DNA-uptake pilus of *Vibrio cholerae* capable of kin-discriminated auto-aggregation

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Abstract

Natural competence for transformation is a widely used and key mode of horizontal gene transfer that can foster rapid bacterial evolution. Competent bacteria take-up DNA from their environment using Type IV pili, a widespread and multi-purpose class of cell surface polymers. However, how pili facilitate DNA-uptake has remained unclear. Here, using direct labelling, we show that in the Gram-negative pathogen *Vibrio cholerae* DNA-uptake pili are highly dynamic and that they retract prior to DNA-uptake. Unexpectedly, these pili can self-interact to mediate auto-aggregation of cells into macroscopic structures. This phenotype is conserved in disease causing pandemic strains. However, extensive strain-to-strain variability in the major pilin subunit PilA, present in environmental isolates, controls the ability of pili to interact without affecting transformation. We go on to show that interactions between pili are highly specific, enabling cells producing pili composed of different PilA subunits to discriminate between one another. On chitin surfaces, a natural habitat of *V. cholerae*, pili connect cells within dense networks, suggesting a model whereby DNA-uptake pili function to promote inter-bacterial interactions during surface colonisation. Moreover, our results provide evidence that type IV pili could provide a simple and potentially widespread mechanism for bacterial kin recognition.

Keywords

Auto-aggregation, DNA uptake, natural transformation, pilus dynamics, type IV pilus, *Vibrio cholerae*

32 **Introduction**

33 How bacteria physically sense and interact with their environment is a fundamental problem
34 in biology. Type IV pili (T4P) are cell surface polymers ideally suited to this task[1,2]. Composed
35 of a single major pilin and assembled by widely distributed and conserved machinery, T4P
36 exhibit extensive functional versatility, with roles in motility, DNA-uptake, surface sensing
37 and adhesion[3-5]. Consequently, T4P are critical virulence factors for a number of important
38 human pathogens including *Vibrio cholerae*, which causes the pandemic diarrhoeal disease
39 cholera[6]. In Gram-negative bacteria pilins are processed at the inner-membrane, extracted
40 by the assembly machinery and polymerised into a helical pilus fibre that exits the cell
41 surface through a gated outer-membrane pore; the secretin[7-11]. A key feature of T4P is their
42 ability to undergo dynamic cycles of extension and retraction[12,13], powered by the action of
43 dedicated extension (*e.g.* PilB) and retraction (*e.g.* PilT) ATPases, which either add or liberate
44 pilin subunits at the base[14,15]. These dynamics are essential for many T4P functions *e.g.*
45 twitching-motility[11,12]. Thus, understanding how T4P function may yield insights valuable for
46 understanding mechanisms of environmental survival and pathogenesis.

47 Despite their multifunctional potential, pandemic strains of *V. cholerae* typically
48 encode three distinct T4P systems – two of which are well characterised. First, toxin co-
49 regulated pili (TCP) serve a dual role as both a receptor for CTXϕ bacteriophage[16], which
50 carries the cholera toxin genes, and as the primary human colonisation factor with multiple
51 essential roles in infection involving adhesion and auto-aggregation on the intestinal cell
52 surface[17-19]. Second, Mannose-sensitive haemagglutinin (MSHA) pili are involved in surface
53 sensing and attachment and thus, are important in the initiation of biofilm formation[20-24].
54 Third, in its natural aquatic environment *V. cholerae* often associates with chitinous
55 surfaces[25], which are nutritious, foster biofilm formation and likely play a role in
56 environmental dissemination and transmission to humans[26-29]. Chitin utilisation triggers
57 competence for natural transformation[30], a widely used mode of horizontal gene transfer

58 that allows bacteria to take up DNA from their environment, and which can thus, foster
59 rapid bacterial evolution[31]. This requires the production of the Chitin-Regulated (ChiRP) or
60 DNA-uptake pilus[30].

61 We previously showed DNA-uptake pili form *bona fide* pili composed of the major
62 subunit PilA and that transformation was dependent on the presumed retraction ATPase
63 PilT[32]. However, the pilus itself is not sufficient for transformation and requires the
64 concerted action of a periplasmic DNA-binding protein, ComEA[32,33]. Upon receipt of
65 transforming DNA ComEA switches from a diffuse to focal localisation[33,34]. These findings,
66 together with work in other organisms, led to a model in which pilus retraction facilitates
67 DNA entry into the periplasm[35], wherein ComEA acts as 'ratchet' to pull in the remaining
68 DNA[33]. Subsequently, DNA transport across the inner-membrane occurs via a spatially
69 coupled channel, ComEC[34,36]. Though this model is well supported by genetic experiments[32]
70 and the similarly combined action of T4P and ComEA in other organisms[37,38], direct evidence
71 is lacking.

72 Here, we visualised the DNA-uptake pilus directly using a cysteine labelling
73 approach, which was recently validated as a tool for labelling pili[39]. As predicted, we
74 demonstrate that the pili are highly dynamic and that these dynamics are PilT-dependent.
75 Unexpectedly, however, we discovered that DNA-uptake pili are also capable of self-
76 interacting, which in liquid culture results in a strong auto-aggregation phenotype.
77 Variability in PilA controls this activity but has no affect on transformation. Remarkably,
78 specific interactions allow pili composed of different PilA subunits to distinguish between
79 one another, enabling a simple mechanism for kin recognition.

80

81 **Results**

82 **Direct observation of pilus dynamics by cysteine labelling**

83 To avoid the limitations imposed by immuno-fluorescent methods, we employed a cysteine
84 labelling approach using a thiol-reactive dye[39,40]. PilA cysteine variants were created along
85 the length of the surface exposed αβ-loop[9,11] and tested for functionality using a previously
86 validated chitin-independent transformation system in which competence induction is
87 arabinose-inducible[41] (Tn*tfoX*; see methods) (Fig. 1A, B and Fig. S1A). A variant, PilA[S67C],
88 was identified that relative to the unmodified parent is fully transformable (Fig.1A),
89 produced at similar levels (Fig. S1B), and does not affect pilus assembly (Fig. S1C), as assayed
90 by a classical shearing method. Since other variants exhibited functional defects they were
91 not studied further.

92 When stained, competent cells producing PilA[S67C] exhibited visible pili (Fig. 1C).
93 On average 27 ± 3 % cells were piliated, with the majority of these displaying 1 or 2 pili per
94 cell (Fig. 1H and I). The length of pili was clustered around 1-2 µm, though pili up to 10 µm in
95 length were also observed (Fig. 1J). Numerous detached pili were also evident in the growth
96 media. Intriguingly, these pili frequently appeared to self-interact, forming large structures
97 composed of networks of pili (Fig. S2A). When examined by time-lapse microscopy cells
98 exhibited rapid pilus dynamics, with multiple assembly and retraction events immediately
99 apparent (Fig. 1K; Movie S1-4). Indeed, within the 1 minute time frame studied 66 ± 3 % cells
100 exhibited pili, with most cells producing 1-2 pili per minute (Fig. 1M). Notably, a small
101 subpopulation of cells was even more dynamic, elaborating ≥5 pili per minute. Consistent
102 with this dynamic behaviour, and in support of the hypothesis that pilus retraction precedes
103 DNA-uptake, when cells were provided with purified genomic DNA it was possible to
104 concurrently visualise pilus retraction followed by DNA-uptake, as monitored by the re-
105 localisation of ComEA-mCherry, which binds incoming DNA in the periplasm (Fig. S3; Movie
106 S5).

107 As expected, deletion of components required for pilus assembly (*e.g.* the assembly
108 ATPase PilB or the secretin PilQ) abolished piliation (Fig. 1D and E). However, despite the
109 absence of obvious pili the cell body still stained. Control experiments in which PilA[S67C],
110 but not PilA[WT], was produced independently of the normal assembly machinery also
111 stained similarly (Fig. S1D), suggesting that this effect results from the dye being taken up
112 non-specifically and retained by the inner-membrane pool of PilA[S67C].

113 As in other species *V. cholerae* encodes two potential retraction ATPases, PilT and
114 PilU. Deletion of *pilU* did not affect piliation (Fig. 1F, H and I), consistent with its
115 dispensability for transformation[32]. In contrast, cells lacking the presumed retraction ATPase
116 PilT were hyper-piliated, with essentially all cells displaying multiple static pili (Fig. G-I;
117 Movie S6-7). Taken together with the dynamics described above, these data are consistent
118 with the presence of multiple assembly complexes scattered across the cell, as previously
119 predicted based on the mobility of PilB and the presence of multiple PilQ foci[32,42]. This might
120 normally serve to facilitate rapid switching of pilus location or else might reflect a need to
121 produce multiple pili under certain conditions. Unexpectedly, cells blocked for retraction
122 were often grouped into small clusters within dense networks of pili as well as occasionally
123 large aggregates of pili-encased cells (Fig. S2B and C). Indeed, when grown in liquid culture
124 cells appeared to auto-aggregate.

125

126 **Competent cells auto-aggregate in the absence of pilus retraction**

127 To test directly whether the aggregation phenotype was an inherent property of hyper-
128 piliated cells or merely an artefact introduced by the cysteine variant, cells containing
129 unmodified *pilA* were examined (Fig. 2A-C). Remarkably, aggregation occurred specifically in
130 competence-induced cells lacking *pilT*, with the formation of large, multi-layered spherical
131 aggregates on the order of 25-100 µm, which went on to form macroscopic aggregates that
132 rapidly sediment (Fig. 2B, +Ara). Quantification of the ratio of cells remaining in solution to

133 those in the settled aggregates (Fig. 2D) revealed that upon competence induction, ≥90% of
134 the retraction deficient cells were present in aggregates (Fig. 2D). Notably, strains producing
135 the PilA[S67C] variant used for labelling behave similarly (Fig. 1B).

136 To rule out the possibility that the deletion of *pilT* has either a polar or otherwise
137 non-specific effect, an intact copy of *pilT* driven by its native promoter was placed at an
138 ectopic locus. Importantly, complementation of Δ*pilT* was sufficient to fully restore the
139 ~1000-fold defect in transformation frequency and abolished the aggregation phenotype
140 (Fig. 2D and E). Complementation also fully counteracted the enhanced motility phenotype
141 of Δ*pilT* (Fig. S4), which occurs due to loss of function in the adhesive MSHA pilus and is in
142 agreement with the previously established shared role of PilT in MSHA pilus function[24,43].

