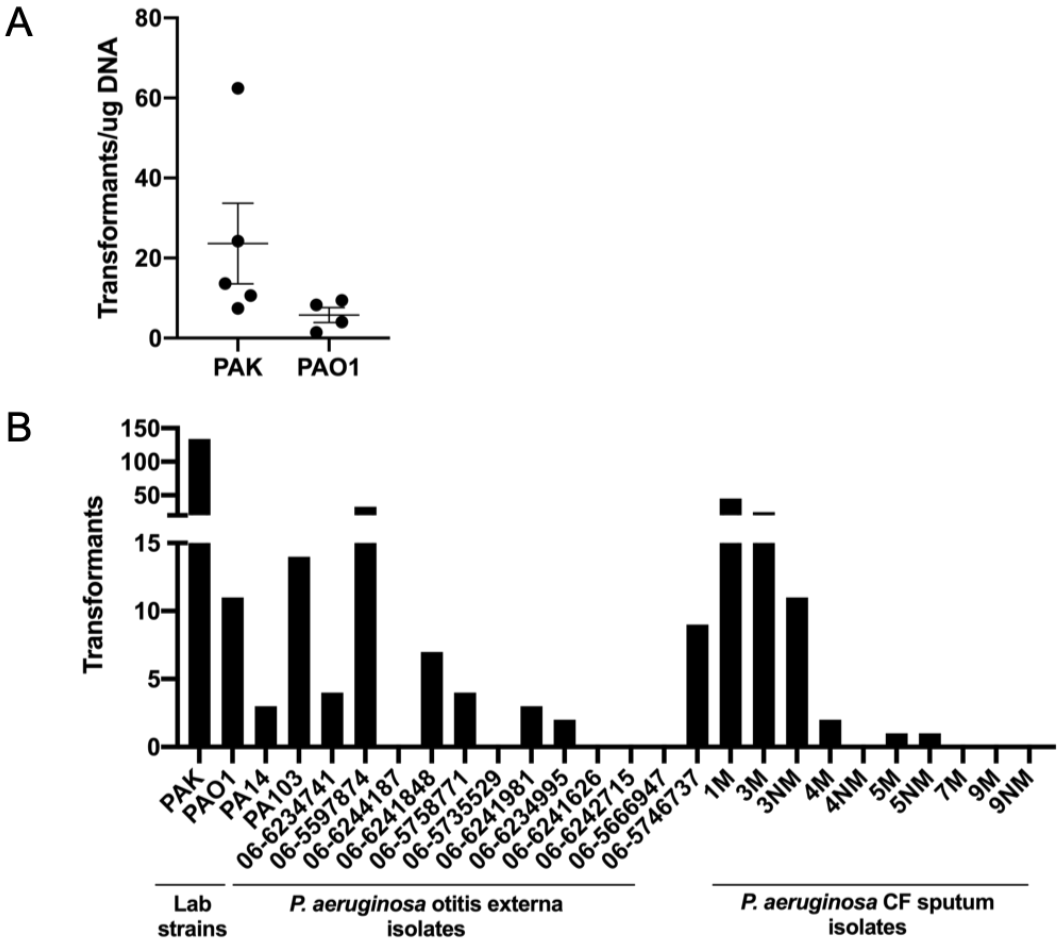

Figure 1. Lab and clinical strains of *P. aeruginosa* are capable of natural transformation of plasmid DNA within colony biofilms. pUCPSK DNA was applied to colony biofilms of (A) *P. aeruginosa* PAK or PAO1, or (B) *P. aeruginosa* lab or clinical strains and incubated for 2 hr. Cells were then harvested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

