How bacteria colonize surfaces and how they distinguish the individuals around them are fundamental biological questions. Type IV pili are a widespread and multipurpose class of cell surface polymers. Here we directly visualize the DNA-uptake pilus of *Vibrio cholerae*, which is produced specifically during growth on its natural habitat—chitinous surfaces. As predicted, these pili are highly dynamic and retract before DNA uptake during competence for natural transformation. Interestingly, DNA-uptake pili can also self-interact to mediate auto-aggregation. This capability is conserved in disease-causing pandemic strains, which typically encode the same major pilus subunit, PilA. Unexpectedly, however, we discovered that extensive strain-to-strain variability in PilA (present in environmental isolates) creates a set of highly specific interactions, enabling cells producing pili composed of different PilA subunits to distinguish between one another. We go on to show that DNA-uptake pili bind to chitinous surfaces and are required for chitin colonization under flow, and that pili capable of self-interaction connect cells on chitin within dense pili networks. Our results suggest a model whereby DNA-uptake pilus function to promote inter-bacterial interactions during surface colonization. Moreover, they provide evidence that type IV pili could offer a simple and potentially widespread mechanism for bacterial kin recognition.

Despite their multifunctional potential, pandemic *V. cholerae* strains typically encode three distinct T4P systems, two of which are well characterized. First, toxin co-regulated pili (TCP) are cell surface polymers ideally suited to this task. Composed of a single major pilin and assembled by widely distributed and conserved machinery, T4P exhibit extensive functional versatility, with roles in motility, DNA uptake, surface sensing and adhesion. Consequently, T4P are critical virulence factors for numerous important human pathogens including *Vibrio* *cholerae*, which causes the pandemic diarrhoeal disease cholera. In Gram-negative bacteria pilins are processed at the inner membrane, extracted by the assembly machinery and polymerized into a helical pilus fibre that exits the cell surface through a gated outer-membrane pore, the secretin. A key feature of T4P is their ability to undergo dynamic cycles of extension and retraction, powered by the action of dedicated extension (for example, PilB) and retraction (for example, PilT) ATPases, which either add or liberate pilin subunits at the base. These dynamics are essential for many T4P functions, for example twitching motility. Thus, understanding how T4P function may yield insights valuable for understanding the mechanisms of environmental survival and pathogenesis.

How bacteria physically sense and interact with their environment is a fundamental problem in biology. Type IV pili (T4P) are cell surface polymers ideally suited to this task. Composed of a single major pilin and assembled by widely distributed and conserved machinery, T4P exhibit extensive functional versatility, with roles in motility, DNA uptake, surface sensing and adhesion. Consequently, T4P are critical virulence factors for numerous important human pathogens including *Vibrio* *cholerae*, which causes the pandemic diarrhoeal disease cholera. In Gram-negative bacteria pilins are processed at the inner membrane, extracted by the assembly machinery and polymerized into a helical pilus fibre that exits the cell surface through a gated outer-membrane pore, the secretin. A key feature of T4P is their ability to undergo dynamic cycles of extension and retraction, powered by the action of dedicated extension (for example, PilB) and retraction (for example, PilT) ATPases, which either add or liberate pilin subunits at the base. These dynamics are essential for many T4P functions, for example twitching motility. Thus, understanding how T4P function may yield insights valuable for understanding the mechanisms of environmental survival and pathogenesis.

Despite their multifunctional potential, pandemic *V. cholerae* strains typically encode three distinct T4P systems, two of which are well characterized. First, toxin co-regulated pili (TCP) serve a dual role, as a receptor for the CTXφ bacteriophage, which carries the cholera toxin genes, and as the primary human colonization factor, with multiple essential roles in infection involving adhesion and auto-aggregation on the intestinal cell surface. Second, Mannose-sensitive haemagglutinin (MSHA) pili are involved in surface sensing and attachment and thus are important in the initiation of biofilm formation. Third, in its natural aquatic environment, *V. cholerae* often associates with chitinous surfaces, which are nutritious, foster biofilm formation and probably play a role in environmental dissemination and transmission to humans in cholera-endemic regions. Chitin utilization triggers competence for natural transformation, a widely used mode of horizontal gene transfer that allows bacteria to take up DNA from their environment and that can thus foster rapid bacterial evolution. This requires production of the chitin-regulated ChiRP or DNA-uptake pili. Importantly, in strains representative of the ongoing seventh cholera pandemic such as A1552, the O1 El Tor clinical isolate used throughout this work, only MSHA pili are produced constitutively under laboratory conditions.

We previously showed that DNA-uptake pili form bona fide pili composed of the major subunit PilA and that transformation is dependent on the presumed retraction ATPase PilT (ref. ). However, the pilus itself is not sufficient for transformation and requires the concerted action of a periplasmic DNA-binding protein, ComEA (refs. ). On receipt of transforming DNA, ComEA switches from a diffuse to focal localization. These findings, together with work in other organisms, led to a model in which pilus retraction facilitates DNA entry into the periplasm, wherein ComEA acts as ‘ratchet’ to pull in the remaining DNA. Subsequently, DNA transport across the inner membrane occurs via a spatially coupled channel, ComEC (ref. ). Although this model is well supported by genetic experiments and the similarly combined action of T4P and ComEC in other organisms, direct evidence has been lacking.

Here, using a recently validated cysteine-labelling approach, we show that, as predicted, DNA-uptake pili are highly dynamic and that these dynamics are PilT-dependent. Unexpectedly, DNA-uptake pili can self-interact, resulting in auto-aggregation. Remarkably, specific interactions between divergent PilA subunits allow pili composed of different PilA subunits to distinguish between one another, enabling a simple mechanism for kin recognition.
Results

Direct observation of pilus dynamics by cysteine labelling. We first identified and validated a fully transformable PilA cysteine variant (PilA[S67C]) using a chitin-independent transformation system in which competence induction is arabinose-inducible34 (TnfoX; see Methods) (Fig. 1a,b and Supplementary Figs. 1 and 2a–c). When stained using a thiolo-reactive dye, competent cells producing PilA[S67C] exhibited visible pili (Fig. 1c). On average 27 ± 3% of cells were pilated, with most displaying one or two pili per cell (Fig. 1h,i). Pili length clustered around 1–2 µm, although pili up to 10 µm in length were also observed (Fig. 1i). Intriguingly, detached pili frequently appeared to self-interact, forming large structures composed of networks of pili (Supplementary Fig. 3a).

When examined by time-lapse microscopy, cells exhibited rapid pili dynamics, with multiple assembly and retraction events immediately apparent (Fig. 1k and Supplementary Videos 1–4). Indeed, within the 1 min time-frame studied, 66 ± 3% of cells exhibited pili, with most cells producing 1–2 pili per minute (Fig. 1m). Notably, some cells were even more dynamic, elaborating ≥5 pili per minute. Consistent with this dynamic behaviour, and in support of the hypothesis that pilus retraction precursors DNA uptake, it was possible to concurrently visualize pilus retraction followed by DNA uptake, by monitoring the re-localization of ComEA-mCherry, which occurs upon DNA binding in the periplasm (Supplementary Fig. 4 and Supplementary Video 5).