143 Time-course experiments indicated that aggregation occurs abruptly, suggesting
144 that rather than gradually increasing in size, large aggregates form via the accumulation of
145 smaller ones (Fig. S5A and B). In support of this hypothesis, when two otherwise isogenic
146 Tn*tfoX* Δ*pilT* strains, one of which constitutively produces GFP, were allowed to aggregate
147 together they formed well-mixed aggregates (Fig. S5E). If, however, they were allowed to
148 aggregate separately before being mixed (Fig. S5C and D), they formed large composite
149 aggregates consisting of a patchwork of labelled and unlabelled cells (Fig. S5F). Finally, the
150 aggregates remained stable and did not disperse, even after prolonged culture overnight.

151

152 **Aggregates form via pilus-pilus interactions**

153 The data so far suggest that hyper-piliated cells auto-aggregate via their DNA-uptake
154 pili. Indeed, deletions affecting DNA-uptake pilus assembly (*pilQ* and *pilA*) were sufficient to
155 abolish aggregation (Fig. 2F). In contrast, deletions targeting the assembly of TCP (*tcpA*) and
156 MSHA (*mshA*) pili, the *Vibrio* polysaccharide matrix (*vpsA*) required for biofilm formation[44] or
157 the flagellum (*flaA*), were dispensable for aggregation, both individually and in combination
158 (Fig. 2F). Similarly, additional genes tested with roles in biofilm formation (*bap1*, *rbmA*,

159 *rbmEF*, *vpsT*)[45,46], adhesion (*gbpA*)[47], cell shape (*crvA*)[48] and an O-linked glycosylase (*VC0393*
160 or $pglL_{Vc}$)[49] that could be involved in pilin modification all aggregated at levels
161 indistinguishable from the unmodified parent (Fig. S6A). Finally, deletion of *VC0502*, an
162 'orphan' type IV pilin encoded on the *Vibrio* seventh pandemic island II, had no effect on
163 transformation, aggregation or motility (Fig. S6B-D). Additional control experiments
164 indicated that aggregation occurred similarly during growth under high salt conditions (*i.e.*
165 LB-S 20 g/L NaCl), which are often used to better reflect the natural aquatic environment of
166 *Vibrio sp*. (Fig. S6E). Likewise, the presence of Bovine serum albumin, which was previously
167 reported to disrupt pilus-pilus interactions in *Neisseria gonorrhoeae*[50], also had no effect
168 (Fig. S6E). Curiously, however, we noticed that the strength of the aggregation phenotype is
169 sensitive to the concentration of divalent cations (*e.g.* $CaCl_2$ or $MgCl_2$) (Fig. S6F), though the
170 significance of this result remains unclear.

171 To investigate how the pilus mediates auto-aggregation *i.e* pilus-pilus, pilus-cell or
172 otherwise, we co-cultured cells producing either dynamic pili (Tn*tfoX*), static pili (Tn*tfoX*,
173 Δ*pilT*), or blocked for both retraction and pilus production (Tn*tfoX*, Δ*pilT*, Δ*pilA*) with a
174 Tn*tfoX* Δ*pilT* strain constitutively producing GFP (Fig. 2G-I) and asked whether these cells
175 were recruited into the GFP-labelled aggregates. Strikingly, intermixed aggregates were only
176 observed when both cells were themselves capable of producing non-retractile pili, and
177 hence aggregating (Fig. 2H), indicating that aggregates form via direct pilus-pilus interactions
178 between DNA-uptake pili.

179

180 **MSHA pili do not mediate aggregation**

181 MSHA pili are important for surface sensing and attachment during the switch to the sessile
182 lifestyle that precedes biofilm formation, and pilus retraction is thought to be required for
183 their function[20-24]. Since MSHA pili are constitutively produced, but retraction deficient cells
184 only aggregate upon competence induction, the results above suggest that MSHA pili are

185 unable to mediate auto-aggregation. To test this hypothesis directly we grew cells
186 engineered to increase the levels of the second messenger c-di-GMP, which has been
187 reported to enhance the assembly of MSHA pili on the cell surface[43]. Control experiments
188 indicated that decreasing c-di-GMP levels cells did not induce aggregation (Fig. S7A and B).
189 In contrast, under elevated c-di-GMP levels, cells formed large clumps that appeared similar
190 to the aggregates described above and had a modest sedimentation phenotype (Fig. S7A
191 and B). However, the assembly of these structures occurred independently of *pilT*, *pilA* and
192 *mshA*, but was abolished by the loss of *vpsA* (Fig. S7C). Thus, we conclude that these
193 structures are not aggregates held together by pili but rather biofilms held together by
194 matrix polysaccharide.
195
196 **PilA from pandemic strains is sufficient for aggregation in a non-pandemic strain**
197 A1552, the strain used throughout this work, is an O1 El Tor clinical isolate representative of
198 the on-going 7th cholera pandemic. To test whether the ability to aggregate is conserved
199 among other pandemic strains we tested 6 additional O1 El Tor strains. In the absence of *pilT*
200 all aggregated to similar levels as A1552, as might be expected given that pandemic strains
201 are closely related (Fig. 3A). Surprisingly, however, N16961 did not aggregate unless its well-
202 characterised *hapR* frame-shift mutation[51], which renders it quorum-sensing (QS) defective,
203 was first repaired (Fig. 3A). Since transcription of the genes required for pilus assembly are
204 not regulated by QS[41] this finding suggests that there may be an additional layer of post-
205 transcriptional control.
206 PilA is known to vary considerably between different environmental strains of *V.*
207 *cholerae*[52], whereas the majority of clinical isolates carry an identical *pilA*. Interesting
208 exceptions are the toxigenic O37-serogroup strains V52 and ATCC25872, which were
209 responsible for limited epidemics of a cholera-like illness in the 1960s[53,54]. These strains are
210 thought to derive from a pandemic O1 classical strain through the exchange of the O-antigen

211 cluster by horizontal gene transfer[55,56]. However, they both encode the same PilA version
212 that is only 50% identical to that typically carried by pandemic strains (Fig. S8). Since V52 is
213 QS deficient, and transformation is QS dependent, we investigated the functionality of this
214 PilA in ATCC25872, which has a functional QS pathway. Indeed, ATCC25872 is transformable
215 at similar levels to that of A1552, and as expected, transformation is PilT-dependent (Fig.
216 3B). However, in contrast to A1552, competence-induced cells lacking *pilT* did not aggregate
217 and were excluded from aggregates formed by A1552 (Fig. 3C-E, H and I).

218 Given that all the other known components required for pilus assembly and
219 transformation are highly conserved in ATCC25872, we tested whether PilA itself was
220 responsible for this phenotype by exchanging the endogenous *pilA* open reading frame for
221 that of A1552 (*pilA*ex). As expected, the ATCC25872 *pilA*ex strain was fully transformable
222 (Fig. 3B). Importantly, however, in the absence of *pilT*, competence-induced cells of this
223 strain were now able to form large aggregates, albeit at lower levels than in A1552 (Fig. 3C, F
224 and G), and intermix within aggregates formed by A1552 (Fig. 3J and K).

225

226 **PilA variability governs auto-aggregation and enables kin-recognition**

227 The results above suggest that the ability to aggregate is dependent on the particular PilA
228 variant carried. Indeed, BLAST analyses of 647 *V. cholerae* genomes deposited in NCBI
229 indicated that PilA exhibits extensive variation, whereas the other proteins encoded within
230 its operon, as well as those from neighbouring genes, are all highly conserved (Fig. S9). Of
231 the 636 intact *pilA* genes identified, the majority (492/636) encode a PilA identical to that of
232 A1552, which is likely due to an over-representation of patient-derived pandemic strains in
233 the database. Next, we extracted the unique PilA coding sequences (56/636) (Fig. S10A) and
234 combined them with those of an in-house collection of various environmental and patient
235 isolates. The resulting phylogenetic tree consists of around 12 distinct groups and was used
236 to sample PilA diversity (Fig. 4A). As expected, the N-terminal ~50 residues, which contain

237 sequences required for membrane trafficking and pre-pilin peptidase recognition, and that
238 goes on to form the long structural alpha helix (α1), are well conserved (Fig. S10A and B). In
239 contrast, apart from a few clusters of key conserved residues, including the C-terminal
240 cysteines that form the characteristic disulphide-bonded loop, the C-terminal domains are
241 highly variable (Fig. S10A and B).

242 To test how PilA variability affects pilus function but avoid the potential problems of
243 working in various strain backgrounds, we inserted new *pilA* alleles at the native *pilA* locus in
244 strain A1552, and used a short (30 bp) duplication of 3' end of the original *pilA* to maintain
245 any regulation of the downstream genes. We validated this *pilA* replacement (*pilA*rep)
246 approach using A1552 PilA (*i.e.* Tn*tfoX*, *pilA*rep[A1552]), which is fully transformable, and in
247 the absence of *pilT* aggregates at levels similar to the unmodified parent (Fig. 4B and C). We
248 then tested 16 different PilA sequences from across the tree (Fig. 4A and Fig. S10B).
249 Interestingly, all were equally capable of supporting transformation (Fig. 4B). In contrast, the
250 ability to aggregate varied depending on the particular PilA. Indeed, in the absence of
251 retraction, 9/16 PilAs supported the aggregation phenotype, though PilA DRC186-4 was
252 intermediate, whereas 7/16 did not detectably aggregate (Fig. 4A and C).

253 Given that aggregation occurs via direct pilus-pilus interactions we hypothesised
254 that the variability between the different aggregation-proficient PilA might allow pili
255 composed of different PilA to distinguish between one another. To test this idea we used
256 the same co-culture approach as before, using strains with and without constitutive GFP
257 production, and examined all possible combinations (Fig. 4D). As expected, cells producing
258 pili composed of an identical PilA always exhibited uniform mixing (Fig. 4D and E).
259 Remarkably, however, in 41/45 possible unique combinations the interactions between
260 different pili were highly specific (Fig. 4D). Indeed, cells of these strains aggregated in a pilin-
261 specific manner, preferentially forming aggregates with cells producing pili composed of the
262 same PilA. This resulted in aggregates that were either almost exclusively fluorescent or

263 non-fluorescent (Fig. 4F). In 4/45 cases a partial cross-interaction was observed, resulting in
264 an intermediate mixing phenotype, with aggregates composed of smaller but still segregated
265 groups of cells (Fig. 4D and G). Overall these data indicate that pili composed of different
266 PilA are able to discriminate between one another, probably via specific PilA-PilA
267 interactions. Moreover, these data demonstrate that PilA variability not only determines the
268 ability to aggregate, but also provides a mechanism for kin-recognition.

