

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

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11 **Abstract**

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

28

29 **Keywords**

30 Auto-aggregation, DNA uptake, natural transformation, pilus dynamics, type IV pilus, *Vibrio*
31 *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 adhesion³⁻⁵. Consequently, T4P are critical virulence factors for a number of important
38 human pathogens including *Vibrio cholerae*, which causes the pandemic diarrhoeal disease
39 cholera⁶. 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 secretin⁷⁻¹¹. 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 bacteriophage¹⁶, 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 surface¹⁷⁻¹⁹. Second, Mannose-sensitive haemagglutinin (MSHA) pili are involved in surface
53 sensing and attachment and thus, are important in the initiation of biofilm formation²⁰⁻²⁴.
54 Third, in its natural aquatic environment *V. cholerae* often associates with chitinous
55 surfaces²⁵, which are nutritious, foster biofilm formation and likely play a role in
56 environmental dissemination and transmission to humans²⁶⁻²⁹. Chitin utilisation triggers
57 competence for natural transformation³⁰, 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 evolution³¹. This requires the production of the Chitin-Regulated (ChiRP) or
60 DNA-uptake pilus³⁰.

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 PilT³². 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 periplasm³⁵, wherein ComEA acts as ‘ratchet’ to pull in the remaining
68 DNA³³. 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 experiments³²
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 pili³⁹. 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 $\alpha\beta$ -loop^{9,11} and tested for functionality using a previously
86 validated chitin-independent transformation system in which competence induction is
87 arabinose-inducible⁴¹ (*TntfoX*; 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 transformation³². 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 μ m, 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 *TntfoX* 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 formation⁴⁴ 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 *pglLvc*)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.* CaCl₂ or MgCl₂) (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 (*TntfoX*), static pili (*TntfoX*,
173 *ΔpilT*), or blocked for both retraction and pilus production (*TntfoX*, *ΔpilT*, *ΔpilA*) with a
174 *TntfoX* *ΔpilT* 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 function²⁰⁻²⁴. 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 surface⁴³. 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 mutation⁵¹, 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 QS⁴¹ 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 (*pilAex*). As expected, the ATCC25872 *pilAex* 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 (α 1), 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 (*pilArep*)
246 approach using A1552 PilA (*i.e.* *TntfoX*, *pilArep[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 Tor⁵⁸⁻⁶⁰. In contrast to the results above and in line with
275 previous work⁵⁸, 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).

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, $\Delta pilT$ 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 *pilArep* 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 *pilArep*[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 *pilArep*[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 DNA⁶¹. 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 defences⁶⁵. 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 humans²⁶ and recovering cholera patients
378 exhibit a strong immune response to PilA⁶⁷. 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 pili⁶⁸. 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; Myxobacteria⁷²). 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 species⁷⁷ 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 work⁷⁸, 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 Peru⁷⁹.

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 described³⁰. 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 methods⁸⁰,
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-Sac28²⁹. 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 described⁸⁵.

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 production⁴¹. 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 TntfoX. In the presence of inducer, strains carrying
455 TntfoX 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 chitin⁴¹. In the absence of inducer, competence genes are not produced and strains
458 are non-transformable⁴¹.

459

460 **Transformation frequency assay**

461 Diverse strains harbouring TntfoX 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 \times /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 FluorTM 488 C₅ 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 μ L of culture was mixed
509 with dye at a final concentration of 25 μ g/mL³⁹ and incubated at RT for 5 min in the dark.
510 Stained cells were harvested by centrifugation (5000 $\times g$; 1 min), washed once with LB, re-
511 suspended in 200 μ L 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 (≥ 72 h), 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 previously⁴¹. 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 quantify 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 ($\Delta flaA$) 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.

580 **References**

581 1 Maier, B. & Wong, G. C. L. How Bacteria Use Type IV Pili Machinery on Surfaces.
582 *Trends in microbiology* **23**, 775-788, doi:10.1016/j.tim.2015.09.002 (2015).

583 2 Persat, A. *et al.* The mechanical world of bacteria. *Cell* **161**, 988-997,
584 doi:10.1016/j.cell.2015.05.005 (2015).

585 3 Berry, J. L. & Pelicic, V. Exceptionally widespread nanomachines composed of type IV
586 pilins: the prokaryotic Swiss Army knives. *FEMS microbiology reviews* **39**, 134-154,
587 doi:10.1093/femsre/fuu001 (2015).

588 4 Giltner, C. L., Nguyen, Y. & Burrows, L. L. Type IV pilin proteins: versatile molecular
589 modules. *Microbiology and molecular biology reviews : MMBR* **76**, 740-772,
590 doi:10.1128/MMBR.00035-12 (2012).

591 5 Melville, S. & Craig, L. Type IV pili in Gram-positive bacteria. *Microbiology and*
592 *molecular biology reviews : MMBR* **77**, 323-341, doi:10.1128/MMBR.00063-12
593 (2013).

594 6 Clemens, J. D., Nair, G. B., Ahmed, T., Qadri, F. & Holmgren, J. Cholera. *Lancet* **390**,
595 1539-1549, doi:10.1016/S0140-6736(17)30559-7 (2017).

596 7 Chang, Y. W. *et al.* Architecture of the *Vibrio cholerae* toxin-coregulated pilus
597 machine revealed by electron cryotomography. *Nature microbiology* **2**, 16269,
598 doi:10.1038/nmicrobiol.2016.269 (2017).

599 8 Chang, Y. W. *et al.* Architecture of the type IVa pilus machine. *Science* **351**, aad2001,
600 doi:10.1126/science.aad2001 (2016).

601 9 Craig, L. *et al.* Type IV pilus structure by cryo-electron microscopy and
602 crystallography: implications for pilus assembly and functions. *Molecular cell* **23**,
603 651-662, doi:10.1016/j.molcel.2006.07.004 (2006).

604 10 Gold, V. A., Salzer, R., Averhoff, B. & Kuhlbrandt, W. Structure of a type IV pilus
605 machinery in the open and closed state. *eLife* **4**, doi:10.7554/eLife.07380 (2015).

606 11 Kolappan, S. *et al.* Structure of the *Neisseria meningitidis* Type IV pilus. *Nature*
607 *communications* **7**, 13015, doi:10.1038/ncomms13015 (2016).

608 12 Merz, A. J., So, M. & Sheetz, M. P. Pilus retraction powers bacterial twitching
609 motility. *Nature* **407**, 98-102, doi:10.1038/35024105 (2000).

610 13 Skerker, J. M. & Berg, H. C. Direct observation of extension and retraction of type IV
611 pili. *Proceedings of the National Academy of Sciences of the United States of America*
612 **98**, 6901-6904, doi:10.1073/pnas.121171698 (2001).

613 14 Jakovljevic, V., Leonardy, S., Hoppert, M. & Sogaard-Andersen, L. PilB and PilT are
614 ATPases acting antagonistically in type IV pilus function in *Myxococcus xanthus*.
615 *Journal of bacteriology* **190**, 2411-2421, doi:10.1128/JB.01793-07 (2008).