As expected, deletion of the components required for pilus assembly (for example, the assembly ATPase PilB or the secretin PilQ) abolished pilation (Fig. 1d,e). However, despite the absence of obvious pili, the cell body still stained. Control experiments indicate this results from non-specific dye uptake and retention by the inner-membrane pool of PilA[S67C] (Supplementary Fig. 2d). Like other species, V. cholerae encodes two potential retraction ATPases, PilT and PilU. Deletion of pilU did not affect pilation (Fig. 1h,i), consistent with its dispensability for transformation27. In contrast, cells lacking the presumed retraction ATPase PilT were hyper-piliated, with essentially all cells displaying multiple static pili (Fig. 1g–l and Supplementary Videos 6 and 7). Taken together with the dynamics described above, these data are consistent with the presence of multiple assembly complexes scattered across the cell, as previously predicted based on the mobility of PilB and the presence of multiple PilQ foci27. This might normally serve to facilitate rapid switching of pilus location or else might reflect a need to produce multiple pili under certain conditions. Unexpectedly, retraction-deficient ΔpilT cells were often grouped into small clusters within dense networks of pili as well as occasionally large aggregates of pili-encased cells (Supplementary Fig. 3b,c). Indeed, when grown in liquid culture, cells appeared to auto-aggregate.

Competent cells auto-aggregate in the absence of pilus retraction. Strikingly, aggregation occurred specifically in competence-induced cells lacking pilT (Fig. 2a–c), with the formation of large, multilayered spherical aggregates on the order of 25–100 µm, which progress to form macroscopic aggregates that rapidly sediment (Fig. 2b, +Ara). Quantification of the ratio of cells remaining in solution to those in the settled aggregates (Fig. 2d) revealed that, following induction of competence, ≥90% of the retraction-deficient cells were present in aggregates (Fig. 2d). Notably, strains producing the PilA[S67C] variant used for labelling behave similarly (Fig. 1b). Importantly, complementation of ΔpilT was sufficient to fully restore the ~1,000-fold transformation defect and abolished the aggregation phenotype (Fig. 2d,e). Complementation also fully counteracted the enhanced motility phenotype of ΔpilT (Supplementary Fig. 5), which occurs due to loss of function in the adhesive MSHA pilus, in agreement with the established shared role of PilT in MSHA pilus function26,31. Finally, time-course experiments indicated that aggregation occurs abruptly via the accretion of smaller aggregates (Supplementary Fig. 6), and that these aggregates remain stable and do not disperse, even after prolonged overnight culture.

Aggregates form via pilus–pilus interactions. The data so far suggest that hyper-piliated cells auto-aggregate via their DNA-uptake pili. Indeed, deletions affecting DNA-uptake pilus assembly (pilQ and pilA) were sufficient to abolish aggregation (Fig. 2f). In contrast, TCP (tcpA) and MSHA (msha) pili, the Vibrio poly-saccharide matrix (vpsA) required for biofilm formation36, or the flagellum (flaA), were dispensable, both individually and in combination (Fig. 2f). Similarly, an array of additional genes responsible for cell surface features were also dispensable (Supplementary Fig. 7a). Moreover, an ‘orphan’ type IV pilin VC0502 encoded on the Vibrio seventh pandemic island II, did not affect transformation, aggregation or motility (Supplementary Fig. 7b–d). Additionally, growth under high salt conditions (LB-S), which better reflects the natural aquatic environment of Vibrio sp. (Supplementary Fig. 7e), or in the presence of BSA, which has been reported to disrupt pilus–pilus interactions in Neisseria gonorrhoeae37, also had no effect (Supplementary Fig. 7e). Curiously, however, the strength of the aggregation phenotype is sensitive to divalent cation concentration (Supplementary Fig. 7f), although the significance of this result remains unclear. Finally, co-culture experiments using GFP/+ cells (Fig. 2g–i) revealed that only when both cells are hyper-piliated do they form well-mixed aggregates, indicating that aggregation occurs via direct pilus–pilus interactions between DNA-uptake pilus. Furthermore, consistent with the results above, MSHA pili do not mediate auto-aggregation, even when artificially upregulated (Supplementary Fig. 8).

PilA from pandemic strains is sufficient for aggregation in a non-pandemic strain. Deletion of pilT in a set of six additional seventh pandemic O1 El Tor isolates revealed that the ability to auto-aggregate is conserved among pandemic strains (Fig. 3a). Surprisingly, N16961 did not aggregate unless its well-characterized hapR frameshift mutation38, which renders it quorum-sensing (QS) defective, was first repaired (Fig. 3a). Because the genes required for pilus assembly are not QS-regulated39 the results above suggest that there may be an additional layer of post-transcriptional control.

PilA varies considerably between environmental strains of V. cholerae40, whereas most clinical isolates encode an identical PilA. Interesting exceptions are the toxigenic O37-serogroup strains V52 and ATCC25872, which were responsible for limited epidemics in the 1960s41,42. They are thought to derive from a pandemic O1-classical progenitor via serogroup conversion42. However, they both encode the same PilA, which is only 50% identical to that typical of pandemic strains (Supplementary Fig. 9). Because ATCC25872, but not V52, is QS-proficient we used it to investigate the functionality of this atypical PilA. Indeed, the transformability of ATCC25872 is comparable to that of A1552, and is PilT-dependent (Fig. 3b). In contrast, however, cells lacking pilT did not auto-aggregate and were excluded from aggregates formed by A1552 (Fig. 3c,e,h,i).

Because the other components required for pilus assembly are all highly conserved, we tested whether PilA itself was responsible for this phenotype by exchanging the endogenous pilA for that of A1552 (ATCC25872-pilAex). As expected, ATCC25872-pilAex was fully transformable (Fig. 3b). Importantly, however, competence-induced cells of this strain lacking pilT were now able to auto-aggregate, albeit at lower levels than in A1552 (Fig. 3c,f,g), and intermix within aggregates formed by A1552 (Fig. 3j,k).

PilA variability governs auto-aggregation and enables kin-recognition. The results above suggest that the ability to aggregate is dependent on the particular PilA variant carried. Indeed, BLAST analyses of 647 V. cholerae genomes deposited in NCBI indicated
Fig. 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\text{foX}). Transformation frequencies are the mean of three independent biological repeats (±s.d.). <d.l., below detection limit. >

**b**, Effect of PilA cysteine variants on the ability of retraction-deficient (Tn\text{foX}, ΔpilT) cells to aggregate. Aggregation is shown as the ratio of the culture optical density at 600 nm (OD$_{600}$) 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\text{foX}, PilA[S67C] and its derivatives: cells of A1552-Tn\text{foX}, PilA[S67C] were grown in the absence (−Ara) and presence (+Ara) of tfoX induction, as indicated, and stained with AF-488-Mal (c; scale bar, 5 μm); cells of A1552-Tn\text{foX}, PilA[S67C] were grown in the presence of tfoX induction and stained with AF-488-Mal in a ΔpilB (d), ΔpilQ (e), ΔpilU (f) and ΔpilT (g) background, as indicated (scale bar, 5 μm).