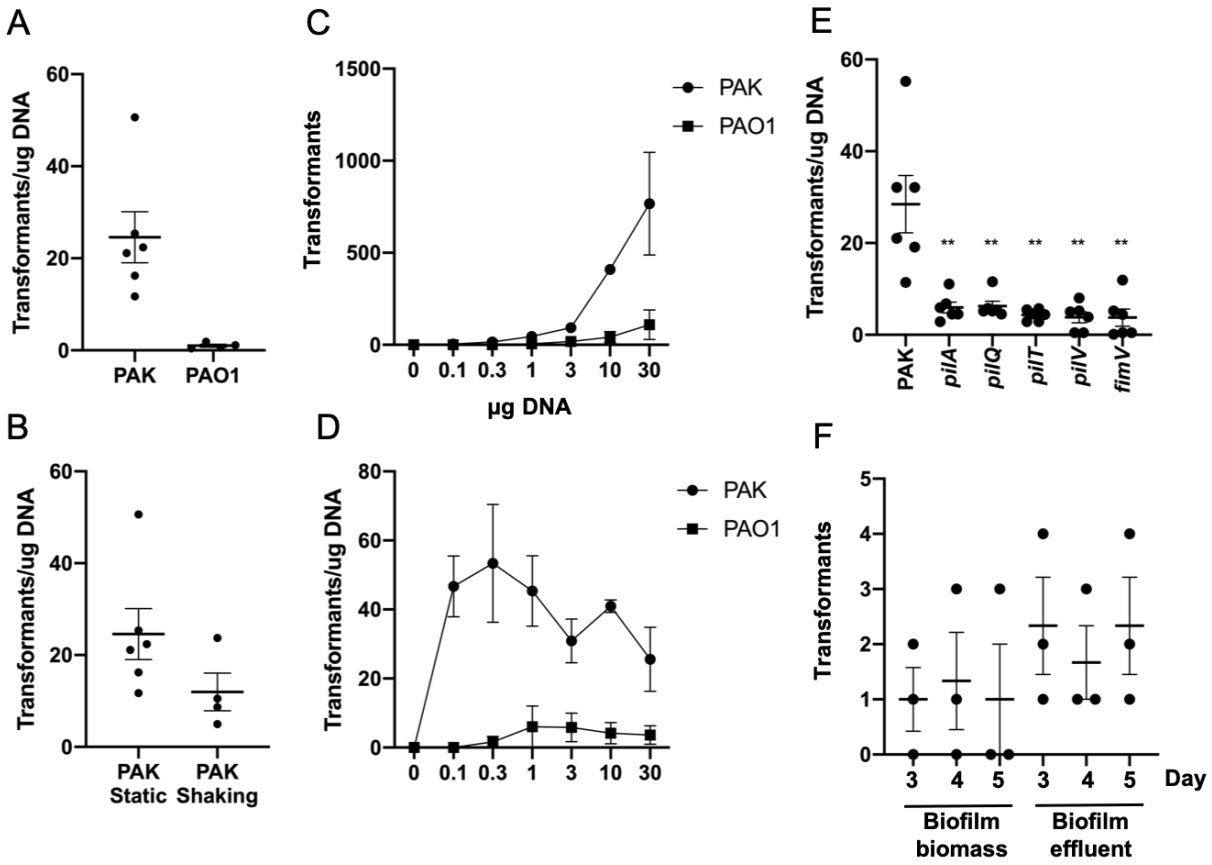

Figure 2. *P. aeruginosa* is capable of natural transformation of plasmid DNA in broth cultures and continuous flow biofilms. Carbenicillin resistant transformants obtained from static (A, C-E) or shaking (B) broth cultures incubated for 24 hr with pUCPSK or (F) from biofilm biomass or effluent of PAO1 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.

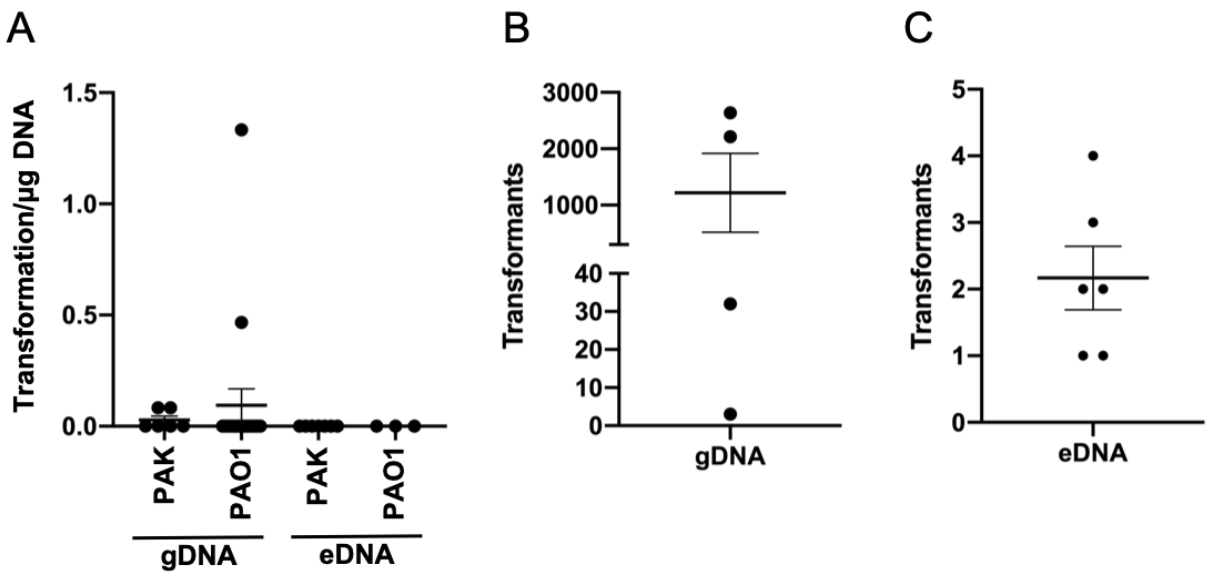

Figure 3. *P. aeruginosa* is capable of natural transformation of chromosomal DNA. Sterile gDNA or eDNA was added to (A) static broth cultures of PAK or PAO1 and incubated for 24 hr or (B) in the media influent of continuous flow biofilms of PAO1 in Tygon tubing and incubated for 5 days. The mean of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