616 15 McCallum, M., Tammam, S., Khan, A., Burrows, L. L. & Howell, P. L. The molecular
617 mechanism of the type IVa pilus motors. *Nature communications* **8**, 15091,
618 doi:10.1038/ncomms15091 (2017).

619 16 Waldor, M. K. & Mekalanos, J. J. Lysogenic conversion by a filamentous phage
620 encoding cholera toxin. *Science* **272**, 1910-1914 (1996).

621 17 Chiang, S. L., Taylor, R. K., Koomey, M. & Mekalanos, J. J. Single amino acid
622 substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization,
623 autoagglutination, and serum resistance. *Molecular microbiology* **17**, 1133-1142
624 (1995).

625 18 Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. Use of *phoA* gene fusions
626 to identify a pilus colonization factor coordinately regulated with cholera toxin.
627 *Proceedings of the National Academy of Sciences of the United States of America* **84**,
628 2833-2837 (1987).

629 19 Kirn, T. J., Lafferty, M. J., Sandoe, C. M. & Taylor, R. K. Delineation of pilin domains
630 required for bacterial association into microcolonies and intestinal colonization by
631 *Vibrio cholerae*. *Molecular microbiology* **35**, 896-910 (2000).

632 20 Chiavelli, D. A., Marsh, J. W. & Taylor, R. K. The mannose-sensitive hemagglutinin of
633 *Vibrio cholerae* promotes adherence to zooplankton. *Applied and environmental*
634 *microbiology* **67**, 3220-3225, doi:10.1128/AEM.67.7.3220-3225.2001 (2001).

635 21 Moorthy, S. & Watnick, P. I. Genetic evidence that the *Vibrio cholerae* monolayer is a
636 distinct stage in biofilm development. *Molecular microbiology* **52**, 573-587,
637 doi:10.1111/j.1365-2958.2004.04000.x (2004).

638 22 Utada, A. S. *et al.* *Vibrio cholerae* use pili and flagella synergistically to effect motility
639 switching and conditional surface attachment. *Nature communications* **5**, 4913,
640 doi:10.1038/ncomms5913 (2014).

641 23 Watnick, P. I., Fullner, K. J. & Kolter, R. A role for the mannose-sensitive
642 hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *Journal of bacteriology*
643 **181**, 3606-3609 (1999).

644 24 Watnick, P. I. & Kolter, R. Steps in the development of a *Vibrio cholerae* El Tor
645 biofilm. *Molecular microbiology* **34**, 586-595 (1999).

646 25 Tamplin, M. L., Gauzens, A. L., Huq, A., Sack, D. A. & Colwell, R. R. Attachment of
647 *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh
648 waters. *Applied and environmental microbiology* **56**, 1977-1980 (1990).

649 26 Blokesch, M. Competence-induced type VI secretion might foster intestinal
650 colonization by *Vibrio cholerae*: Intestinal interbacterial killing by competence-
651 induced *V. cholerae*. *BioEssays : news and reviews in molecular, cellular and*
652 *developmental biology* **37**, 1163-1168, doi:10.1002/bies.201500101 (2015).

653 27 Colwell, R. R. *et al.* Reduction of cholera in Bangladeshi villages by simple filtration.
654 *Proceedings of the National Academy of Sciences of the United States of America*
655 **100**, 1051-1055, doi:10.1073/pnas.0237386100 (2003).

656 28 Huq, A. *et al.* A simple filtration method to remove plankton-associated *Vibrio*
657 *cholerae* in raw water supplies in developing countries. *Applied and environmental*
658 *microbiology* **62**, 2508-2512 (1996).

659 29 Meibom, K. L. *et al.* The *Vibrio cholerae* chitin utilization program. *Proceedings of the*
660 *National Academy of Sciences of the United States of America* **101**, 2524-2529
661 (2004).

662 30 Meibom, K. L., Blokesch, M., Dolganov, N. A., Wu, C. Y. & Schoolnik, G. K. Chitin
663 induces natural competence in *Vibrio cholerae*. *Science* **310**, 1824-1827,
664 doi:10.1126/science.1120096 (2005).

665 31 Johnston, C., Martin, B., Fichant, G., Polard, P. & Claverys, J. P. Bacterial
666 transformation: distribution, shared mechanisms and divergent control. *Nature*
667 *reviews. Microbiology* **12**, 181-196, doi:10.1038/nrmicro3199 (2014).

668 32 Seitz, P. & Blokesch, M. DNA-uptake machinery of naturally competent *Vibrio*
669 *cholerae*. *Proceedings of the National Academy of Sciences of the United States of*
670 *America* **110**, 17987-17992, doi:10.1073/pnas.1315647110 (2013).

671 33 Seitz, P. *et al.* ComEA is essential for the transfer of external DNA into the periplasm
672 in naturally transformable *Vibrio cholerae* cells. *PLoS genetics* **10**, e1004066,
673 doi:10.1371/journal.pgen.1004066 (2014).

674 34 Seitz, P. & Blokesch, M. DNA transport across the outer and inner membranes of
675 naturally transformable *Vibrio cholerae* is spatially but not temporally coupled. *mBio*
676 **5**, doi:10.1128/mBio.01409-14 (2014).

677 35 Wolfgang, M. *et al.* PilT mutations lead to simultaneous defects in competence for
678 natural transformation and twitching motility in pilated *Neisseria gonorrhoeae*.
679 *Molecular microbiology* **29**, 321-330 (1998).

680 36 Draskovic, I. & Dubnau, D. Biogenesis of a putative channel protein, ComEC, required
681 for DNA uptake: membrane topology, oligomerization and formation of disulphide
682 bonds. *Molecular microbiology* **55**, 881-896, doi:10.1111/j.1365-2958.2004.04430.x
683 (2005).

684 37 Gangel, H. *et al.* Concerted spatio-temporal dynamics of imported DNA and ComE
685 DNA uptake protein during gonococcal transformation. *PLoS pathogens* **10**,
686 e1004043, doi:10.1371/journal.ppat.1004043 (2014).

687 38 Laurenceau, R. *et al.* A type IV pilus mediates DNA binding during natural
688 transformation in *Streptococcus pneumoniae*. *PLoS pathogens* **9**, e1003473,
689 doi:10.1371/journal.ppat.1003473 (2013).

690 39 Ellison, C. K. *et al.* Obstruction of pilus retraction stimulates bacterial surface
691 sensing. *Science* **358**, 535-538, doi:10.1126/science.aan5706 (2017).

692 40 Blair, K. M., Turner, L., Winkelman, J. T., Berg, H. C. & Kearns, D. B. A molecular
693 clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320**, 1636-1638,
694 doi:10.1126/science.1157877 (2008).

695 41 Lo Scudato, M. & Blokesch, M. The regulatory network of natural competence and
696 transformation of *Vibrio cholerae*. *PLoS genetics* **8**, e1002778,
697 doi:10.1371/journal.pgen.1002778 (2012).

698 42 Metzger, L. C. & Blokesch, M. Composition of the DNA-uptake complex of *Vibrio*
699 *cholerae*. *Mobile genetic elements* **4**, e28142, doi:10.4161/mge.28142 (2014).