**h–j**, Quantification of pilation in snapshot imaging (error bars represent the mean of three repeats ±s.d.): percentage of piliated cells in the indicated backgrounds (h: n=2,000 cells per strain per repeat); histogram of number of pili per cell in piliated cells in WT parent (A1552-Tn\text{foX}, PilA[S67C]), ΔpilU (A1552-Tn\text{foX}, PilA[S67C], ΔpilU) and ΔpilT (A1552-Tn\text{foX}, PilA[S67C], ΔpilT) backgrounds, as indicated (k: n=300 cells per strain per repeat); histogram of pilus length in cells of A1552-Tn\text{foX}, PilA[S67C] (j: n=500–600 cells per repeat).

**k,l**, Time-lapse series of pilus dynamics in WT parent (A1552-Tn\text{foX}, PilA[S67C]) (k) and ΔpilT (A1552-Tn\text{foX}, PilA[S67C], ΔpilT) (l) backgrounds. Cells were stained with AF-488-Mal and imaged at 10 s intervals for 1 min. Throughout, upper panels show phase-contrast (PC) images; lower panels show fluorescence (dye) images. Time in seconds (s) is indicated. Scale bars, 5 μm.

**m**, Histogram showing quantification of pili produced per cell per minute in the WT parent (A1552-Tn\text{foX}, PilA[S67C]) background. Error bars represent the mean of three repeats (±s.d.). n≈500–700 cells per repeat.
that PilA exhibits extensive variation, whereas the other proteins encoded within its operon, as well as those from neighbouring genes, are all highly conserved (Supplementary Fig. 10). Of the 636 intact pilA genes identified, the majority (492/636) encode an ‘A1552-type’ PilA, probably due to over-representation of patient-derived pandemic strains in the database. Next, we extracted the unique PilA coding sequences (56/636) (Supplementary Fig. 11a) and combined them with those of an in-house collection of various environmental and patient isolates. The resulting phylogenetic tree consists of ~12 distinct groups and was used to sample PilA diversity (Fig. 4a). As expected, the N-terminal domains, required for membrane trafficking and pre-pilin peptidase recognition, and diversity (Fig. 4a). As expected, the N-terminal domains, required for membrane trafficking and pre-pilin peptidase recognition, and

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Fig. 2 | Competent cells auto-aggregate in the absence of pilus retraction. a-c, PC microscopy of cells of strains A1552-Tn\textit{tfoX} (a), A1552-Tn\textit{tfoX}, \textit{\Delta pilT} (b) and A1552-Tn\textit{tfoX}, \textit{\Delta pilT}, pil\textit{T}+ (c) grown in the absence (--\textit{Ara}) and presence (+\textit{Ara}) of \textit{tfoX} induction, as indicated. Pi\textit{T} complementation (\textit{pilT}+) was achieved by placing an intact copy of \textit{pilT} driven by its native promoter at a neutral ectopic locus. Scale bar, 25 \textmu m. d,e, Aggregation and transformation frequency of cells of strains A1552-Tn\textit{tfoX}, A1552-Tn\textit{tfoX}, \textit{\Delta pilT} and A1552-Tn\textit{tfoX}, \textit{\Delta pilT}, pil\textit{T}+, grown in the absence (--) and presence (+) of \textit{tfoX} induction, as indicated. A1552 (without inducible \textit{tfoX}) was used as a negative control. In d, aggregation is shown as the ratio of the culture OD<sub>600</sub> before and after vortexing. Values are the mean of three repeats (±s.d.). In 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 OD<sub>600</sub> before and after vortexing, in the absence (--) and presence (+) of \textit{tfoX} induction, as indicated. Δ4 = Δ\textit{tcpA}, Δ\textit{mshA}, Δ\textit{vpsA}, Δ\textit{flaA} quadruple mutant. Values are the mean of three repeats (±s.d.). g-i, Co-culture of fluorescent \textit{\Delta pilT} cells (A1552-Tn\textit{tfoX}, \textit{\Delta pilT}, GFP+) producing green fluorescent protein (GFP) and non-fluorescent cells of the WT parent (A1552-Tn\textit{tfoX}) (g), \textit{\Delta pilT} (A1552-Tn\textit{tfoX}, \textit{\Delta pilT}) (h) and \textit{\Delta pilA}, \textit{\Delta pilT} (A1552-Tn\textit{tfoX}, \textit{\Delta pilA}, \textit{\Delta pilT}) (i), grown in the presence of \textit{tfoX} induction. Merged images show GFP in green and PC in red. Scale bar, 25 \textmu m. j, Schematic showing the experimental setup, with the pilation status (lines) and retraction status (arrows) of the cells indicated.
Fig. 3 | A1552 PilA is sufficient for aggregation in a non-pandemic strain. a, Aggregation of representative seventh pandemic strains of *V. cholerae*, including the effect of *hapR*<sup>Tes</sup> on N16961, in a Tn*foX* and a Tn*foX*, ΔpilT<sup>Tes</sup> background, as indicated. Aggregation is shown as the ratio of the culture OD<sub>600</sub> before and after vortexing, in the presence of *foX* induction. Values are the mean of three repeats (±s.d.). b, c, Transformation frequency and aggregation of *V. cholerae* strain ATCC25872-Tn*foX* compared to that of A1552-Tn*foX*: chitin-independent transformation assay (b; transformation frequencies are the mean of three repeats (±s.d.)); aggregation is shown as the ratio of the culture OD<sub>600</sub> before and after vortexing, in the absence (−Ara) and presence (+Ara) of *foX* induction, as indicated (c; values are the mean of three repeats (±s.d.)). d–g, PC microscopy of ATCC25872-Tn*foX*, ΔvpsA cells carrying their native pilA (*pilA* WT) (d, e) and A1552 pilA (*pilA*ex) (f, g), in a pilT<sup>+</sup> (d, f) and ΔpilT (e, g) background, as indicated. Strains were cultured in the absence (−Ara) and presence (+Ara), as indicated. Scale 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*foX*, ΔpilT, GFP<sup>+</sup> producing GFP, and non-fluorescent cells of ATCC25872-Tn*foX*, ΔvpsA carrying their native pilA (*pilA* WT) (h, i) and A1552 PilA (*pilA*ex) (j, k), in a pilT<sup>+</sup> (h, j) and ΔpilT (i, k) background, as indicated. Cells were grown in the presence of *foX* induction. Merged images show GFP in green and PC in red. Scale bar, 25 µm.
inserted new pilA alleles at the native pilA locus in strain A1552, using a short (30 bp) duplication of the 3’ end of the original pilA to maintain any downstream regulation. We validated this pilA replacement (pilArep) approach using A1552-pilA (that is, TnfromX, pilArep[A1552]), which is fully transformable, and in cells lacking pilT fully auto-aggregates (Fig. 4b,c). We then tested 16 different PilA sequences from across the tree (Fig. 4a and Supplementary Fig. 11b). Interestingly, all supported transformation equally (Fig. 4b).