269 The TCP is also capable of aggregation under conditions mimicking virulence
270 induction[18,57]. Indeed, in strains artificially induced for virulence TCP-mediated aggregates
271 readily formed that appeared similar to those described in this work (Fig. S11A and C). As
272 expected, they did not intermix with aggregates formed by DNA-uptake pili (Fig. S11B-D). Of
273 note, however, is that TCP are largely limited to pandemic lineages with 2 variants of its
274 major pilin TcpA: Classical and El Tor[58-60]. In contrast to the results above and in line with
275 previous work[58], a classical and El Tor strain formed uniformly mixed TCP aggregates (Fig.
276 S11E), indicating that the different TcpA do not discriminate between one another.

277

278 **The unusual tail of ATCC25872/V52 PilA is an inbuilt inhibitor of aggregation**

279 To rule out the possibility that strains unable to aggregate simply fail to make sufficient
280 numbers of pili, we made labelable *pilA*rep cysteine variants of A1552 PilA (*pilA*rep[A1552;
281 S67C]) and 2 non-aggregating alleles (*i.e. pilA*rep[Sa5Y; S67C] and *pilA*rep[V52; N67C]). All
282 constructs behaved similarly to the equivalent non-cysteine variant, though the
283 *pilA*rep[Sa5Y; S67C] has a modest transformation defect (Fig. S12A and B). Both the
284 PilA[Sa5Y] and PilA[V52] cysteine variants were piliated at similar levels to the A1552 control
285 and in the absence of *pilT* were hyper-piliated, although pili composed of PilA[Sa5Y] were
286 generally very short (Fig. 5A and B). The hyper-piliation was especially clear for the V52
287 cysteine variant and provides direct evidence that the inability of some alleles to aggregate
288 is likely not due to a failure to produce sufficient numbers of pili but reflects a specific

289 property of the pilin itself. Notably, sheared pili of the *pilA*rep[A1552; S67C] variant
290 assembled into large structures, as above, whereas those of the others did not (Fig. S12C).
291 Strains V52 and ATCC25872 encode an identical PilA with an unusual repetitive C-
292 terminal extension (SGSGSGSGSGSGSGSGSGSGN), or 'tail'. Among the PilA sequences analysed
293 here, there were 9 examples of this type of tail clustered into 2 well-separated phylogenetic
294 groups (Fig. S8). Moreover, of the *pilA*rep PilAs unable to support aggregation 3/7 contain a
295 tail (Fig. S8B). Thus, to test if the tail affected V52 PilA function we created a truncated
296 variant *i.e. pilA*rep[V52Δtail], which was similarly transformable to the equivalent tailed
297 version (Fig. 5C). Remarkably, however, removal of the tail restored the ability to aggregate
298 in the absence of *pilT*, at levels indistinguishable from that of the PilA[A1552] controls (Fig.
299 5D), and demonstrated a similar ability to 'recognise' itself in co-culture experiments (Fig.
300 5E). These data suggest that the tail inhibits the ability of pili to aggregate, possibly by
301 masking the site of pilus-pilus interaction. Indeed, a strain in which this tail was transplanted
302 onto PilA of A1552 (*i.e. pilA*rep[A1552+tail] remained highly transformable but was unable
303 to aggregate (Fig. 5C and D). However, since efforts to label this PilA variant have so far been
304 unsuccessful we cannot exclude another, non-specific effect.
305
306 **DNA-uptake pili form networks on chitin surfaces**
307 The data so far indicates that interactions between pili can mediate intercellular contacts.
308 However, in liquid culture this relies on blocking pilus retraction. We reasoned that if these
309 interactions are relevant to the normal ecology of *V. cholerae* then we might be able to
310 detect them under more realistic conditions. Therefore, we visualised pili produced by
311 otherwise WT cells upon cultivation on chitinous surfaces (Fig. 6A). Strikingly, under these
312 conditions of natural induction, cells colonising the chitin surface were generally found
313 embedded within dense networks of pili that were often overlaid by larger pili structures
314 (Fig. 6A). In contrast to the chitin-independent liquid culture experiments, cells on the chitin

315 surface often appeared to possess multiple pili simultaneously (Fig. 6B), though the crowded
316 nature of the cells precluded direct quantification. Furthermore, since the washing steps
317 required for pilus staining tend to remove cells, but not pili, from the surface, the number of
318 cells engaged in these networks is likely underestimated. Control experiments confirmed
319 that labelling on surfaces is specific and that these structures are DNA-uptake pili (Fig. S13A-
320 C). Notably, Δ*pilT* cells are heavily hyper-piliated under these natural induction conditions
321 (Fig. S13D), indicating that the phenotypes observed in liquid culture are not an artefact of
322 artificial competence induction.

323 Next, using the *pilA*rep cysteine variants described above, we directly compared the
324 pili assembled on chitin surfaces by cells producing the aggregation-proficient PilA[A1552]
325 with those producing the aggregation-deficient PilA[V52]. Consistent with its functionality in
326 the various assays above, cells carrying *pilA*rep[A1552; S67C] were also found within similar
327 pili networks (Fig. 6C and E). In contrast, such networks were never observed for cells
328 carrying *pilA*rep[V52; N67C] (Fig. 6D and F). Instead, the surfaces were coated in what
329 appeared to be mostly individual pili. This difference was particularly clear at later time-
330 points when cells presumably began to exhaust the chitin surface. Cells producing
331 PilA[A1552] covered surfaces with extensive and long-range networks of pili that were
332 maintained within detached pieces of biofilm (Fig. S14A and C). In contrast, for cells
333 producing PilA[V52] the surfaces were coated with individual pili and never large networks
334 (Fig. S14B and D). In summary, these data suggest that the aggregation phenotypes
335 observed in liquid culture experiments report the natural ability of pili to interact on chitin
336 surfaces.

337

338 **Discussion**

339 Here we demonstrate that DNA-uptake pili are highly dynamic, that these dynamics are PilT-
340 dependent, and that cells lacking *pilT* are minimally transformable, providing direct evidence

341 for the longstanding model, whereby pilus retraction facilitates DNA-uptake. Indeed, our
342 results on pilus dynamics are in close agreement with those recently reported by Ellison *et*
343 *al.*, who notably, went on to demonstrate that the pilus binds directly to DNA[61]. The major
344 finding of this work, however, is that DNA-uptake pili are able to interact and distinguish
345 between one another in a specific manner depending on their sequence. In liquid culture
346 when retraction is blocked this manifests as an exaggerated auto-aggregation phenotype.
347 Since only a subpopulation of cells is piliated at any one time, and these pili are dynamic,
348 blocking retraction likely facilitates auto-aggregation by producing a homogenous
349 population of hyper-piliated cells, thereby increasing the chances of interactions between
350 pili. Work in *Neisseria meningitidis*, which auto-aggregates naturally at low levels but is
351 dramatically enhanced by the deletion of *pilT*, supports this idea[62,63]. Importantly, however,
352 under natural induction conditions on chitin surfaces, cells producing pili capable of
353 aggregation elaborate multiple pili and form dense pili networks in an otherwise unmodified
354 background, indicating that the chitin surface likely promotes interactions between pili. The
355 crowded surface environment might inherently foster these interactions. Alternatively, the
356 altered physiology of cells growing on chitin might also impact pilus assembly via effects on
357 the extension/retraction motors.

358 The TCP of *V. cholerae* is induced during virulence and is essential for
359 colonisation[18,57,64]. Under laboratory conditions TCP production results in auto-aggregation
360 similar to that described here[17,19,58]. Moreover, in an infection model cells on the intestinal
361 cell surface were found encased within networks of TCP, that were suggested to protect
362 cells from host defences[65]. Given the similarities between the two systems, especially our
363 observation of dense pili networks on colonised chitin surfaces, we propose that the DNA-
364 uptake pilus might play an analogous role in the aquatic environment. Indeed, as
365 hypothesised elsewhere[29,66], since pilus production is dependent on an intact chitin-
366 utilisation pathway, colonisation mechanisms using DNA-uptake pili would be inherently

367 selective for (i) nutritious chitinous surfaces and (ii) favour the recruitment and retention of
368 productive cells while excluding non-productive cells unable to make pili. Thus, intercellular
369 interactions between DNA-uptake pili could act at multiple stages of colonisation, but might
370 be particularly advantageous during early stages to bring smaller chitin particles together,
371 and thus provide resistance to protozoan grazers, as well as at later stages to keep cells
372 together during biofilm dispersal. It remains possible, however, that these interactions
373 represent an ancient colonisation mechanism that has since been replaced. Arguing against
374 this idea is the observation that all pandemic strains retain the same interaction-proficient
375 PilA even though other environmental strains have been free to vary their PilA without
376 impacting its ability to mediate transformation. Interestingly, ingestion of colonised chitin
377 particles is thought to facilitate transmission to humans[26] and recovering cholera patients
378 exhibit a strong immune response to PilA[67]. One possibility that should be investigated is
379 that the networks of DNA-uptake pili we observed on chitin surfaces protect cells during this
380 process.

381 In *Neisseria gonorrhoeae*, artificially varying the density or post-translation
382 modification state of pili leads to a form of cell sorting based on differential interaction
383 forces between pili[68]. This effect is likely related to aggregate dispersal during its infective
384 lifestyle but does not permit specific recognition *per se*[69]. In contrast, the discovery here that
385 the natural variability of PilA controls the ability of pili to self-interact, and creates highly
386 specific interactions, provides a direct mechanism for kin recognition. The best-studied
387 examples of kin recognition in microorganisms all involve adhesins and some form of
388 aggregation (*e.g.* Flo1; *Saccharomyces cerevisiae*[70], TgrB1-TgrC1; *Dictyostelium discoideum*[71],
389 and TraA; Myxobacteria[72]). In evolutionary terms, these recognition mechanisms are
390 classified as 'greenbeards' because the cue, recognition of the cue and the resulting
391 cooperative activity are all encoded by the same gene[73,74]. The ability of DNA-uptake pili to
392 recognise and interact with pili composed of the same kind of PilA fits this classification and

393 is therefore a specific form of greenbeard recognition[73,74]. However, this form of recognition
394 implies close identity only at the greenbeard locus and so is better referred to as kind
395 recognition[73,74].