700 43 Jones, C. J. *et al.* C-di-GMP Regulates Motile to Sessile Transition by Modulating
701 MshA Pili Biogenesis and Near-Surface Motility Behavior in *Vibrio cholerae*. *PLoS*
702 *pathogens* **11**, e1005068, doi:10.1371/journal.ppat.1005068 (2015).

703 44 Fong, J. C., Syed, K. A., Klose, K. E. & Yildiz, F. H. Role of Vibrio polysaccharide (vps)
704 genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis.
705 *Microbiology* **156**, 2757-2769, doi:10.1099/mic.0.040196-0 (2010).

706 45 Absalon, C., Van Dellen, K. & Watnick, P. I. A communal bacterial adhesin anchors
707 biofilm and bystander cells to surfaces. *PLoS pathogens* **7**, e1002210,
708 doi:10.1371/journal.ppat.1002210 (2011).

709 46 Berk, V. *et al.* Molecular architecture and assembly principles of *Vibrio cholerae*
710 biofilms. *Science* **337**, 236-239, doi:10.1126/science.1222981 (2012).

711 47 Kirn, T. J., Jude, B. A. & Taylor, R. K. A colonization factor links *Vibrio cholerae*
712 environmental survival and human infection. *Nature* **438**, 863-866,
713 doi:10.1038/nature04249 (2005).

714 48 Bartlett, T. M. *et al.* A Periplasmic Polymer Curves *Vibrio cholerae* and Promotes
715 Pathogenesis. *Cell* **168**, 172-185 e115, doi:10.1016/j.cell.2016.12.019 (2017).

716 49 Gebhart, C. *et al.* Characterization of exogenous bacterial oligosaccharyltransferases
717 in *Escherichia coli* reveals the potential for O-linked protein glycosylation in *Vibrio*
718 *cholerae* and *Burkholderia thailandensis*. *Glycobiology* **22**, 962-974,
719 doi:10.1093/glycob/cws059 (2012).

720 50 Biais, N., Ladoux, B., Higashi, D., So, M. & Sheetz, M. Cooperative retraction of
721 bundled type IV pili enables nanonewton force generation. *PLoS biology* **6**, e87,
722 doi:10.1371/journal.pbio.0060087 (2008).

723 51 Joelsson, A., Liu, Z. & Zhu, J. Genetic and phenotypic diversity of quorum-sensing
724 systems in clinical and environmental isolates of *Vibrio cholerae*. *Infection and*
725 *immunity* **74**, 1141-1147, doi:10.1128/IAI.74.2.1141-1147.2006 (2006).

726 52 Aagesen, A. M. & Häse, C. C. Sequence analyses of type IV pili from *Vibrio cholerae*,
727 *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Microbial ecology* **64**, 509-524,
728 doi:10.1007/s00248-012-0021-2 (2012).

729 53 Aldova, E., Laznickova, K., Stepankova, E. & Lietava, J. Isolation of nonagglutinable
730 vibrios from an enteritis outbreak in Czechoslovakia. *The Journal of infectious*
731 *diseases* **118**, 25-31 (1968).

732 54 Chun, J. *et al.* Comparative genomics reveals mechanism for short-term and long-
733 term clonal transitions in pandemic *Vibrio cholerae*. *Proceedings of the National*
734 *Academy of Sciences of the United States of America* **106**, 15442-15447,
735 doi:10.1073/pnas.0907787106 (2009).

736 55 Blokesch, M. & Schoolnik, G. K. Serogroup conversion of *Vibrio cholerae* in aquatic
737 reservoirs. *PLoS pathogens* **3**, e81, doi:10.1371/journal.ppat.0030081 (2007).

738 56 Li, M., Shimada, T., Morris, J. G., Jr., Sulakvelidze, A. & Sozhamannan, S. Evidence for
739 the emergence of non-O1 and non-O139 *Vibrio cholerae* strains with pathogenic
740 potential by exchange of O-antigen biosynthesis regions. *Infection and immunity* **70**,
741 2441-2453 (2002).

742 57 DiRita, V. J., Neely, M., Taylor, R. K. & Bruss, P. M. Differential expression of the ToxR
743 regulon in classical and E1 Tor biotypes of *Vibrio cholerae* is due to biotype-specific
744 control over toxT expression. *Proceedings of the National Academy of Sciences of*
745 *the United States of America* **93**, 7991-7995 (1996).

746 58 Jude, B. A. & Taylor, R. K. The physical basis of type 4 pilus-mediated microcolony
747 formation by *Vibrio cholerae* O1. *Journal of structural biology* **175**, 1-9,
748 doi:10.1016/j.jsb.2011.04.008 (2011).

749 59 Lim, M. S. *et al.* *Vibrio cholerae* El Tor TcpA crystal structure and mechanism for
750 pilus-mediated microcolony formation. *Molecular microbiology* **77**, 755-770,
751 doi:10.1111/j.1365-2958.2010.07244.x (2010).

752 60 Rhine, J. A. & Taylor, R. K. TcpA pilin sequences and colonization requirements for
753 O1 and O139 *Vibrio cholerae*. *Molecular microbiology* **13**, 1013-1020 (1994).

754 61 Ellison, C. K. *et al.* Retraction of DNA-bound type IV competence pili initiates DNA
755 uptake during natural transformation in *Vibrio cholerae*. *Nature microbiology* **3**, 773-
756 780, doi:10.1038/s41564-018-0174-y (2018).

757 62 Hélaine, S. *et al.* PilX, a pilus-associated protein essential for bacterial aggregation, is
758 a key to pilus-facilitated attachment of *Neisseria meningitidis* to human cells.
759 *Molecular microbiology* **55**, 65-77, doi:10.1111/j.1365-2958.2004.04372.x (2005).

760 63 Imhaus, A. F. & Duménil, G. The number of *Neisseria meningitidis* type IV pili
761 determines host cell interaction. *The EMBO journal* **33**, 1767-1783,
762 doi:10.15252/embj.201488031 (2014).

763 64 Nielsen, A. T. *et al.* A bistable switch and anatomical site control *Vibrio cholerae*
764 virulence gene expression in the intestine. *PLoS pathogens* **6**, e1001102,
765 doi:10.1371/journal.ppat.1001102 (2010).

766 65 Krebs, S. J. & Taylor, R. K. Protection and attachment of *Vibrio cholerae* mediated by
767 the toxin-coregulated pilus in the infant mouse model. *Journal of bacteriology* **193**,
768 5260-5270, doi:10.1128/JB.00378-11 (2011).

769 66 Shime-Hattori, A. *et al.* Two type IV pili of *Vibrio parahaemolyticus* play different
770 roles in biofilm formation. *FEMS microbiology letters* **264**, 89-97,
771 doi:10.1111/j.1574-6968.2006.00438.x (2006).

772 67 Hang, L. *et al.* Use of in vivo-induced antigen technology (IVIAT) to identify genes
773 uniquely expressed during human infection with *Vibrio cholerae*. *Proceedings of the*
774 *National Academy of Sciences of the United States of America* **100**, 8508-8513,
775 doi:10.1073/pnas.1431769100 (2003).