In contrast, the ability to aggregate varied depending on the particular PilA. Indeed, in retraction-deficient cells 9/16 PilAs could auto-aggregate, though pilArep[DRC186–4] was intermediate, whereas 7/16 did not detectably aggregate (Fig. 4a,c).

Given that aggregation occurs via direct pilus–pilus interactions, we hypothesized that the variability between the different aggregation-proficient PilA might allow pilis composed of different PilA to distinguish between one another. To test this idea we again used...
a co-culture approach, using strains +/- constitutive GFP production, and examined all possible combinations (Fig. 4d). As expected, cells producing pili composed of an identical PilA always exhibited uniform mixing (Fig. 4d,e). Remarkably, however, in 41/45 possible unique combinations the interactions between different pili were highly specific (Fig. 4d). Indeed, cells of these strains aggregated in a pilin-specific manner, preferentially forming aggregates with cells producing pili composed of the same PilA, resulting in aggregates that were either almost exclusively fluorescent or non-fluorescent (Fig. 4f). In 4/45 cases a partial cross-interaction was observed, resulting in an intermediate mixing phenotype, with aggregates composed of smaller but still segregated groups of cells (Fig. 4d,g). Overall, these data indicate that pili composed of different PilA are able to discriminate between one another, probably via specific PilA–PilA interactions. Moreover, these data demonstrate that PilA variability not only determines the ability to aggregate, but also provides a mechanism for kin-recognition.

Finally, upon virulence induction, TCP mediate auto-aggregation
v, forming aggregates like those described here. As expected, however, the two distinct types of aggregate do not intermix (Supplementary Fig. 12a–d). Moreover, TCP are largely limited to pandemic lineages with two variants of its major pilin TcpA: classical and El Tor t. In contrast to the results above, and as previously reported v, classical and El Tor strains formed uniformly mixed TCP aggregates, indicating that the TcpA variants do not discriminate between one another (Supplementary Fig. 12e).

The unusual tail of ATCC25872/V52 PilA is an inbuilt inhibitor of aggregation. To exclude the possibility that non-aggregating strains simply fail to make sufficient numbers of pili, pilA(Cys) variants of A1552-PilA (pilArep[A1552; S67C]) and two non-aggregating alleles (pilArep[Sa5Y; S67C]/[V52; N67C]) were constructed. These behaved similarly to the equivalent non-cysteine variant, although pilArep[Sa5Y; S67C] has a modest transformation defect (Supplementary Fig. 13a,b). Both variants were piliated at similar levels to the A1552 control and in ∆pilT were hyper-piliated, although Sa5Y pilis were generally very short (Fig. 5a,b). Hyper-piliation was especially clear for V52 pilis, providing direct evidence (Fig. 5c). Remarkably, however, in 41/45 possible cell pairs producing pili composed of V52-PilA (that is, pilArep[∆pilT; V52]) did not affect transformation (Fig. 5c). Conversely, however, in cells lacking pilT it restored the ability to aggregate at levels indistinguishable from that of the A1552-PilA controls (Fig. 5d), and also demonstrated the ability to ‘recognize’ itself in co-culture experiments (Fig. 5e).

These data suggest that the tail inhibits the ability of pili to aggregate, possibly by masking the site of pilus–pilus interaction. Indeed, a strain in which this tail was transplanted onto A1552-PilA (that is, pilArep[A1552 + tail]) remained highly transformable but was unable to aggregate (Fig. 5c,d). However, because efforts to label this PilA variant have so far been unsuccessful we cannot exclude another, non-specific effect.

DNA-uptake pili form networks on chitin surfaces. The data so far indicate that interactions between pili can mediate intercellular contacts. However, in liquid culture this requires artificial ftoX expression and the deletion of pilT. To investigate whether these interactions are relevant to the normal ecology of V. cholerae, we visualized DNA-uptake pili produced by otherwise WT cells following cultivation on chitinous surfaces, upon which ftoX is naturally induced. Strikingly, cells colonizing the chitin surface produced large numbers of pili that frequently appeared to self-interact, forming dense networks of larger pili structures that overlie and interconnect the cells (Fig. 6a). Indeed, these structures were particularly evident at later time points when extensive, mesh-like networks of pili extended across the entire chitin surface (Fig. 6b). Notably, although cells had now begun departing from the surface, these pili networks were retained within the detached pieces of biofilm (Fig. 6c). Control experiments confirmed that labelling on surfaces is specific and that these structures are DNA-uptake pili (Supplementary Fig. 14a). Moreover, although cells often appeared to possess multiple pili simultaneously (Supplementary Fig. 15a), upon adding exogenous DNA the majority of cells readily underwent DNA-uptake events, indicating that pilus retraction remains robust under these conditions (Supplementary Fig. 15b,c). Finally, using the pilA(Cys) variants described above, we directly compared the pili assembled on chitin surfaces by cells producing the aggregation-proficient A1552-PilA with those producing the aggregation-deficient V52-PilA. As expected, pilArep[A1552; S67C] cells were also found within pili networks similar to those described above (Fig. 6d,e and Supplementary Fig. 16). In contrast, such networks were never observed for pilArep[V52; S67C] surfaces. Faces were instead covered in what appeared to be mostly individual pili (Fig. 6f and Supplementary Fig. 16).

An important caveat is that because the washing steps required for pilus staining tend to remove cells, but not pili, from the surface, the number of cells engaged in these networks is probably underestimated (Supplementary Fig. 14b). Given this observation, we hypothesized that DNA-uptake pili might bind to the chitin surface directly. Indeed, empty chitin beads incubated with either purified pili (Fig. 6h,i) or those naturally sheared off during liquid culture (Supplementary Fig. 17) rapidly became coated with pili. Moreover, attachment of A1552-PilA pili produced large self-interacting networks of pili (Fig. 6h and Supplementary Fig. 17). In contrast, pili composed of V52-PilA bound to the surface as individual filaments (Fig. 6i and Supplementary Fig. 17). Next, because DNA-uptake pili can bind directly to chitin surfaces we tested whether they play a role in chitin colonization. However, in agreement with previous work, the ∆pilA cells had only a modest colonization defect, especially compared to cells unable to assemble MSHA pili (Supplementary Fig. 18). In stark contrast, when we repeated these experiments under conditions of continuous mixing to approximate the flow encountered in the natural environment, cells lacking pilA displayed a severe colonization defect comparable to that of ∆mshA and were outcompeted by the WT (Fig. 6j–l and Supplementary Fig. 19). Thus, these data support a model whereby DNA-uptake pili act downstream of MSHA pili, which are required for the initial attachment step, acting to maintain or reinforce chitin colonization. Indeed, all but one (TP-PilA) of the 16 pilA alleles studied here supported robust chitin colonization under these conditions (Supplementary Fig. 20).