396 An important question going forward will be to understand what drives PilA diversity
397 and how this is related to the type VI secretion system, which acts to kill non-kin
398 bacteria[75,76]. Similarly, the apparent acquisition of an inhibitor of pilus interactions by some
399 PilA (*e.g.* ATCC25872/V52) hints that the ability to interact may not always be beneficial.
400 Therefore, future work should focus on how the pili networks we observed on chitin
401 surfaces contribute to the ecology of *V. cholerae*, especially under environmental conditions.
402 Indeed, we still know relatively little about the natural lifestyle of *V. cholerae* on chitin, in
403 part due to the inherent technical difficulties associated with manipulating these surfaces.
404 Nevertheless, the demonstration that in liquid media, specific interactions between pili
405 composed of different major pilins is sufficient to enable segregation, provides a robust
406 proof-of-concept that T4P have the ability to function as a recognition mechanism. Finally,
407 the fact that (i) T4P are widespread, (ii) auto-aggregation via T4P has been reported in
408 multiple species[77] and (iii) the major pilin subunit often varies[4,52], raises the possibility that
409 specific interactions between T4P might be quite common and therefore represent an
410 important contribution to bacterial kin recognition worthy of continued investigation.

411

412 **Materials and Methods**

413 **Bacterial strains and plasmids**

414 The bacterial strains used in this study are shown in Supplementary Table S1, together with
415 the plasmids used and their construction. A1552, the *V. cholerae* strain used throughout this
416 work[78], is a toxigenic O1 El Tor Inaba strain representative of the on-going 7th cholera
417 pandemic, and was derived from a traveller entering the United States after being infected
418 on a commercial aeroplane that took off in Peru[79].

419

420 **General methods**

421 Bacterial cultures were grown aerobically at 30˚C or 37˚C, as required. Liquid medium used
422 for growing bacterial strains was Lysogeny Broth (LB-Miller; 10 g/L NaCl, Carl Roth,
423 Switzerland) and solid medium was LB agar. Where indicated, LB-S contained 20 g/L NaCl.
424 Ampicillin (Amp; 100 µg/mL), gentamicin (Gent; 50 µg/mL), kanamycin (Kan; 75 µg/mL),
425 streptomycin (Str; 100 µg/mL) and rifampicin (Rif; 100 µg/mL) were used for selection in *E.*
426 *coli* and *V. cholerae*, as required. To induce expression from the P_{BAD} promoter, cultures
427 were grown in media supplemented with 0.2% L-arabinose. Natural transformation of *V.*
428 *cholerae* on chitin flakes was done in 0.5x DASW (Defined artificial seawater), supplemented
429 with vitamins (MEM, Gibco) and 50 mM HEPES, as previously described[30]. Counter-selection
430 of phenylalanyl-tRNA synthetase (*pheS**) insertions (Trans2 method; see below) was done
431 on medium supplemented with 20 mM 4-chloro-phenylalanine (cPhe; Sigma-Aldrich,
432 Switzerland). Thiosulfate citrate bile salts sucrose (TCBS; Sigma-Aldrich, Switzerland) agar
433 was used to counter-select for *E. coli* following bacterial mating. SacB-based counter-
434 selection was done on NaCl-free medium containing 10 % sucrose.

435

436 **Strain construction**

437 DNA manipulations and *E. coli* transformations were carried out using standard methods[80],
438 and all constructs were verified by PCR and Sanger sequencing (Microsynth AG, Switzerland).
439 Genetic engineering of *V. cholerae* was done using a combination of natural transformation
440 and FLP-recombination; Trans-FLP[81,82], *pheS**-based counter-selection; Trans2[83,84], and allelic
441 exchange using bi-parental mating and the counter-selectable plasmid pGP704-Sac28[29]. The
442 mini-Tn7 transposon carrying *araC* and various P_{BAD}-driven genes was integrated into the
443 large chromosome by tri-parental mating, as previously described[85].

444

445 **Chitin-independent competence induction**

446 Chitin oligosaccharides resulting from growth on chitin trigger natural competence induction
447 via the production of a master regulator, TfoX[29,30,86-88]. High cell-density, as sensed by
448 quorum sensing via HapR, results in the production of an intermediate regulator QstR, which
449 acts in concert with TfoX to regulate the transcription of a subset of the competence
450 genes[30,41,89]. Therefore, to induce natural competence in liquid culture we used a well
451 characterised and already validated chitin-independent approach that results in low levels of
452 TfoX production[41]. This approach is based on the integration of a mini-Tn7 transposon into
453 the large chromosome of *V. cholerae* containing an arabinose-inducible copy of *tfoX* (*i.e.*
454 *araC*, P_{BAD}-*tfoX*), which we refer to as Tn*tfoX*. In the presence of inducer, strains carrying
455 Tn*tfoX* turn on the expression of the competence genes according to the known regulatory
456 pathways and upon reaching high cell-density are transformable at levels similar to those
457 seen on chitin[41]. In the absence of inducer, competence genes are not produced and strains
458 are non-transformable[41].

459

460 **Transformation frequency assay**

461 Diverse strains harbouring Tn*tfoX* were tested for transformation using a chitin-independent
462 transformation frequency assay, as previously described[32,41]. Briefly, overnight cultures were
463 back-diluted 1:100 in fresh media with and without arabinose, as indicated, and grown 3h at
464 30˚C with shaking (180 rpm). 0.5 mL aliquots of the cultures were mixed with 1 µg genomic
465 DNA (GC#135; A1552-lacZ-Kan) in 1.5 mL eppendorf tubes and incubated 5h at 30˚C with
466 shaking (180 rpm), prior to serial dilution in PBS (Phosphate buffered saline) and
467 enumeration after overnight growth on LB media in the absence and presence of kanamycin.
468 Transformation frequency was calculated as the number of transformants divided by the
469 total number of bacteria.

470

471 **Pilus shearing assay**

472 Cultures were grown for 6h at 30˚C with shaking (180 rpm) in 25 mL LB + 0.2% arabinose
473 within a 125 mL Erlenmeyer flask. To shear pili from the cell surface, 10 mL culture was
474 removed, vortexed at max speed for 1 min, and cells removed by three sequential
475 centrifugation steps (10min; 4000 x *g*; 4˚C). To the resulting supernatant saturated
476 ammonium sulphate was added to 40% and incubated on ice for 1h. Precipitated proteins
477 were recovered by centrifugation (30min; 20,000 x *g*; 4˚C) and washed once with PBS.
478 Samples were then re-suspended in 2x Laemmli buffer, boiled (15min; 95˚C) and stored at -
479 20˚C until needed. To compare PilA levels between samples the re-suspension volume was
480 normalised according to the optical density of the starting culture. Total protein controls
481 were intact cell lysates. The relative amount of PilA in each sample was determined by
482 Western blotting.

483

484 **Aggregation assay**

485 Overnight cultures were back-diluted 1:100 in the absence and presence of arabinose, as
486 needed, and grown in 14 mL round bottom polystyrene test tubes (Falcon, Corning) on a
487 carousel style rotary wheel (40 rpm) at 30˚C. After 6h growth, aggregates were allowed to
488 settle by standing the tube at RT for 30 min. The optical density at 600 nm ($O.D._{600}$) of the
489 culture was then measured before and after mechanical disruption (vortex max speed; ~5
490 sec), which served to disperse any settled aggregates and return them to solution.
491 Aggregation is expressed as the ratio of the $O.D._{600}$ Pre/Post-vortexing. For time-course
492 experiments the standing time was reduced to 5 min. To visualise aggregates by microscopy
493 overnight cultures were back-diluted either 1:100 individually or 1:200 when mixed, as
494 needed, and were grown for 4h, as described above.

495

496 **Microscopy**

497 Cells were mounted on microscope slides coated with a thin agarose pad (1.2% w/v in PBS),
498 covered with a #1 cover-slip, and were observed using a Zeiss Axio Imager M2 epi-
499 fluorescence microscope attached to an AxioCam MRm camera and controlled by Zeiss
500 AxioVision software. Image acquisition was done using a Plan-Apochromat 100×/1.4 Ph3 oil
501 objective illuminated by an HXP120 lamp. Images were analysed and prepared for
502 publication using ImageJ (http://rsb.info.nih.gov/ij).

503

504 **Pilus staining and quantification**

505 Alexa Fluor™ 488 C_5 Maleimide (AF-488-Mal; Thermo Fisher Scientific; Cat# A10254) was
506 dissolved in DMSO, aliquoted and stored at -20˚C protected from light. Cultures used for
507 staining were grown for 3.5h at 30˚C on the rotary wheel, as above, in the absence and
508 presence of competence induction, as required. To stain cells 100 µL of culture was mixed
509 with dye at a final concentration of 25 µg/mL[39] and incubated at RT for 5 min in the dark.
510 Stained cells were harvested by centrifugation (5000 x *g*; 1 min), washed once with LB, re-
511 suspended in 200 µL LB and imaged immediately. For quantification of piliation in snapshot
512 imaging approximately 2000 cells per strain were analysed in each of three independent
513 repeats. A subset of this data set was also analysed for the number of pili per cell and pilus
514 length, as indicated in the text. For time-lapse analysis, images were acquired at RT at 10 sec
515 intervals for 1 min (*i.e.* 7 frames). To analyse the number of pili produced per cell, per min, 5
516 fields of cells were analysed for each of the three independent repeats, yielding an analysis
517 of 1947 cells in total.