776 68 Oldewurtel, E. R., Kouzel, N., Dewenter, L., Henseler, K. & Maier, B. Differential
777 interaction forces govern bacterial sorting in early biofilms. *eLife* **4**,
778 doi:10.7554/eLife.10811 (2015).

779 69 Chamot-Rooke, J. *et al.* Posttranslational modification of pili upon cell contact
780 triggers *N. meningitidis* dissemination. *Science* **331**, 778-782,
781 doi:10.1126/science.1200729 (2011).

782 70 Smukalla, S. *et al.* FLO1 is a variable green beard gene that drives biofilm-like
783 cooperation in budding yeast. *Cell* **135**, 726-737, doi:10.1016/j.cell.2008.09.037
784 (2008).

785 71 Hirose, S., Benabentos, R., Ho, H. I., Kuspa, A. & Shaulsky, G. Self-recognition in
786 social amoebae is mediated by allelic pairs of tiger genes. *Science* **333**, 467-470,
787 doi:10.1126/science.1203903 (2011).

788 72 Pathak, D. T., Wei, X., Dey, A. & Wall, D. Molecular recognition by a polymorphic cell
789 surface receptor governs cooperative behaviors in bacteria. *PLoS genetics* **9**,
790 e1003891, doi:10.1371/journal.pgen.1003891 (2013).

791 73 Strassmann, J. E., Gilbert, O. M. & Queller, D. C. Kin discrimination and cooperation
792 in microbes. *Annual review of microbiology* **65**, 349-367,
793 doi:10.1146/annurev.micro.112408.134109 (2011).

794 74 Wall, D. Kin Recognition in Bacteria. *Annual review of microbiology* **70**, 143-160,
795 doi:10.1146/annurev-micro-102215-095325 (2016).

796 75 Borgeaud, S., Metzger, L. C., Scignari, T. & Blokesch, M. The type VI secretion
797 system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* **347**, 63-67,
798 doi:10.1126/science.1260064 (2015).

799 76 Ho, B. T., Dong, T. G. & Mekalanos, J. J. A view to a kill: the bacterial type VI
800 secretion system. *Cell host & microbe* **15**, 9-21, doi:10.1016/j.chom.2013.11.008
801 (2014).

802 77 Trunk, T., Khalil, H. S. & Leo, J. C. Bacterial autoaggregation. *AIMS Microbiology* **4**,
803 140-164, doi:<http://dx.doi.org/10.3934/microbiol.2018.1.140> (2018).

804 78 Yildiz, F. H. & Schoolnik, G. K. Role of *rpoS* in stress survival and virulence of *Vibrio*
805 *cholerae*. *Journal of bacteriology* **180**, 773-784 (1998).

806 79 Blokesch, M. A quorum sensing-mediated switch contributes to natural
807 transformation of *Vibrio cholerae*. *Mobile genetic elements* **2**, 224-227,
808 doi:10.4161/mge.22284 (2012).

809 80 Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular Cloning: A Laboratory Manual.
810 *Cold Spring Harbor: Cold Spring Harbor Laboratory Press* (1989).

811 81 De Souza Silva, O. & Blokesch, M. Genetic manipulation of *Vibrio cholerae* by
812 combining natural transformation with FLP recombination. *Plasmid* **64**, 186-195,
813 doi:10.1016/j.plasmid.2010.08.001 (2010).

814 82 Marvig, R. L. & Blokesch, M. Natural transformation of *Vibrio cholerae* as a tool -
815 optimizing the procedure. *BMC microbiology* **10**, 155, doi:10.1186/1471-2180-10-
816 155 (2010).

817 83 Van der Henst, C. *et al.* Molecular insights into *Vibrio cholerae*'s intra-amoebal host-
818 pathogen interactions. *Nature communications*, doi:10.1038/s41467-018-05976-x
819 (2018).

820 84 Gurung, I., Berry, J. L., Hall, A. M. J. & Pelicic, V. Cloning-independent markerless
821 gene editing in *Streptococcus sanguinis*: novel insights in type IV pilus biology.
822 *Nucleic acids research* **45**, e40, doi:10.1093/nar/gkw1177 (2017).

823 85 Bao, Y., Lies, D. P., Fu, H. & Roberts, G. P. An improved Tn7-based system for the
824 single-copy insertion of cloned genes into chromosomes of gram-negative bacteria.
825 *Gene* **109**, 167-168 (1991).

826 86 Yamamoto, S. *et al.* Regulation of natural competence by the orphan two-
827 component system sensor kinase ChiS involves a non-canonical transmembrane
828 regulator in *Vibrio cholerae*. *Molecular microbiology* **91**, 326-347,
829 doi:10.1111/mmi.12462 (2014).

830 87 Dalia, A. B., Lazinski, D. W. & Camilli, A. Identification of a membrane-bound
831 transcriptional regulator that links chitin and natural competence in *Vibrio cholerae*.
832 *mBio* **5**, e01028-01013, doi:10.1128/mBio.01028-13 (2014).
833 88 Yamamoto, S. *et al.* Identification of a chitin-induced small RNA that regulates
834 translation of the *tfoX* gene, encoding a positive regulator of natural competence in
835 *Vibrio cholerae*. *Journal of bacteriology* **193**, 1953-1965, doi:10.1128/JB.01340-10
836 (2011).
837 89 Lo Scudato, M. & Blokesch, M. A transcriptional regulator linking quorum sensing
838 and chitin induction to render *Vibrio cholerae* naturally transformable. *Nucleic acids*
839 *research* **41**, 3644-3658, doi:10.1093/nar/gkt041 (2013).
840 90 Kearse, M. *et al.* Geneious Basic: an integrated and extendable desktop software
841 platform for the organization and analysis of sequence data. *Bioinformatics* **28**,
842 1647-1649, doi:10.1093/bioinformatics/bts199 (2012).