In summary, DNA-uptake pili bind chitin directly and are required for chitin colonization under flow. Moreover, these data demonstrate that the aggregation phenotypes observed in liquid culture experiments report the natural ability of pili to self-interact and form networks on chitin surfaces. Finally, because the ability of DNA-uptake pili to bind chitin and their ability to self-interact appear to be distinct functions, the data suggest that those capable of both probably have additional functions in chitin colonization.

Discussion
Here we demonstrate that DNA-uptake pili are highly dynamic, that these dynamics are PilT-dependent, and that cells lacking pilT are
minimally transformable, providing direct evidence for the long-standing model whereby pilus retraction facilitates DNA uptake. Indeed, our results on pilus dynamics are in close agreement with those recently reported by Ellison et al.\textsuperscript{47}, who notably went on to demonstrate that the pilus binds directly to DNA\textsuperscript{47}. The major finding of this work, however, is that DNA-uptake pili are able to interact and distinguish between one another in a sequence-specific manner. In liquid culture, when retraction is deficient via the deletion of pilT, this manifests as an exaggerated auto-aggregation phenotype, which we have used as a convenient tool to reveal and then investigate the ability of pili to self-interact. Because only a subpopulation of cells is piliated at any one time, and these pili are dynamic, eliminating retraction probably facilitates auto-aggregation by producing a homogenous population of hyper-piliated cells, thereby increasing the chances of interactions between pili. Work in \textit{Neisseria meningitidis}, which auto-aggregates naturally at low levels...
**Fig. 6** | DNA-uptake pili form networks on chitin surfaces. 

a,b, DNA-uptake pili composed of A1552-PilA form networks naturally on chitin surfaces. Chitin beads were stained with AF-488-Mal after incubation for either 48 h (a) or 72 h (b) in defined artificial seawater (DASW) with cells of A1552-PilA[S67C], as indicated. 

c, A piece of detached biofilm-like material. Note the retention of the pilus networks. Scale bars, 25 µm. 

d-g, The ability to form pilus networks is dependent on the ability to self-interact. Chitin beads were stained with AF-488-Mal after incubation for 48 h in DASW with cells of either A1552-pilArep[A1552; S67C] (d) or A1552-pilArep[V52; N67C] (f), as indicated. 

e, g, Enlargements of the boxed regions of the surfaces shown in d, f. Note the absence of large pili networks in g. Scale bars, 25 µm (d, f) and 5 µm (e, g). 

h, i, DNA-uptake pili bind to chitin surfaces. Chitin beads were stained with AF-488-Mal after incubation for 1 h with purified DNA-uptake pili composed of either A1552-PilA[S67C] (h) or V52-PilA[N67C] (i), as indicated. Scale bar, 25 µm. 

j-l, DNA-uptake pili are required for chitin colonization under flow. Chitin beads were imaged after incubation in DASW for 48 h under conditions of continuous mixing with GFP+ cells of strains A1552-GFP (WT-GFP) (j), A1552-GFP, ΔpilA (ΔpilA-GFP) (k) and A1552-GFP, ΔmshA (ΔmshA-GFP) (l), as indicated. Scale bar, 50 µm.
but is dramatically enhanced by the deletion of pilT, supports this idea. Importantly, however, the ability of pili to self-interact in artificial liquid culture conditions reflects a natural ability to interact on chitin surfaces. Indeed, under natural induction conditions on chitin surfaces, cells producing pili capable of aggregation elaborate multiple pili and form dense pili networks in an otherwise unmodified background, indicating that the chitin surface probably promotes interactions between pili. The proximity of cells to each other in the crowded surface environment might inherently foster these interactions. Alternatively, the altered physiology of cells growing on chitin might also impact pilus assembly via effects on the extension/retraction motors.

Our discovery that DNA-uptake pili bind chitin and are required for chitin colonization under flow suggests an important role in the aquatic environment. Consequently, we propose that the primary function of DNA-uptake pili in the environment is probably not for natural transformation but rather for chitin colonization. Indeed, as hypothesized elsewhere, because pilius production is dependent on an intact chitin-utilization pathway, colonization mechanisms using DNA-uptake pili would (1) be inherently selective for nutritious chitinous surfaces and (2) favour the recruitment and retention of productive cells while excluding non-productive cells unable to make pili. In contrast, MSHA pili are produced constitutively and bind biotic and abiotic surfaces similarly. Furthermore, the ability to interact not only with the surface but to mediate selective interactions with other cells would confer additional benefits that could act at multiple stages of colonization. This might be particularly advantageous during early stages to bring smaller chitin particles together, and thus provide resistance to protozoan grazers, as well as at later stages to keep cells together during biofilm dispersal and thus, by allowing cells to arrive at a new niche in greater number, aid in persistence and dissemination. However, further work will be necessary to rule out the possibility that these interactions represent an ancient colonization mechanism that has since been replaced. Nevertheless, the energetic cost of producing the pili networks we observe on chitin, the existence of a set of highly specific interactions, and the fact that in contrast to environmental isolates, pandemic strains all retain the same interaction-proficient PilA, argue for an ongoing and important role.

Interestingly, auto-aggregation by TCP during virulence is essential for host colonization. Furthermore, TCP networks encasing cells on the intestinal cell surface have been suggested to protect cells from host defences. Given the similarities between the two systems, especially our observation of dense pili networks on colonized chitin surfaces, we propose that the DNA-uptake pili might play an analogous role in the aquatic environment. Indeed, ingestion of colonized chitin particles is thought to facilitate transmission to humans in chola-endemic areas, and recovering cholera patients exhibit a strong immune response to PilA. Thus, future work should investigate whether the networks of DNA-uptake pili we observed on chitin surfaces protect cells during this process.

In N. gonorrhoeae, artificially varying the density or post-translational modification state of pili leads to a form of cell sorting based on differential interaction forces between pili. This effect is probably related to aggregate dispersal during its infective lifestyle, but does not permit specific recognition per se. In contrast, the discovery here that natural PilA variability controls the ability of pili to self-interact, and creates highly specific interactions, provides a direct mechanism for kin recognition. The best-studied examples of kin recognition in microorganisms all involve adhesins and some form of aggregation (for example, Flo1, Saccharomyces cerevisiae; TgrB1-TgrC1, Dictyostelium discoideum; and TraA, Myxobacteria). In evolutionary terms, these recognition mechanisms are classified as ‘greenbeards’ because the cue, recognition of the cue and the resulting cooperative activity are all encoded by the same gene. The ability of DNA-uptake pili to recognize and interact with pili composed of the same kind of PilA fits this classification and is therefore a specific form of greenbeard recognition. However, this form of recognition implies close identity only at the greenbeard locus and so is better referred to as kind recognition.