518

519 **Imaging on chitin surfaces**

520 Visualisation of pilus production on chitin surfaces was done using chitin beads (NEB;
521 Cat#S6651), which have previously been validated as a useful analogue for the natural chitin

522 surface[41,75]. Prior to use, chitin beads were washed 5x with 0.5x DASW + 50 mM HEPES +
523 vitamins. Colonisation of chitin beads was done in 12-well culture plates (Cellstar) by mixing
524 0.1mL washed O/N culture with 0.1 mL washed beads in a final volume of 1 mL 0.5x DASW +
525 50 mM HEPES + vitamins, and incubated at 30˚C; 48h. Pilus staining was done as described
526 above except that the final re-suspension volume was reduced to 50 µL. Stained beads were
527 mounted directly on glass microscope slides, covered with a #1 cover-slip and imaged using
528 either a Plan-Apochromat 100x/1.4 Ph3 or Plan-Neofluar 40x/1.3 Ph3 oil objective, as
529 needed. To image cells after prolonged incubation (≥72h), plates were incubated within a
530 homemade humidified chamber. To avoid damaging the beads manipulations were done
531 using wide bore tips.
532
533 **Western blotting**
534 Cell lysates were prepared by suspending harvested cells in 2x Laemmli buffer (100 µL buffer
535 per O.D. unit) before boiling at 95˚C for 15 min. Proteins were separated by SDS-PAGE using
536 a 15% resolving gel and blotted onto PVDF membranes using a wet-transfer apparatus.
537 Immuno-detection was performed as described previously[41]. Primary anti-PilA antibodies
538 were raised in rabbits against synthetic peptides of A1552 PilA (Eurogentec, Belgium;
539 #1510525) and used at a dilution of 1:5000. Anti-Rabbit IgG HRP (Sigma; Cat# A9169) diluted
540 1:5000 was used as a secondary antibody. Sample loading was verified with anti-RNA
541 Sigma70-HRP (BioLegend; Cat# 663205) diluted 1:10,000.
542
543 **Motility assay**
544 To quantity motility phenotypes 2 µL overnight culture was spotted soft LB agar (0.3%)
545 plates (two technical replicates) and incubated at RT for 24h prior to photography. The
546 swarming diameter (cm) was measured and is expressed as the mean of three independent

547 biological repeats. A flagellin-deficient (Δ*flaA*) non-motile strain was used as a negative
548 control.
549
550 **Bioinformatics of PilA diversity**
551 *Vibrio cholerae* genomes were obtained from NCBI (National Center for Biotechnology
552 Information), and are listed in Supplementary File S1. Geneious software (10.2.3)[90] was used
553 to perform custom BLAST analyses and identify *pilA*. Unique PilA sequences were extracted
554 and combined with the PilA sequences from strain A1552 and a collection of environmental
555 isolates, as deduced by Sanger sequencing (Supplementary File S2), as indicated in the text.
556 PilA sequences were aligned with Muscle and a consensus neighbour-joining tree
557 constructed using the Jukes-Cantor substitution model, resampled with 100 bootstrap
558 replicates. MshA from strain A1552 was used as an outgroup.
559
560 **Reproducibility**
561 All data shown are representative of the results of at least three independent biological
562 repeats.
563
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573

574 **Author contributions**

575 Conception, design and analysis: D.W.A and M.B. Performed research: D.W.A, S.S, C.S and

576 M.B. Wrote the manuscript: D.W.A and M.B.

577

578 **Declaration of Interests**

579 The authors declare no competing interests.

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Figure 1

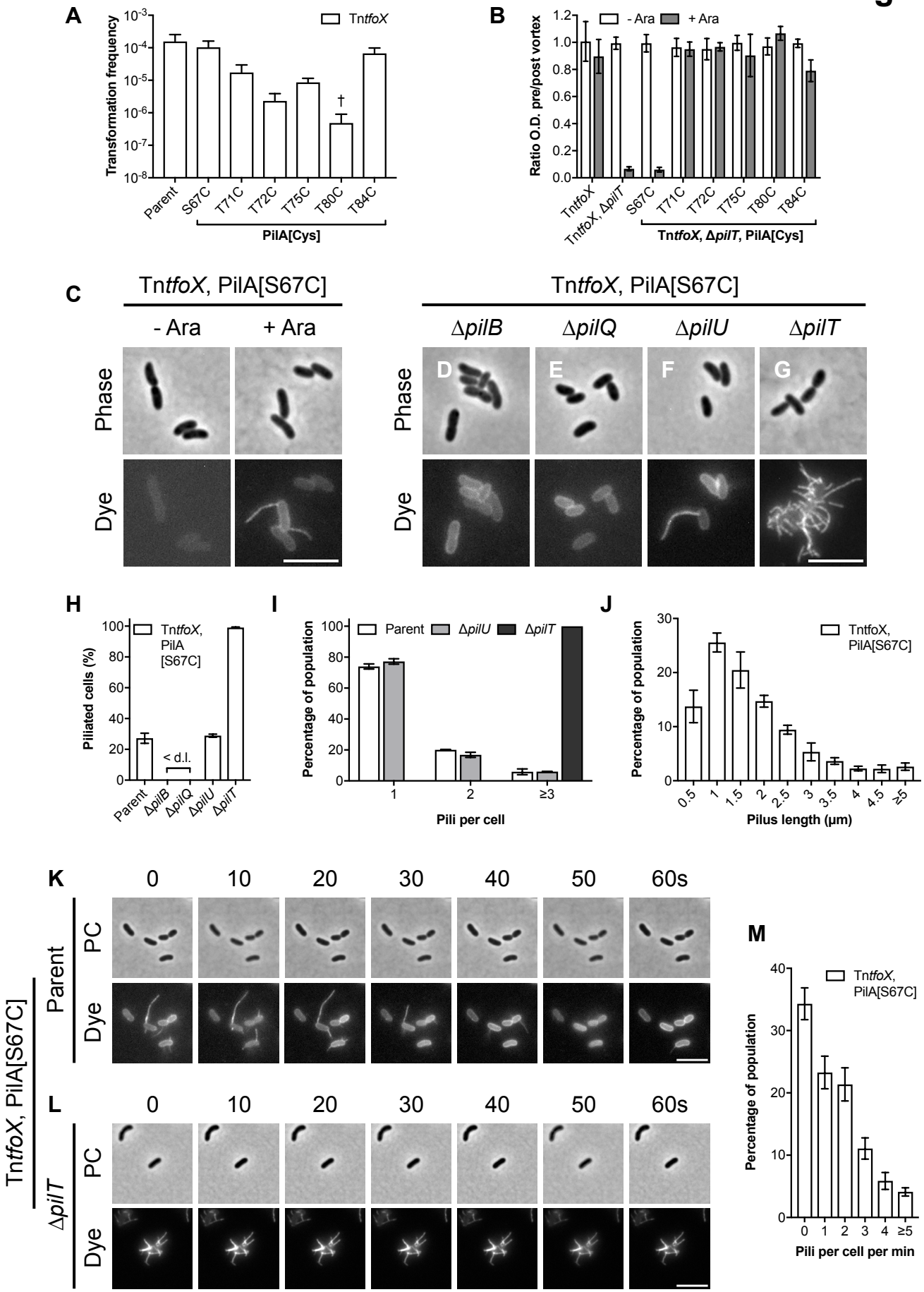
A
Transformation frequency
TntfoX
Parent
S67C
T71C
T72C
T75C
T80C
T84C
PilA[Cys]
B
Ratio O.D. pre/post vortex
- Ara
+ Ara
TntfoX
TntfoX, ΔpilT
TntfoX, ΔpilT, PilA[Cys]
C
TntfoX, PilA[S67C]
- Ara
+ Ara
ΔpilB
ΔpilQ
ΔpilU
ΔpilT
Phase
Dye
D
E
F
G
H
Piliated cells (%)
< d.l.
I
Percentage of population
Pili per cell
J
Pilus length (μm)
K
L
PC
Parent
ΔpilT
M
Pili per cell per min

Figure 1. Direct observation of dynamic DNA-uptake pili.

(**A**) Functionality of PilA cysteine variants (PilA[Cys]) in a chitin-independent transformation assay using strains carrying an arabinose-inducible copy of *tfoX* (Tn*tfoX*). Transformation frequencies are the mean of three independent biological repeats (+S.D.). < d.l., below detection limit. †, < d.l. in one repeat.

(**B**) Effect of PilA cysteine variants on the ability of retraction deficient (Tn*tfoX*, Δ*pilT*) cells to aggregate. Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing, in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated. Values are the mean of three independent biological repeats (±S.D.).

(**C**-**G**) Snapshot imaging of pili in cells of A1552-Tn*tfoX*, PilA[S67C] and its derivatives. (**C**) Cells of A1552-Tn*tfoX*, PilA[S67C] were grown in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated, and stained with AF-488-Mal. Bar = 5 µm. (D-E) Cells of A1552-Tn*tfoX*, PilA[S67C] were grown in the presence of *tfoX* induction and stained with AF-488-Mal in a (**D**) Δ*pilB*, (**E**) Δ*pilQ*, (**F**) Δ*pilU* and (**G**) Δ*pilT* background, as indicated. Bar = 5 µm.

(**H**-**J**) Quantification of piliation in snapshot imaging. Bars represent the mean of three repeats (±S.D.). (**H**) Percentage of piliated cells in the indicated backgrounds. *n* = *c.a.* 2000 cells per strain per repeat. (**I**) Histogram of number of pili per cell in piliated cells in WT parent (A1552-Tn*tfoX*, PilA[S67C]), Δ*pilU* (A1552-Tn*tfoX*, PilA[S67C], Δ*pilU*) and Δ*pilT* (A1552-Tn*tfoX*, PilA[S67C], Δ*pilT*) backgrounds, as indicated. *n* = *c.a.* 300 cells per strain per repeat. (**J**) Histogram of pilus length in cells of A1552-Tn*tfoX*, PilA[S67C]. *n* = *c.a.* 500-600 cells per repeat.

(**K**-**L**) Time-lapse series of pilus dynamics in (**K**) WT parent (A1552-Tn*tfoX*, PilA[S67C]) and (**L**) Δ*pilT* (A1552-Tn*tfoX*, PilA[S67C], Δ*pilT*) backgrounds. Cells were stained with AF-488-Mal and imaged at 10s intervals for 1 minute. Upper panels show phase-contrast (PC), lower panels show fluorescence (dye). Time in seconds (s), as indicated. Bars = 5 µm.

(**M**) Histogram showing quantification of pili produced per cell per min in the WT parent (A1552-Tn*tfoX*, PilA[S67C]) background. Bars represent the mean of three repeats (±S.D.). *n* = *c.a.* 500-700 cells per repeat.