Figure 1

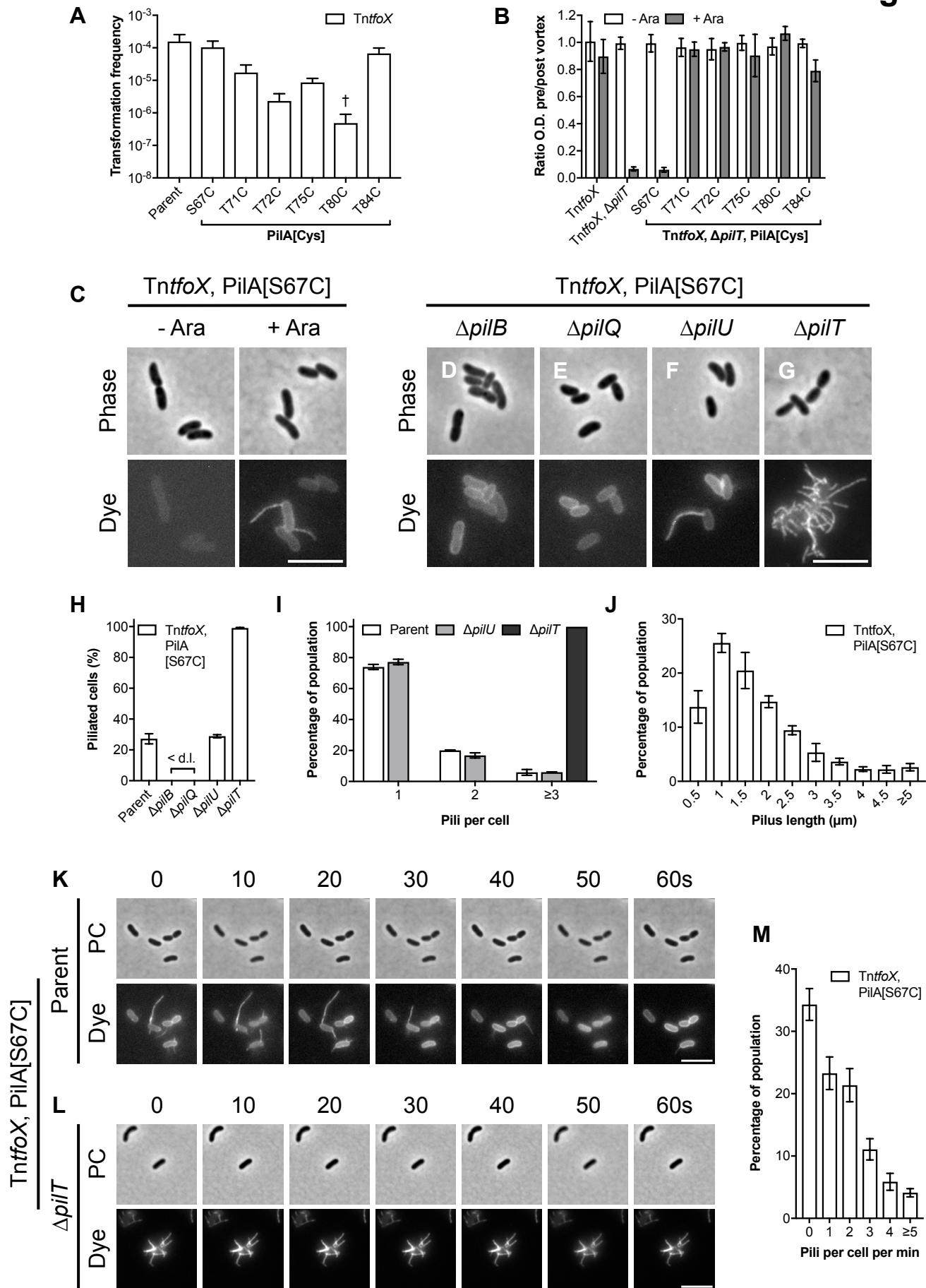


Figure 1. Direct observation of dynamic DNA-uptake pili.

(A) Functionality of PilA cysteine variants (PilA[Cys]) in a chitin-independent transformation assay using strains carrying an arabinose-inducible copy of *tfoX* (TntfoX). 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 (TntfoX, 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 (\pm S.D.).

(C-G) Snapshot imaging of pili in cells of A1552-TntfoX, PilA[S67C] and its derivatives. **(C)** Cells of A1552-TntfoX, PilA[S67C] were grown in the absence (- Ara) and presence (+ Ara) of *tfoX* induction, as indicated, and stained with AF-488-Mal. Bar = 5 μ m. **(D-E)** Cells of A1552-TntfoX, 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 μ m.

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

(K-L) Time-lapse series of pilus dynamics in **(K)** WT parent (A1552-TntfoX, PilA[S67C]) and **(L)** Δ pilT (A1552-TntfoX, 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 μ m.

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

Figure 2

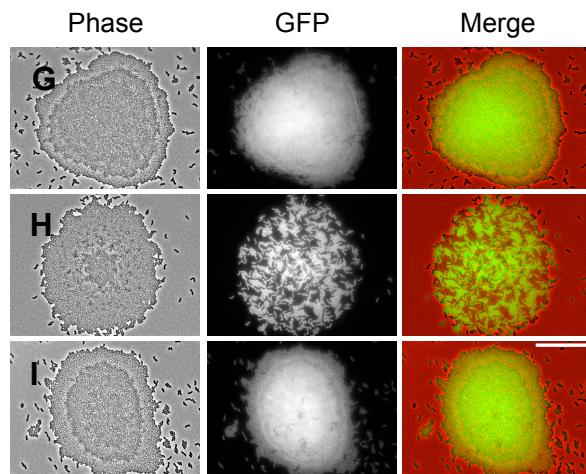
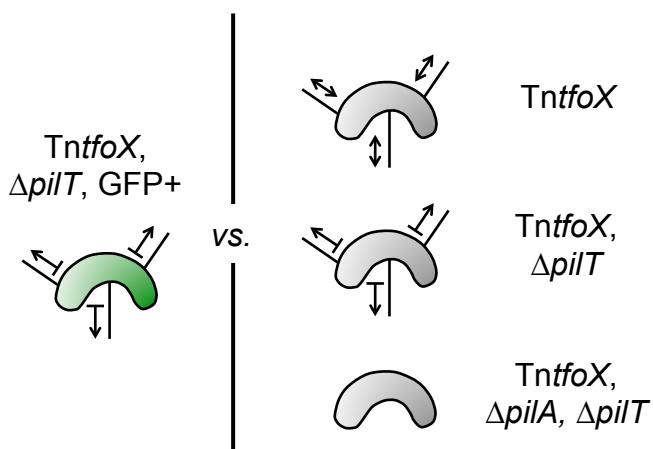
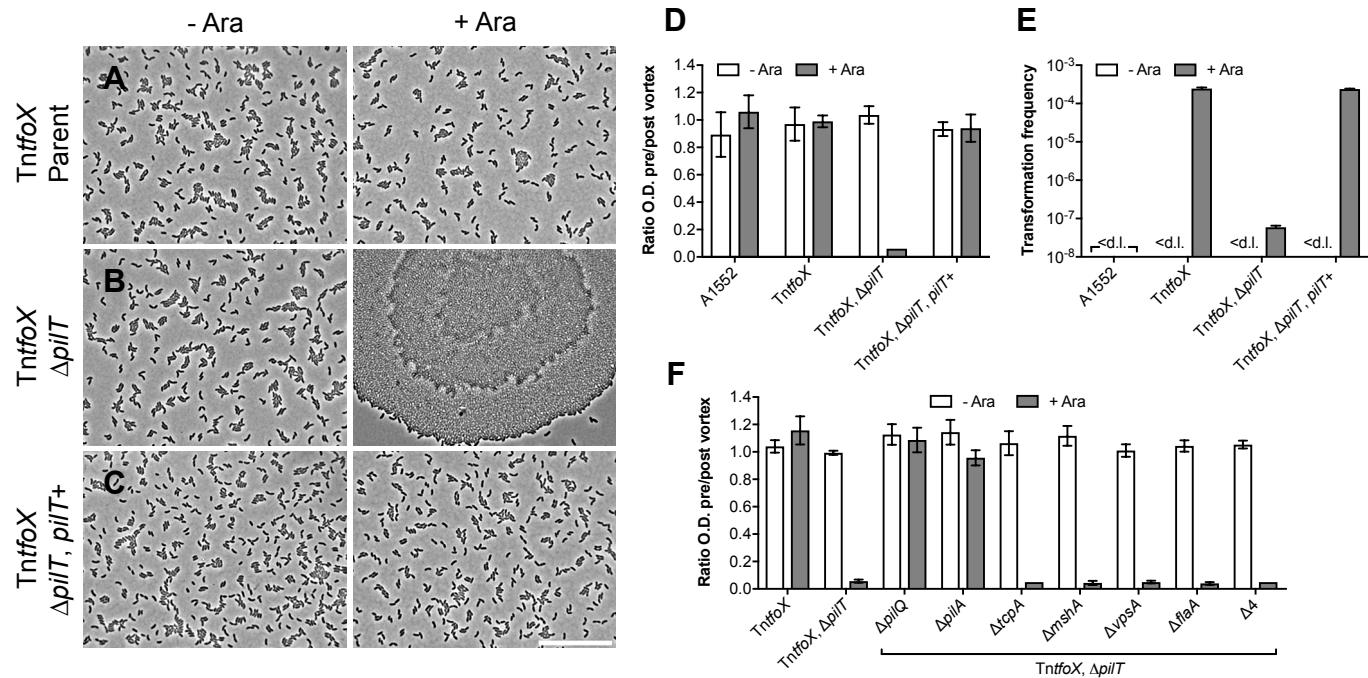