An important question going forward will be to understand what drives PilA diversity and how this is related to the type VI secretion system, which acts to kill non-kin bacteria. Similarly, do those strains that lack the ability to self-interact employ alternative mechanisms (for example, upregulation of biofilm production) to colonize chitin surfaces? Indeed, the apparent acquisition of an inhibitor of pilus interactions by some PilA (for example, ATCC25872/V52) hints that the ability to interact may not always be beneficial or else may reflect an adaptation to a specific niche. Therefore, future work should focus on how the pili networks we observed on chitin surfaces contribute to the ecology of V. cholerae, especially under environmental conditions. Indeed, we still know relatively little about its natural lifestyle on chitin, partly due to the inherent technical difficulties associated with manipulating these surfaces. Nevertheless, the demonstration in liquid media that specific interactions between pili composed of different major pilins are sufficient to enable segregation provides a robust proof of concept that T4P have the capacity to function as a recognition mechanism. Finally, given that (1) T4P are widespread, (2) auto-aggregation via T4P has been reported in multiple species and (3) the major pilin subunit often varies, there exists the possibility that specific interactions between T4P could be relatively common and therefore represent an important contribution to bacterial recognition worthy of continued investigation.

Methods

Bacterial strains and plasmids. The bacterial strains used in this study are provided in Supplementary Table 1, together with the plasmids used and their construction. 1552, the V. cholerae strain used throughout this work, is a fully sequenced toxigenic O1 El Tor Inaba strain representative of the ongoing seventh cholera pandemic, and was derived from a traveller entering the United States after being infected on a commercial aeroplane that took off in Peru.

General methods. Bacterial cultures were grown aerobically at 30 °C or 37 °C, as required. The liquid medium used for growing bacterial strains was lysogenic broth (LB-Miller, 10 g l NaCl, Carl Roth) and the solid medium was LB agar. Where indicated, LB-S contained 20 g l NaCl. Ampicillin (Amp, 100 µg ml−1), tetracycline (Tet, 10 µg ml−1), kanamycin (Kan, 75 µg ml−1), streptomycin (Str, 100 µg ml−1) and rifampicin (Rif, 100 µg ml−1) were used for selection, as required.

To induce expression from the P promoter, cultures were grown in media supplemented with 0.2% l-arabinose. Natural transformation of V. cholerae on chitin flakes was done in 0.5x DAW, supplemented with vitamins (MEM, Gibco) and 30 mM HEPES, as previously described. Counter-selection of phytohalinyl-tRNA synthetase (pheS) insertions (Trans2 method, see below) was performed on medium supplemented with 20 mM 4-chloro-phenylalanine (cPhe, Sigma-Aldrich). Thiocitrate citrate bile salts sucrose (TCBS, Sigma-Aldrich) agar was used to counter-select for Escherichia coli following bacterial mating. SacB-based counter-selection was done on NaCl-free medium containing 10% sucrose.

Strain construction. DNA manipulations and E. coli transformations were carried out using standard methods, and all constructs were verified by PCR and Sanger sequencing (Megabiont AG). Genetic engineering of V. cholerae was done using a combination of natural transformation and FLP-recombination; TransFLP[47, Trans3[48], and allelic exchange using bi-parental mating and the counter-selectable plasmid pGPT704-Sac2[49]. The mini-Tn7 transposon carrying arac and various P promoter-driven genes was integrated into the large chromosome by tri-parental mating, as previously described.

Chitin-independent competence induction. Chitin oligosaccharides resulting from growth on chitin trigger natural competence induction via the production of the regulator, TfoX, a master regulator, TfoX, which acts to kill non-kin bacteria. The presence of chitin in liquid culture we used a well characterized and already validated chitin-independent approach that results in low levels of TfoX production. This approach is based on the integration of a mini-Tn7 transposon into the large chromosome of V. cholerae containing an arabinose-inducible copy of tfoX (that is, arac, P arac-tfoX), which we refer to as TfoX. In the presence of inducer, strains carrying TfoX turn on the expression
of the competence genes according to the known regulatory pathways and, upon reaching high cell density, are transformable at levels similar to those seen on chitin\(^5\). In the absence of inducer, competence genes are not produced and strains are non-transformable\(^6\).

**Transformation frequency assay.** Diverse strains harbouring Tntf6X were tested for transformation using a chitin-independent transformation frequency assay, as previously described\(^7\)\(^\text{—}\)\(^8\). Briefly, overnight cultures were back-diluted 1:100 in fresh medium with and without arabinose, as indicated, and grown for 3 h at 30 °C with shaking (180 r.p.m.). Then, 0.5 ml aliquots of the cultures were mixed with 1 µg genomic DNA (derived from strain A1552-lacZ-Kan) in 1.5 ml Eppendorf tubes and incubated for 5 h at 30 °C with shaking (180 r.p.m.), before serial dilution in phosphatebuffered saline (PBS) and enumeration after overnight growth on LB medium in the absence and presence of kanamycin. Transformation frequency was calculated as the number of transformants divided by the total number of bacteria.

**Pilus shearing assay.** Cultures were grown for 6 h at 30 °C with shaking (180 r.p.m.) in 25 ml LB + 0.2% arabinose within a 125 ml Erlenmeyer flask. To shear pili from the cell surface, 10 ml culture was removed, vortexed at max speed for 1 min, and cells were removed by three sequential centrifugation steps (10 min, 4,000 g, 4 °C). To the resulting supernatant saturated ammonium sulfate was added to 40% and incubated on ice for 1 h. Precipitated proteins were recovered by centrifugation (30 min, 20,000 g, 4 °C) and washed once with PBS. Samples were then resuspended in 2× Laemmli buffer, boiled (15 min, 95 °C), and stored at −20 °C until needed. To compare PilA levels between samples, semi-supernatant volumes were normalized according to the OD of the starting culture. Total protein controls were intact cell lysates. The relative amount of PilA in each sample was determined by western blotting.

**Aggregation assay.** Overnight cultures were back-diluted 1:100 in the absence and presence of arabinose, as needed, and grown in 14 ml round-bottomed polystyrene test tubes (Falcon) on a carousel style rotary wheel (40 r.p.m.) at 30 °C. After 6 h growth, aggregates were allowed to settle by standing the tube at room temperature for 30 min. The OD\(_{600}\) of the culture was measured before and after mechanical disruption (vortex max speed, ∼5 s), which served to disperse any settled aggregates and return them to solution. Aggregation is expressed as the ratio of the OD\(_{600}\) pre- and post-vortexing. For time-course experiments the standing time was reduced to 5 min. To visualize aggregates by microscopy, overnight cultures were back-diluted either 1:100 individually or 1:200 when mixed, as needed, and were grown for 4 h, as described above.