Figure 2

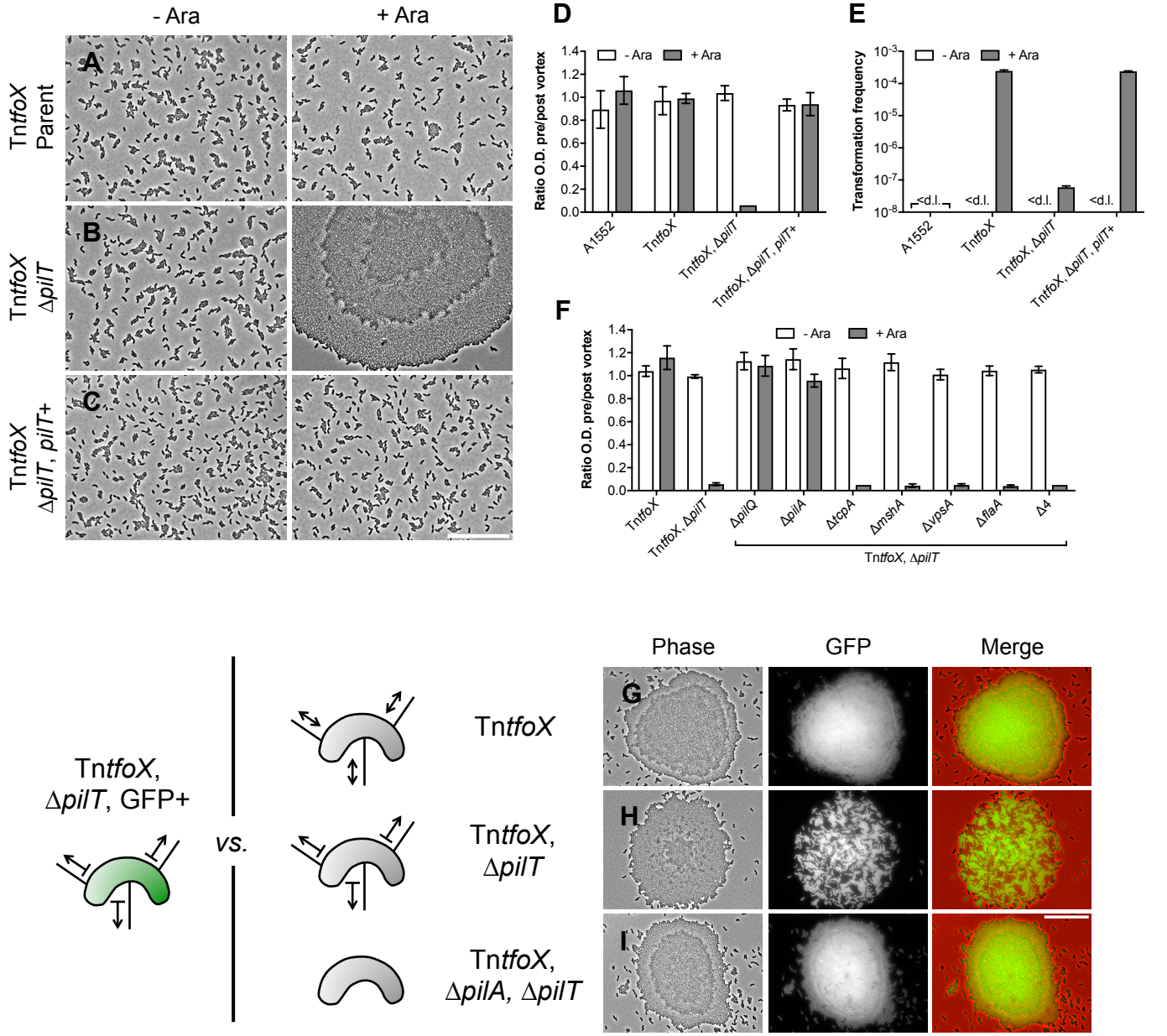
- Ara
+ Ara
A
B
C
TntfoX Parent
TntfoX ΔpilT
TntfoX ΔpilT, pilT+
D
Ratio O.D. pre/post vortex
- Ara
+ Ara
A1552
TntfoX
TntfoX, ΔpilT
TntfoX, ΔpilT, pilT+
E
Transformation frequency
<d.l.
F
ΔpilQ
ΔpilA
ΔtcpA
ΔmshA
ΔvpsA
ΔflaA
Δ4
TntfoX, ΔpilT
TntfoX, ΔpilT, GFP+
vs.
TntfoX
TntfoX, ΔpilT
TntfoX, ΔpilA, ΔpilT
Phase
GFP
Merge
G
H
I

Figure 2. Competent cells auto-aggregate in the absence of pilus retraction.

(**A**-**C**) Phase-contrast microscopy of cells of strains (**A**) A1552-Tn*tfoX*, (**B**) A1552-Tn*tfoX*, Δ*pilT* and (**C**) A1552-Tn*tfoX*, Δ*pilT*, *pilT*+, grown in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated. Scale bar = 25 µm.

(**D**-**E**) Aggregation and transformation frequency of cells of strains A1552-Tn*tfoX*, A1552-Tn*tfoX*, Δ*pilT* and A1552-Tn*tfoX*, Δ*pilT*, *pilT*+, grown in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated. A1552 (without inducible *tfoX*) was used as a negative control. (**D**) Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing. Values are the mean of three repeats (±S.D.). (**E**) Chitin-independent transformation frequency assay. Values are the mean of three repeats (+S.D.). < d.l., below detection limit.

(**F**) Effect of various deletion backgrounds on the ability of retraction deficient cells to aggregate. Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing, in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated. Δ4 = Δ*tcpA*, Δ*mshA*, Δ*vpsA*, Δ*flaA* quadruple mutant. Values are the mean of three repeats (±S.D.).

(**G**-**I**) Co-culture of fluorescent Δ*pilT* cells (A1552-Tn*tfoX*, Δ*pilT*, GFP+) producing GFP and non-fluorescent cells of the (**G**) WT parent (A1552-Tn*tfoX*), (**H**) Δ*pilT* (A1552-Tn*tfoX*, Δ*pilT*) and (**I**) Δ*pilA*, Δ*pilT* (A1552-Tn*tfoX*, Δ*pilA*, Δ*pilT*), grown in the presence of *tfoX* induction. Merged images show GFP in green and phase-contrast in red. Bar = 25 µm.

Figure 3

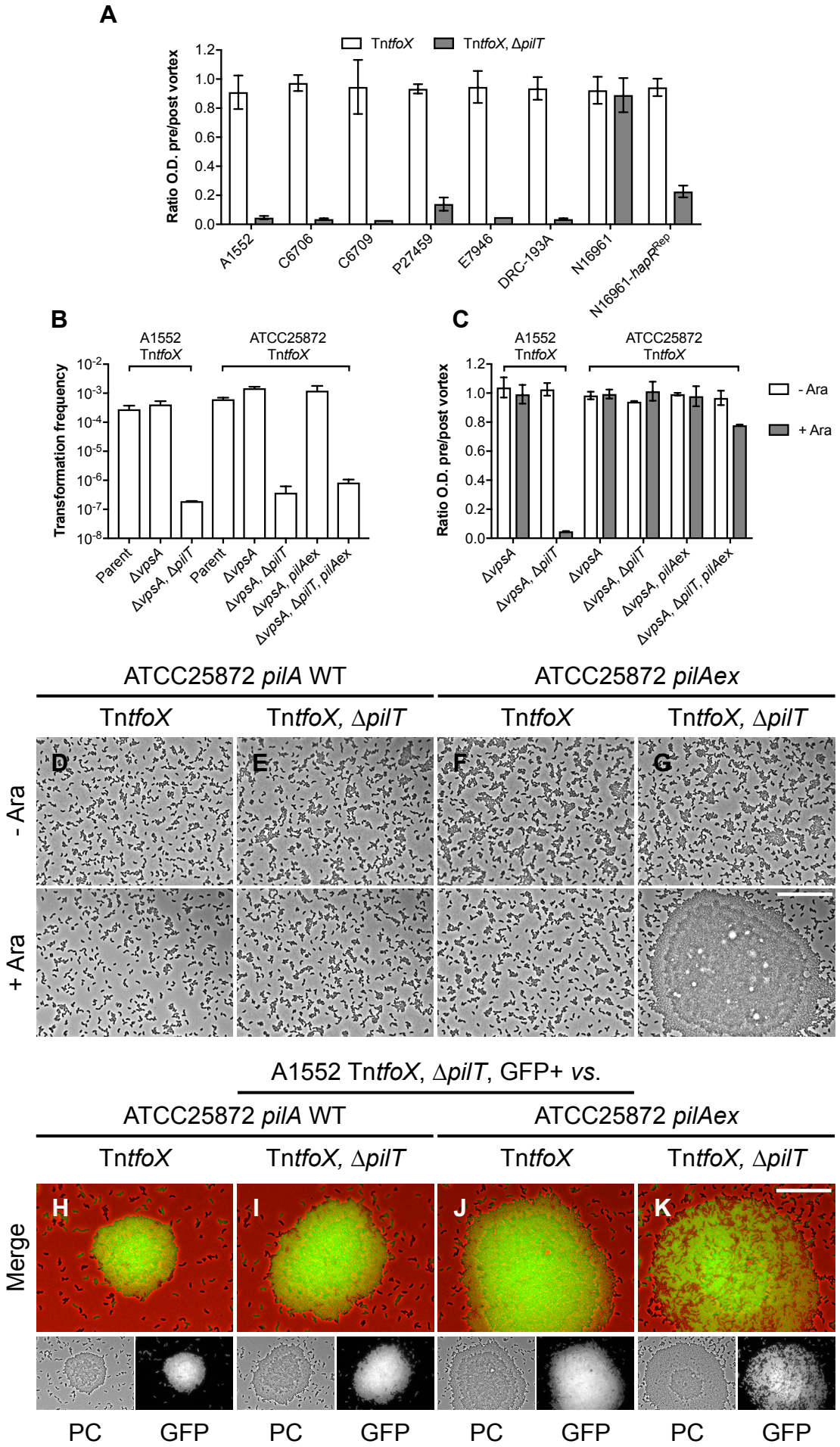
A
TntfoX
TntfoX, ΔpilT
Ratio O.D. pre/post vortex
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A1552
C6706
C6709
P27459
E7946
DRC-193A
N16961
N16961-hapR^Rep
B
A1552 TntfoX
ATCC25872 TntfoX
Transformation frequency
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
Parent
ΔvpsA
ΔvpsA, ΔpilT
Parent
ΔvpsA
ΔvpsA, ΔpilT
ΔvpsA, pilAex
ΔvpsA, ΔpilT, pilAex
C
A1552 TntfoX
ATCC25872 TntfoX
Ratio O.D. pre/post vortex
- Ara
+ Ara
ΔvpsA
ΔvpsA, ΔpilT
ΔvpsA
ΔvpsA, ΔpilT
ΔvpsA, pilAex
ΔvpsA, ΔpilT, pilAex
ATCC25872 pilA WT
ATCC25872 pilAex
TntfoX
TntfoX, ΔpilT
TntfoX
TntfoX, ΔpilT
- Ara
+ Ara
D
E
F
G
A1552 TntfoX, ΔpilT, GFP+ vs.
ATCC25872 pilA WT
ATCC25872 pilAex
TntfoX
TntfoX, ΔpilT
TntfoX
TntfoX, ΔpilT
Merge
H
I
J
K
PC
GFP
PC
GFP
PC
GFP
PC
GFP

Figure 3. A1552 PilA is sufficient for aggregation in a non-pandemic strain.