Figure 2. Competent cells auto-aggregate in the absence of pilus retraction.

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

(D-E) Aggregation and transformation frequency of cells of strains A1552-TntfoX, A1552-TntfoX, Δ pilT and A1552-TntfoX, 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 (\pm 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. Δ 4 = Δ tcpA, Δ mshA, Δ vpsA, Δ flaA quadruple mutant. Values are the mean of three repeats (\pm S.D.).

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

Figure 3

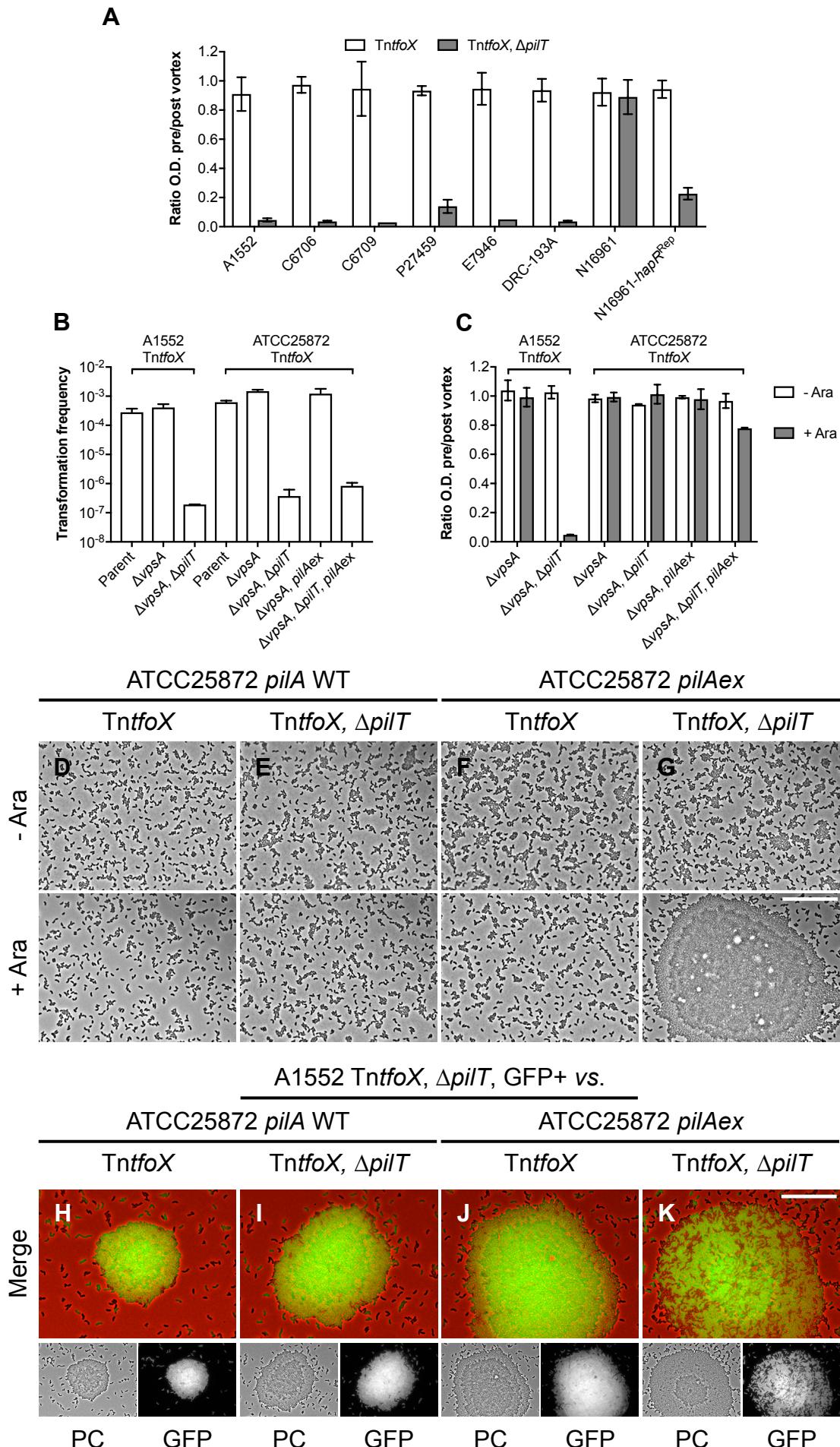


Figure 3. A1552 PilA is sufficient for aggregation in a non-pandemic strain.

(A) Aggregation of representative 7th pandemic strains of *V. cholerae*, including the effect of *hapR*^{Rep} on N16961, in a *TntfoX* and a *TntfoX*, *ΔpilT* 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 (\pm S.D.).

(B-C) Transformation frequency and aggregation of *V. cholerae* strain ATCC25872-*TntfoX* compared to that of A1552-*TntfoX*. **(B)** Chitin-independent transformation assay. Transformation frequencies are the mean of three repeats (\pm 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 (\pm S.D.).

(D-G) Phase-contrast microscopy of ATCC25872-*TntfoX*, *ΔvpsA* 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)** *ΔpilT* background, as indicated. Strains were cultured in the absence (- Ara) and presence (+ Ara), as indicated. Bar = 25 μ m. 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-*TntfoX*, *ΔpilT*, GFP+, producing GFP, and non-fluorescent cells of ATCC25872-*TntfoX*, *ΔvpsA* 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)** *ΔpilT* 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 μ m.