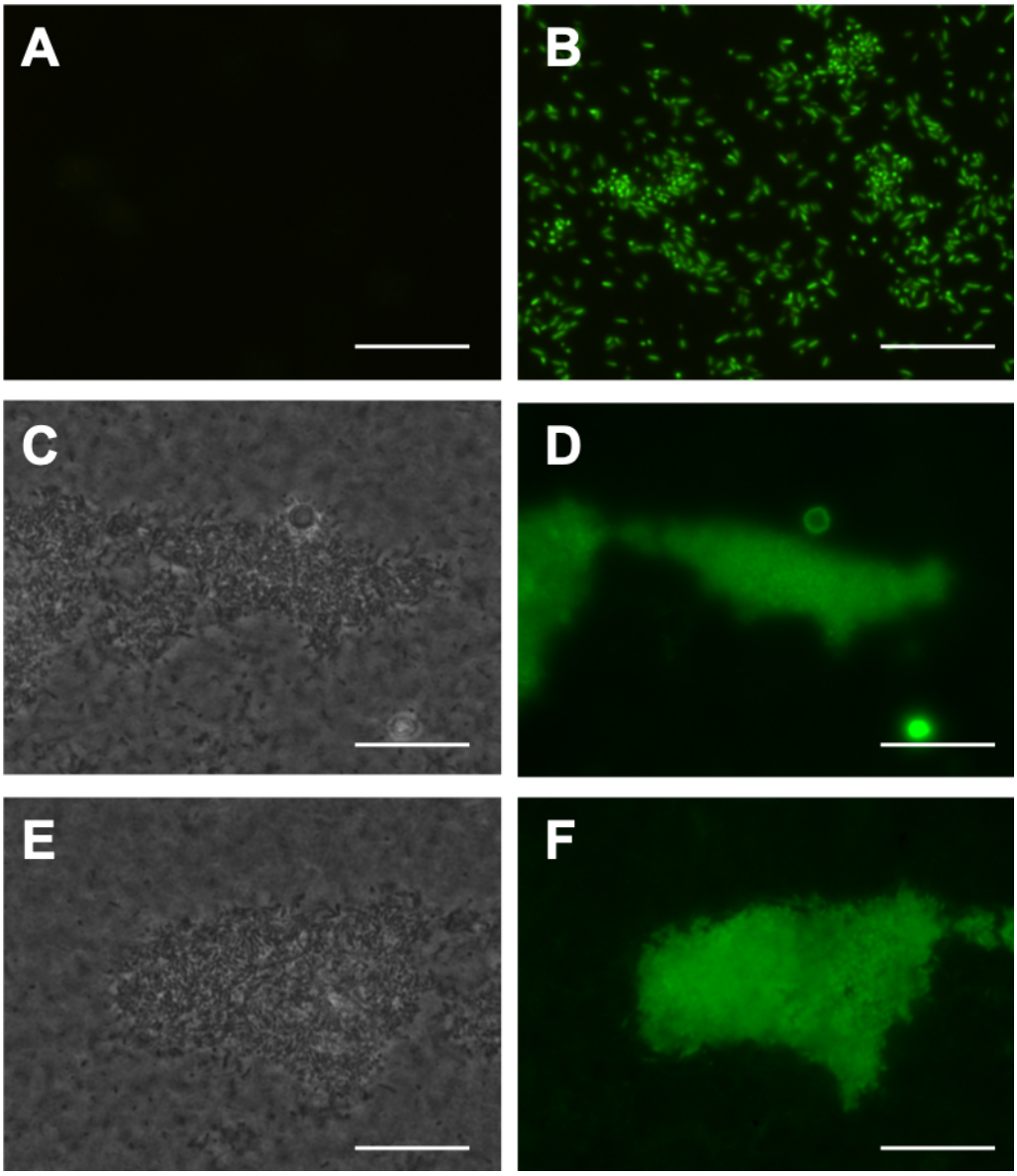

Figure 4. *P. aeruginosa* cultured in continuous flow biofilms can stably integrate and express antibiotic resistance and *gfp* gene cassettes by natural transformation of exogenous chromosomal DNA. Sterile $PAO1_{GFP}$ gDNA was added to the media influent of continuous flow biofilms of PAO1 in Tygon 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.