(**A**) Aggregation of representative 7th pandemic strains of *V. cholerae*, including the effect of *hapR*^{Rep} on N16961, in a Tn*tfoX* and a Tn*tfoX*, Δ*pilT* background, as indicated. Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing, in the presence of *tfoX* induction. Values are the mean of three repeats (±S.D.).

(**B**-**C**) Transformation frequency and aggregation of *V. cholerae* strain ATCC25872-Tn*tfoX* compared to that of A1552-Tn*tfoX*. (**B**) Chitin-independent transformation assay. Transformation frequencies are the mean of three repeats (+S.D.). (**C**) Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing, in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated. Values are the mean of three repeats (±S.D.).

(**D**-**G**) Phase-contrast microscopy of ATCC25872-Tn*tfoX*, Δ*vpsA* cells carrying (**D** and **E**) their native *pilA* (*pilA* WT) and (**F** and **G**) A1552 *pilA* (*pilA*ex), in a (**D** and **F**) *pilT*+ and (**E** and **G**) Δ*pilT* background, as indicated. Strains were cultured in the absence (- Ara) and presence (+ Ara), as indicated. Bar = 25 µm. Note that ATCC25872 derivatives were co-deleted for *vpsA* to rule out any compounding effects of biofilm formation.

(**H**-**K**) Co-culture of fluorescent cells of A1552-Tn*tfoX*, Δ*pilT*, GFP+*,* producing GFP, and non-fluorescent cells of ATCC25872-Tn*tfoX*, Δ*vpsA* carrying (**H** and **I**) their native *pilA* (*pilA* WT) and (**J** and **K**) A1552 PilA (*pilA*ex), in a (**H** and **J**) *pilT*+ and (**I** and **K**) Δ*pilT* background, as indicated. Cells were grown in the presence of *tfoX* induction. Merged images show GFP in green and phase-contrast (PC) in red. Bar = 25 µm.

Figure 4

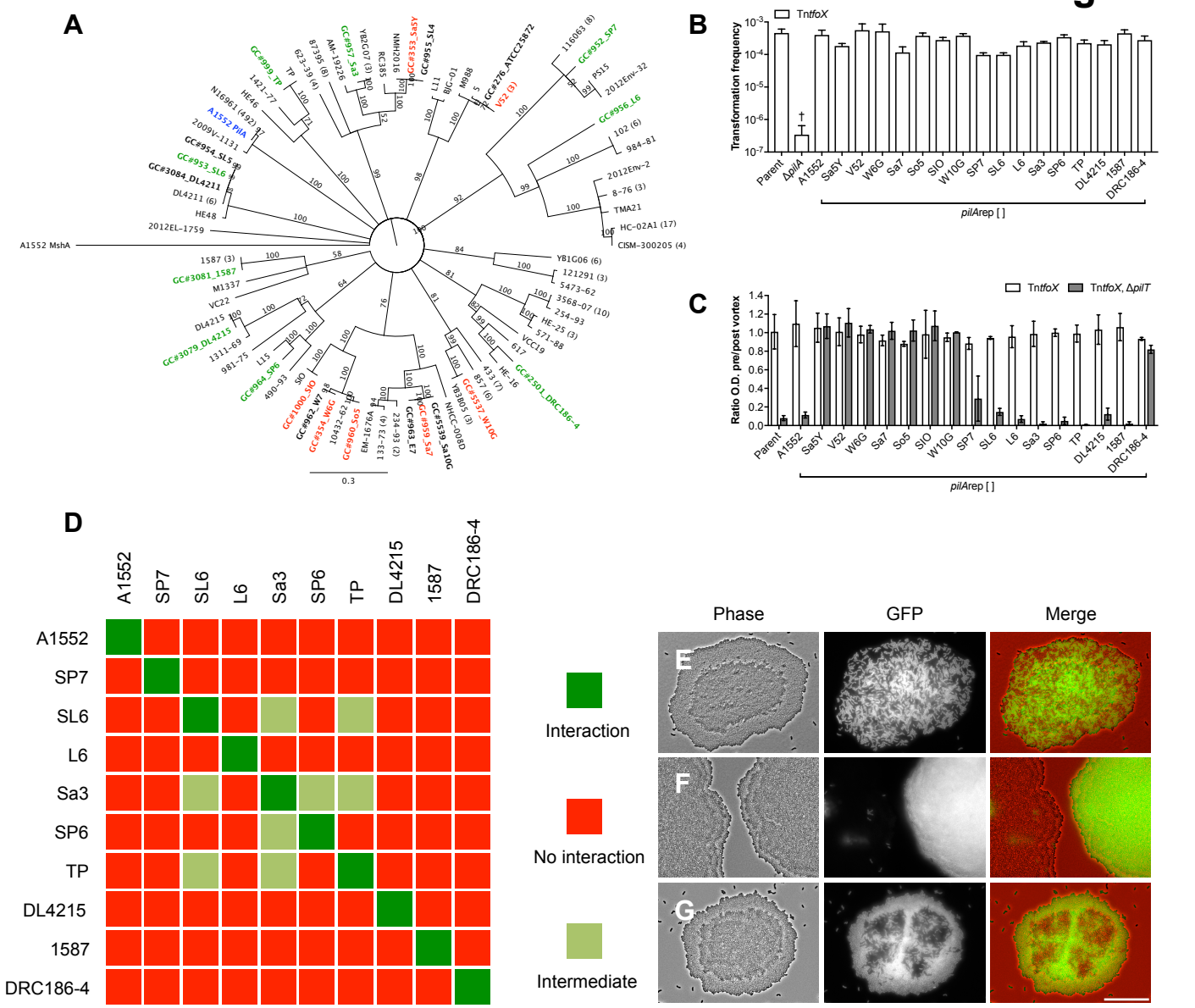
A
B
C
D
TntfoX
TntfoX, ΔpilT
Transformation frequency
Ratio O.D. pre/post vortex
pilArep [ ]
Parent
ΔpilA
A1552
Sa5Y
V52
W6G
Sa7
So5
SIO
W10G
SP7
SL6
L6
Sa3
SP6
TP
DL4215
1587
DRC186-4
Interaction
No interaction
Intermediate
Phase
GFP
Merge
E
F
G

Figure 4. PilA variability governs auto-aggregation and enables kin-recognition.

(**A**) Consensus neighbour-joining phylogenetic tree of PilA. The tree consists of the 56 unique PilA sequences identified from NCBI, 22 sequences from an in-house collection of various environmental and patient isolates (bold), and A1552 PilA (blue) and MshA (outgroup). Values shown are consensus support values (%). Aggregation capable (green) and incapable (red) PilAs tested in the *pilA*rep experiments are highlighted. Note that ATCC25872 and V52 PilA are identical.

(**B**-**C**) Functionality of A1552-Tn*tfoX*, *pilA*rep[A1552] and 16 different PilAs assessed by transformation frequency and aggregation. (**B**) Chitin-independent transformation frequency assay. WT parent (A1552-Tn*tfoX*) and Δ*pilA* (A1552-Tn*tfoX*, Δ*pilA*) strains served as positive and negative controls. Transformation frequencies are the mean of three repeats (+S.D.). †, <d.l. in one repeat. (**C**) Aggregation was determined for the WT parent and each *pilA*rep strain in a *pilT*+ (A1552-Tn*tfoX*) and Δ*pilT* (A1552-Tn*tfoX*, Δ*pilT*) background, as indicated. Aggregation is shown as the ratio of the culture optical density (O.D. $_{600}$) before and after vortexing, in the presence of *tfoX* induction. Values are the mean of three repeats (±S.D.).

(**D**) Interaction matrix showing the results of a pairwise analysis of all possible interactions between aggregation proficient PilA. Interactions were tested by co-culturing non-fluorescent cells of the relevant Tn*tfoX*, Δ*pilT*, pilA*rep*[] strains with a fluorescent (GFP+) derivative of each strain. Cells were grown in the presence of *tfoX* induction. Self-self combinations served as controls.

(**E**-**G**) Representative examples of (**E**) interaction, resulting in well-mixed aggregates (**F**) no interaction, resulting in fluorescent or non-fluorescent aggregates and (**G**) intermediate interactions, resulting in patterned aggregates. Cells were grown in the presence of *tfoX* induction. Merged images show GFP in green and phase-contrast (PC) in red. Bar = 25 µm.