Figure 4

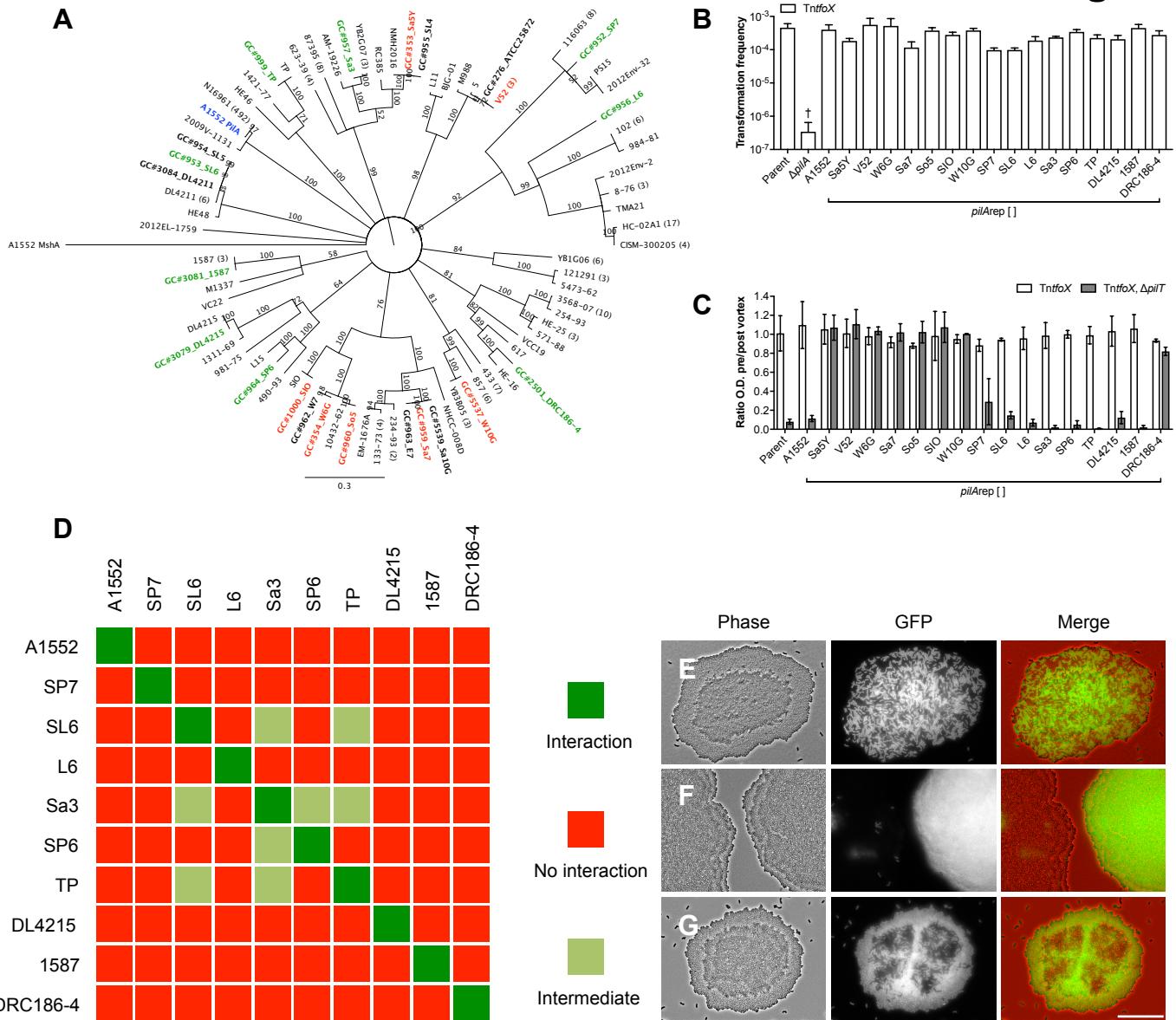


Figure 4. PilA variability governs auto-aggregation and enables kin-recognition.

(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.

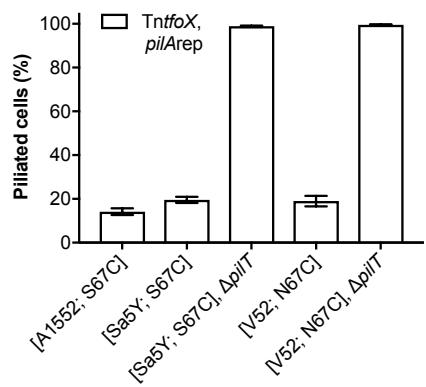
(B-C) Functionality of A1552-TntfoX, *pilA*rep[A1552] and 16 different PilAs assessed by transformation frequency and aggregation. **(B)** Chitin-independent transformation frequency assay. WT parent (A1552-TntfoX) and Δ *pilA* (A1552-TntfoX, Δ *pilA*) strains served as positive and negative controls. 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-TntfoX) and Δ *pilT* (A1552-TntfoX, 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 (\pm 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 TntfoX, 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.

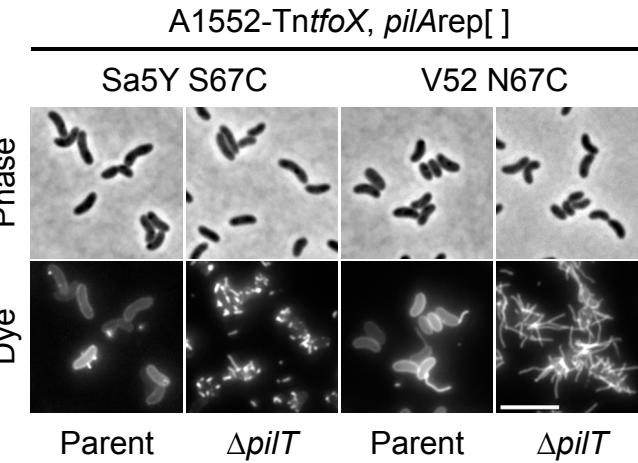
(E-G) Representative examples of **(E)** interaction, resulting in well-mixed aggregates **(F)** no interaction, resulting in fluorescent or non-fluorescent aggregates and **(G)** intermediate interactions, resulting in patterned aggregates. Cells were grown in the presence of *tfoX* induction. Merged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

Figure 5

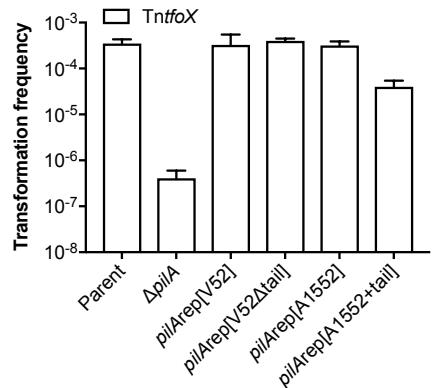
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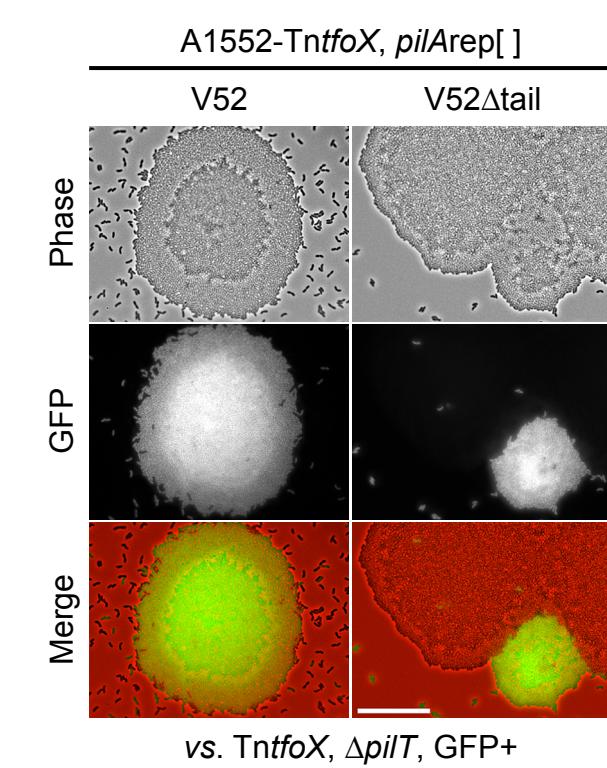
B