**Microscopy.** Cells were mounted on microscope slides coated with a thin agarose pad (1.2% wt/vol in PBS), covered with a #1 coverslip, and were observed using a Zeiss Axio Imager M2 epi-fluorescence microscope attached to an AxioCam MRm camera and controlled by Zeiss AxioVision software. Image acquisition was done using a Plan-Apochromat x100/NA 1.4 Ph3 oil objective illuminated by an HXP120 lamp. Images were analysed and prepared for publication using ImageJ (http://rsb.info.nih.gov/ij).

**Design and validation of PilA cysteine variants.** To avoid the limitations imposed by immunofluorescent methods, we used a cysteine labelling approach using a thiol-reactive dye\(^7\)\(^\text{—}\)\(^8\). PilA cysteine variants were created along the length of the pilus surface-exposed loop\(^5\),\(^7\) (Supplementary Fig. 1) and tested for functionality of the competence genes according to the known regulatory pathways and, upon induction, competence genes were not produced and strains were non-transformable\(^3\). In the absence of inducer, competence genes are not produced and strains reaching high cell density, are transformable at levels similar to those seen on chitin\(^3\). Chitin binding activity of DNA-uptake pili. Cultures of strains encoding PilA cysteine variants were grown under chitin-independent competence induction for 5 h at 30 °C on a rotary wheel, as described above. To shear pili from the cell surface, 10 ml culture was removed, vortexed at max speed for 1 min, and cells were washed by centrifugation (5 min, 5,000 g). Next, either 0.1 ml of sheared pili or 0.1 ml of untreated cell culture, as indicated in the text, were mixed with 0.1 ml washed chitin beads in 0.5× DAWX + 50 mM HEPEs + vitamins and incubated flat in a 1.5 ml Eppendorf tube with shaking (30 °C, 180 r.p.m., 1 h). Bound pili were then washed with AF-488 Mal, as described above, washed once with 0.8 ml LB and resuspended in a final volume of 0.1 ml LB.

**Western blotting.** Cell lysates were prepared by suspending harvested cells in 2× Laemmli buffer (100 µl buffer per OD unit) before boiling at 95 °C for 15 min. Proteins were separated by SDS–PAGE using a 15% resolving gel and blotted onto polyvinylidene difluoride membranes using a wet-transfer apparatus. Immunodetection was performed as described previously\(^9\). Primary anti-PiLA antibodies were raised in rabbits against synthetic peptides of A1552 PilA (Eurogentec, #1510525) and used at a dilution of 1:5,000. Anti-rabbit IgG horseradish peroxidase (HRP) (Sigma, cat. no. A9169) diluted 1:5,000 was used as a secondary antibody. Sample loading was verified with Direct-Blot HRP anti-E. coli RNA Sigma70 (Biolegend, cat. no. 663205) diluted 1:1,000.

**Motility assay.** To quantify motility phenotypes, 2 µl of an overnight culture was spotted onto soft LB agar (0.3%) plates (two technical replicates) and incubated at room temperature for 24 h before photography. The swimming diameter (cm) was measured and is expressed as the mean of three independent biological repeats.

**Bioinformatics of PilA diversity.** V. cholerae genomes were obtained from NCBI (National Center for Biotechnology Information), and are listed in Supplementary File 1. Generous software (10.2.3)\(^\text{—}\)\(^\text{—}\) (http://www.gnu.org/software/multalign) was used to perform custom BLAST analyses and identify pilA. Unique PilA sequences were extracted and combined with the PilA sequences from strain A1552 and a collection of environmental isolates\(^10\),\(^\text{—}\)\(^12\) as deduced by Sanger sequencing (Supplementary File 2), as indicated in the text. PilA sequences were aligned with Muscle and a consensus neighbour-joining tree was constructed using the Jones–Kantor substitution model, resampled with 100 bootstrap replicates. MshA from strain A1552 was used as an outgroup.

**Statistics and reproducibility.** All data are representative of the results of three independent biological repeats. All experiments were repeated independently three times, with similar results. Bar graphs display the mean value, error bars display the standard deviation, and dot plots are overlaid to indicate the distribution of the individual data points.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The data that support the findings of this study are available from the corresponding authors upon request.

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**References**

1. Maier, B. & Wong, G. C. L. How bacteria use type IV pili machinery on surfaces. *Trends Microbiol.* **23**, 775–788 (2015).

2. Berry, J. L. & Pellici, V. Exceptionally widespread nanomachines composed of type IV pilins: the prokaryotic Swiss Army knives. *FEBS Microbiol. Rev.* **39**, 134–154 (2015).

3. Gilner, C. L., Nguyen, Y. & Burrows, L. L. Type IV pilin proteins: versatile molecular modules. *Microbiol. Mol. Biol. Rev.* **76**, 740–772 (2012).

4. 5. 6. 7. 8. 9. 10. 11. 12.
31. Gangel, H. et al. Concerted spatio-temporal dynamics of imported DNA and nanocomplexes in the Vibrio cholerae genome. *Nat. Commun.* 7, 10301 (2016).

32. Laurenceau, R. et al. A type IV pilus mediates DNA binding during natural transformation in *Streptococcus pneumoniae*. *PLoS Pathog.* 9, e1003473 (2013).

33. Ellison, C. K. et al. Obstruction of pilus retraction stimulates bacterial surface sensing. *Science* 358, 535–538 (2017).

34. Lo Scurotto, M. & Blokesch, M. The regulatory network of natural competence and transformation of *Vibrio cholerae*. *PLoS Genet.* 8, e1002778 (2012).

35. Jones, C. J. et al. C-di-GMP regulates motile to sessile transition by modulating MshA pilus biogenesis and near-surface motility behavior in *Vibrio cholerae*. *PLoS Pathog.* 11, e1005068 (2015).

36. Fung, J. C., Syed, K. A., Klose, K. E. & Yildiz, F. H. Role of Vibrio poly-ssaccharide (vps) genes in VPS production, biofilm formation and Vibrio cholerae pathogenesis. *Microbiology* 156, 2757–2769 (2010).

37. Biais, N., Ladoux, B., Higashi, D., So, M. & Sheetz, M. Cooperative retraction of bundled type IV pili enables nanowotonet force generation. *PLoS Biol.* 6, e17 (2008).

38. Joëlsens, A., Liu, Z. & Zhu, J. Genetic and phenotypic diversity of quorum-sensing systems in clinical and environmental isolates of *Vibrio cholerae*. *Infect. Immun.* 74, 1141–1147 (2006).