Figure 5

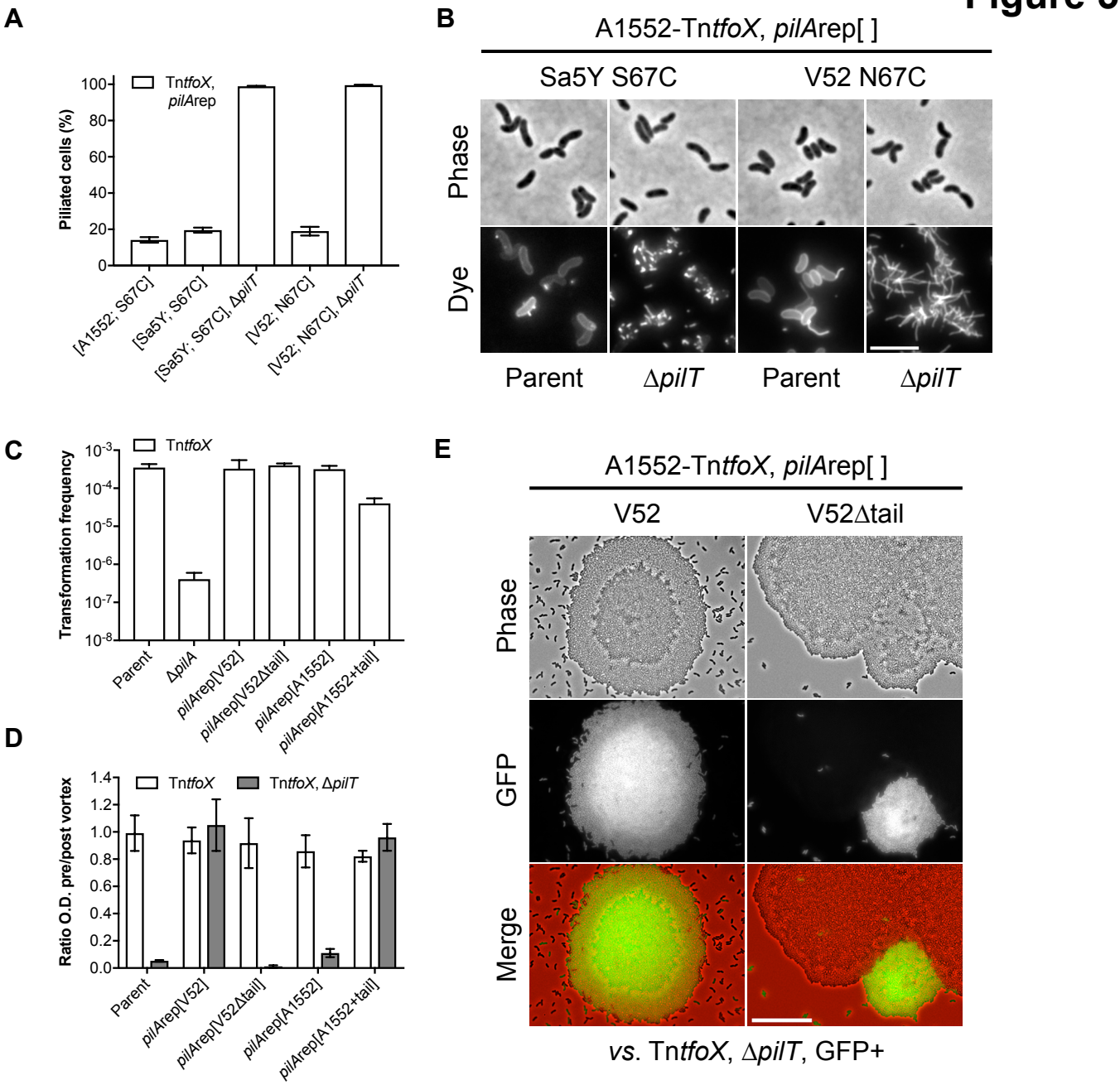
A
Piliated cells (%)
TntfoX, pilArep
100
80
60
40
20
0
[A1552; S67C]
[Sa5Y; S67C]
[Sa5Y; S67C], ΔpilT
[V52; N67C]
[V52; N67C], ΔpilT
B
A1552-TntfoX, pilArep[ ]
Sa5Y S67C
V52 N67C
Phase
Dye
Parent
ΔpilT
Parent
ΔpilT
C
Transformation frequency
TntfoX
10-3
10-4
10-5
10-6
10-7
10-8
Parent
ΔpilA
pilArep[V52]
pilArep[V52Δtail]
pilArep[A1552]
pilArep[A1552+tail]
D
Ratio O.D. pre/post vortex
TntfoX
TntfoX, ΔpilT
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Parent
pilArep[V52]
pilArep[V52Δtail]
pilArep[A1552]
pilArep[A1552+tail]
E
A1552-TntfoX, pilArep[ ]
V52
V52Δtail
Phase
GFP
Merge
vs. TntfoX, ΔpilT, GFP+

Figure 5. The unusual tail of ATCC25872/V52 PilA inhibits aggregation.

(**A**) Quantification of piliation in snapshot imaging of cells of strains A1552-Tn*tfoX*, *pilA*rep[A1552; S67C], A1552-Tn*tfoX*, *pilA*rep[Sa5Y; S67C] and A1552-Tn*tfoX*, *pilA*rep[V52; N67C], in the indicated backgrounds. Cells were grown with *tfoX* induction and pili stained with AF-488-Mal. Bars represent the mean of three repeats (±S.D.). *n* = *c.a.* 2000 cells per strain per repeat. (**B**) Direct observation of pili stained with AF-488-Mal in cells carrying *pilA*rep[Sa5Y; S67C] and *pilA*rep[V52; N67C], in a WT parent (A1552-Tn*tfoX*) and Δ*pilT* (A1552-Tn*tfoX*, Δ*pilT*) background, as indicated. Bar = 5 µm.

(**C**-**D**) Functionality of *pilA*rep tail variants assessed by natural transformation and aggregation. (**C**) Chitin-independent transformation assay. WT parent (A1552-Tn*tfoX*) and Δ*pilA* (A1552-Tn*tfoX*, Δ*pilA*) strains served as positive and negative controls. Transformation frequencies are the mean of three repeats (+S.D.). (**D**) Aggregation was determined for the WT parent and each *pilA*rep strain in a *pilT*+ (A1552-Tn*tfoX*) and Δ*pilT* (A1552-Tn*tfoX*, Δ*pilT*) background, as indicated. Aggregation is shown as the ratio of the culture optical density ($O.D._{600}$) before and after vortexing, in the presence of *tfoX* induction. Values are the mean of three repeats (±S.D.).

(**E**) Co-culture of fluorescent cells of A1552-Tn*tfoX*, Δ*pilT*, GFP+, producing GFP, and non-fluorescent cells of either *pilA*rep[V52] (A1552-Tn*tfoX*, Δ*pilT*, *pilA*rep[V52]) or *pilA*rep[V52Δtail] (A1552-Tn*tfoX*, Δ*pilT*, *pilA*rep[V52Δtail]), as indicated. Note that ATCC25872 and V52 PilA are identical. Cells were grown in the presence of *tfoX* induction. Merged images show GFP in green and phase-contrast in red. Bar = 25 µm.

Figure 6

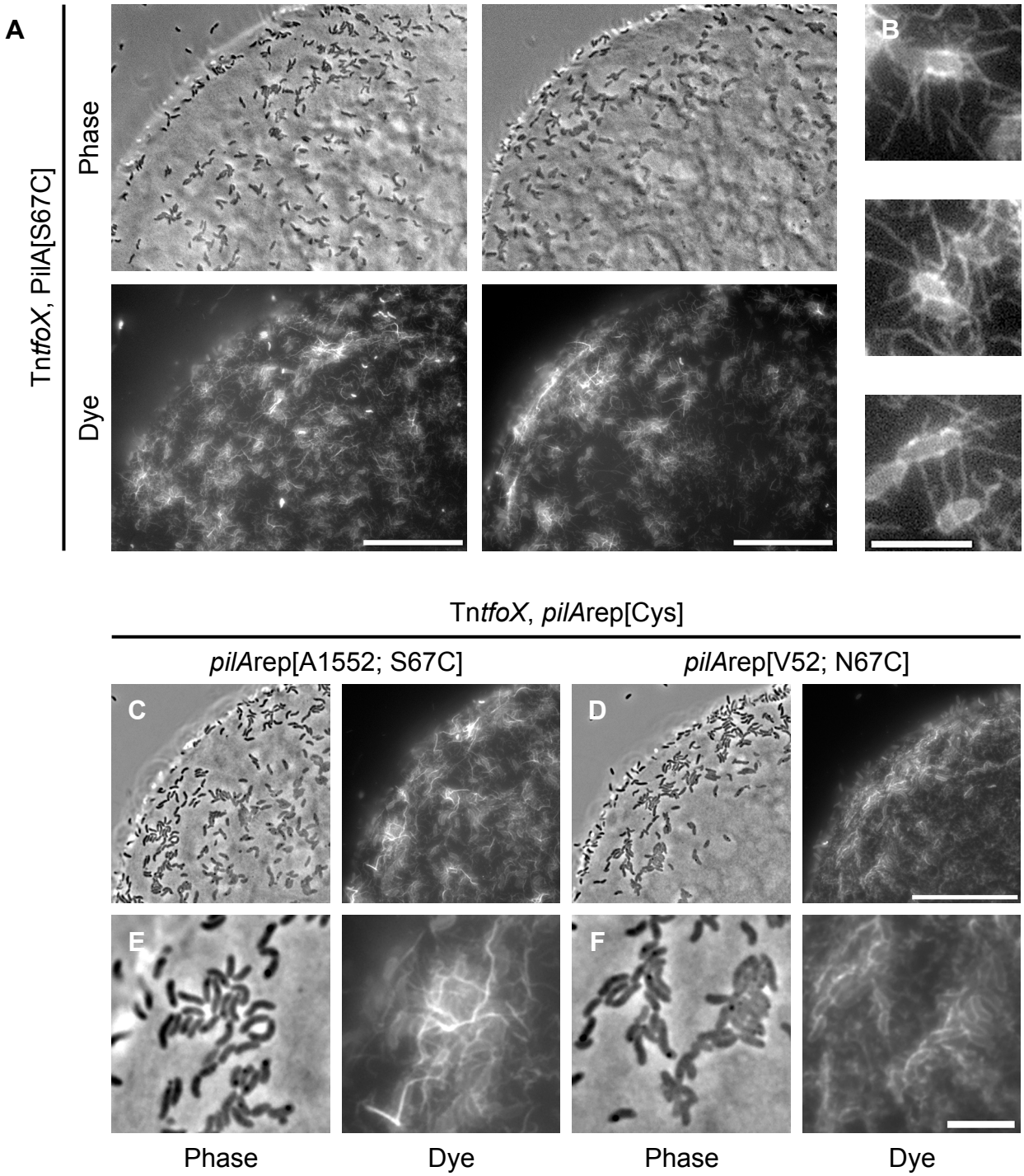
A
Tn*tfoX*, PilA[S67C]
Phase
Dye
B
Tn*tfoX*, *pilA*rep[Cys]
*pilA*rep[A1552; S67C]
*pilA*rep[V52; N67C]
C
D
E
F
Phase
Dye
Phase
Dye

Figure 6. DNA-uptake pili form networks on chitin surfaces.

(**A-B**) Chitin beads were stained with AF-488-Mal after incubation for 48h in defined artificial seawater (DASW) with cells of A1552-Tn*tfoX*, PilA[S67C], as indicated. The figure depicts two separate examples of colonised surfaces, bars = 25 µm. (**B**) Insets show enlarged examples of cells producing multiple pili. Bar = 5 µm.

(**C**-**F**) Chitin beads were stained with AF-488-Mal after incubation for 48h in DASW with cells of either (**C**) A1552-Tn*tfoX*, *pilA*rep[A1552; S67C] or (**D**) A1552-Tn*tfoX*, *pilA*rep[V52; N67C], as indicated. Bar = 25 µm. Panels (**E** and **F**) show enlargements of the surfaces shown in (**C** and **D**). Note the absence of large pili networks in (**F**). Bar = 5 µm.