C



E



D

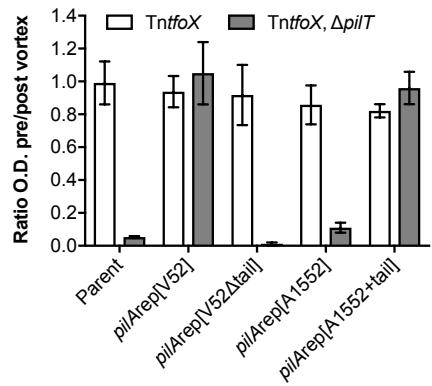


Figure 5. The unusual tail of ATCC25872/V52 PilA inhibits aggregation.

(A) Quantification of piliation in snapshot imaging of cells of strains A1552-TntfoX, *pilArep*[A1552; S67C], A1552-TntfoX, *pilArep*[Sa5Y; S67C] and A1552-TntfoX, *pilArep*[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 (\pm S.D.). $n = c.a.$ 2000 cells per strain per repeat. **(B)** Direct observation of pili stained with AF-488-Mal in cells carrying *pilArep*[Sa5Y; S67C] and *pilArep*[V52; N67C], in a WT parent (A1552-TntfoX) and Δ *pilT* (A1552-TntfoX, Δ *pilT*) background, as indicated. Bar = 5 μ m.

(C-D) Functionality of *pilArep* tail variants assessed by natural transformation and aggregation. **(C)** Chitin-independent transformation assay. WT parent (A1552-TntfoX) and Δ *pilA* (A1552-TntfoX, 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 *pilArep* strain in a *pilT*+ (A1552-TntfoX) and Δ *pilT* (A1552-TntfoX, 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 (\pm S.D.).

(E) Co-culture of fluorescent cells of A1552-TntfoX, Δ *pilT*, GFP+, producing GFP, and non-fluorescent cells of either *pilArep*[V52] (A1552-TntfoX, Δ *pilT*, *pilArep*[V52]) or *pilArep*[V52 Δ tail] (A1552-TntfoX, Δ *pilT*, *pilArep*[V52 Δ tail]), 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 μ m.

Figure 6

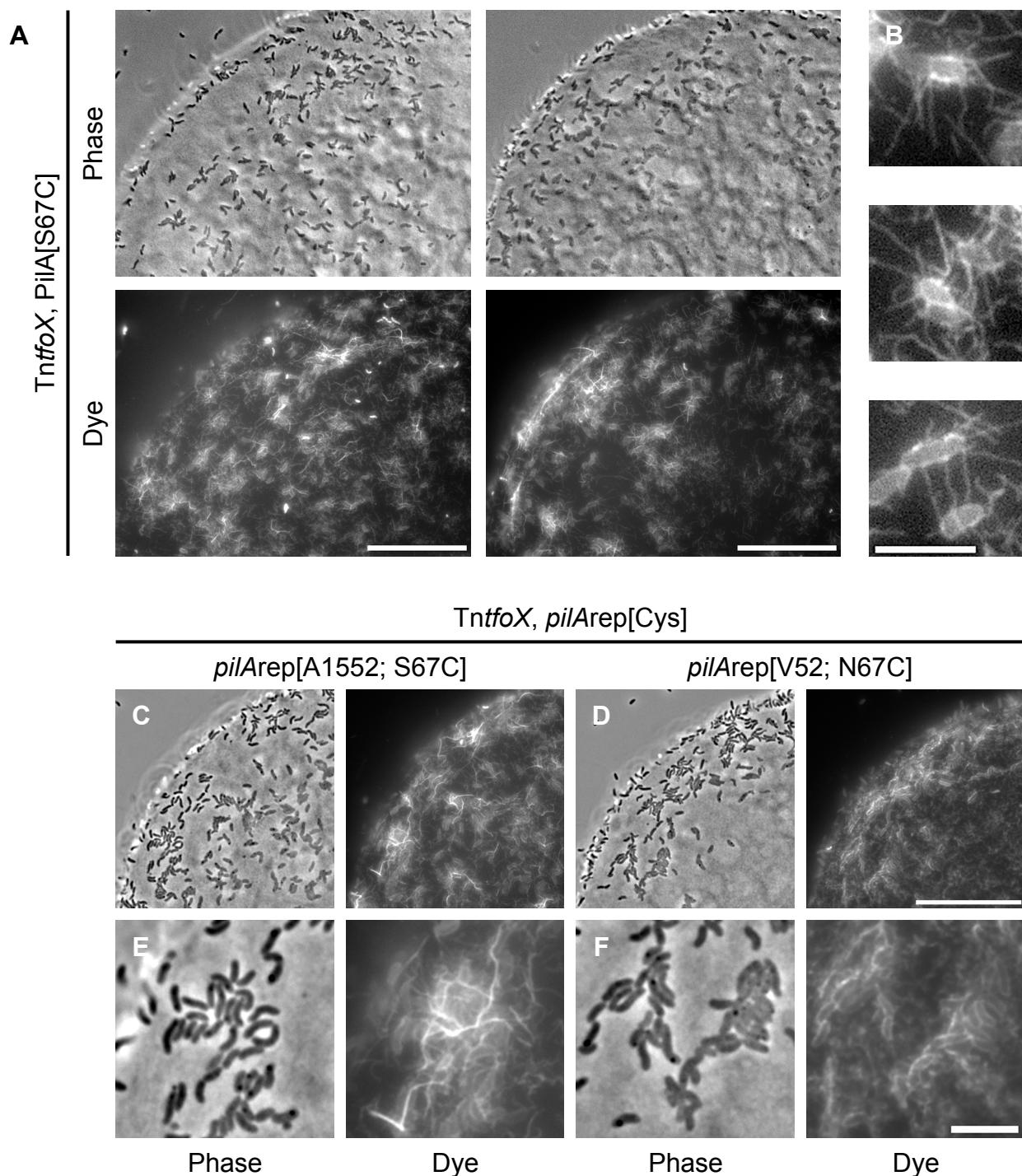


Figure 6. DNA-uptake pili form networks on chitin surfaces.

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

(C-F) Chitin beads were stained with AF-488-Mal after incubation for 48h in DASW with cells of either **(C)** A1552-TntfoX, *pilArep*[A1552; S67C] or **(D)** A1552-TntfoX, *pilArep*[V52; N67C], as indicated. Bar = 25 μ m. 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 μ m.