39. Aagesen, A. M. & Halse, B. C. Surface analyses of type IV pili from *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Microb. Ecol.* 64, 319–524 (2012).

40. Aldova, E., Lazznickova, K., Stepankova, E. & Lietava, J. Isolation of nonagglutinable vibrios from an enteritis outbreak in Czechoslovakia. *J. Infect. Dis.* 118, 25–31 (1968).

41. Chun, J. et al. Comparative genomics reveals mechanism for short-term and long-term temporal clonal transition in the pandemic *Vibrio cholerae*. *Proc. Natl Acad. Sci. USA* 106, 15442–15447 (2009).

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

43. DiBaise, R. J., Neely, M., Taylor, R. K. & Bruss, P. M. Differential expression of the ToxT regulon in classical and El Tor biotypes of *Vibrio cholerae* due to biotype-specific control over toxT expression. *Proc. Natl Acad. Sci. USA* 93, 7991–7995 (1996).

44. Jude, B. A. & Taylor, R. K. The physical basis of type IV pili-mediated microcolony formation by *Vibrio cholerae* O1. *J. Struct. Biol.* 175, 1–9 (2011).

45. Lim, M. S. et al. *Vibrio cholerae* El Tor TcpA crystal structure and mechanism for pilus-mediated microcolony formation. *Microb. Mol. Biol. Rev.* 77, 755–770 (2010).

46. Rhine, J. A. & Taylor, R. K. TcpA pilin sequences and colonization requirements for O1 and O139 *Vibrio cholerae*. *Microb. Mol. Biol. Rev.* 13, 1013–1020 (1999).

47. Ellison, C. K. et al. Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in *Vibrio cholerae*. *Nat. Microbiol.* 3, 773–780 (2018).

48. Hélation, S. et al. PiliX, a pilus-associated protein essential for bacterial aggregation, is a key to pilus-facilitated attachment of Neisseria meningitidis to human cells. *Microb. Mol. Biol. Rev.* 65, 65–77 (2005).

49. Shime-Hattori, A. et al. Two type IV pilus of *Vibrio parahaemolyticus* play different roles in biofilm formation. *FEMS Microbiol. Lett.* 264, 89–97 (2007).

50. Krebs, S. & Taylor, R. K. Protection and attachment of *Vibrio cholerae* mediated by the toxin-coregulated pilus in the infant mouse model. *J. Infect. Dis.* 194, 15444–15450 (2006).

51. Hirose, S., Benabentos, R., Ho, H. I., Kuspa, A. & Shaulsky, G. Self-sensing systems in clinical and environmental isolates of *Vibrio cholerae*. *Syst. Biol.* 57, 535–538 (2017).

52. Pathak, D. T., Wei, X., Dey, A. & Wall, D. Molecular recognition by a variable green beard gene that drives biofilm-like aggregation, is a key to pilus-facilitated attachment of *Vibrio cholerae* to human cells. *Microb. Mol. Biol. Rev.* 73, 1544–15450 (2009).

53. Cremer, S., Benabentos, R., Ho, H. I., Kuspa, A. & Shaulsky, G. Self-sensing systems in clinical and environmental isolates of *Vibrio cholerae*. *Syst. Biol.* 57, 535–538 (2017).

54. Oldenburg, J. E. & Queller, D. C. Kin discrimination and cooperation in budding yeast. *Science* 333, 467–470 (2010).

55. Pathak, D. T., Wei, X., Dey, A. & Wall, D. Molecular recognition by a polymorphic cell surface receptor governs cooperative behaviors in bacteria. *PLoS Genet.* 9, e1003891 (2013).

56. Strassmann, J. E., Gilbert, O. M. & Queller, D. C. Kin discrimination and cooperation in microbes. *Annu. Rev. Microbiol.* 65, 349–367 (2011).

57. Wall, D. Kin recognition in bacteria. *Annu. Rev. Microbiol.* 70, 43–60 (2016).

58. Borjesson, S., Metzger, L. C., Scirigna, T. S. & Blokesch, M. The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* 347, 63–67 (2015).

59. Trunk, T., Khalil, H. S. & Leo, J. C. Bacterial autoaggregation. *AIMS Microbiol.* 4, 140–164 (2018).
61. Yildiz, F. H. & Schoolnik, G. K. Role of rpoS in stress survival and virulence of Vibrio cholerae. J. Bacteriol. 180, 773–784 (1998).
62. Matthey, N., Drebès Dörz, N. C. & Blokesch, M. Long-read-based genome sequences of pandemic and environmental Vibrio cholerae strains. Microbiol. Resour. Announc. 7, e01374-19 (2018).
63. Blokesch, M. A quorum sensing-mediated switch contributes to natural transformation of Vibrio cholerae. Mol. Genet. Elements 2, 224–227 (2012).
64. Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989).
65. De Souza Silva, O. & Blokesch, M. Genetic manipulation of Vibrio cholerae by combining natural transformation with FLP recombination. Plasmid 64, 186–195 (2010).
66. Marvig, R. L. & Blokesch, M. Natural transformation of Vibrio cholerae as a tool—optimizing the procedure. BMC Microbiol. 10, 155 (2010).
67. Blokesch, M. TransFLP—a method to genetically modify Vibrio cholerae based on natural transformation and FLP recombination. J. Vis. Exp. 68, e3761 (2012).
68. Van der Henst, C. et al. Molecular insights into Vibrio cholerae’s intraamoebal host-pathogen interactions. Nat. Commun. 9, 3460 (2018).
69. Gurung, I., Berry, J. L., Hall, A. M. J. & Pelicic, V. Cloning-independent markerless gene editing in Streptococcus sanguinis: novel insights in type IV pilus biology. Nucleic Acids Res. 45, e40 (2017).
70. Bao, Y., Lies, D. P., Fu, H. & Roberts, G. P. An improved Tin7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109, 167–168 (1991).
71. Yamamoto, S. et al. Regulation of natural competence by the orphan two-component system sensor kinase ChiS involves a non-canonical transmembrane regulator in Vibrio cholerae. Mol. Microbiol. 91, 326–347 (2014).
72. Dalia, A. B., Laziński, D. W. & Camilli, A. Identification of a membrane-bound transcriptional regulator that links chitin and natural competence in Vibrio cholerae. mBio 5, e01028-01013 (2014).
73. Yamamoto, S. et al. Identification of a chitin-induced small RNA that regulates translation of the fioX gene, encoding a positive regulator of natural competence in Vibrio cholerae. J. Bacteriol. 193, 1953–1965 (2011).
74. Lo Scrudato, M. & Blokesch, M. A transcriptional regulator linking quorum sensing and chitin induction to render Vibrio cholerae naturally transformable. Nucleic Acids Res. 41, 3644–3658 (2013).
75. Jaskólska, M., Stuttmann, S., Stoudmann, C. & Blokesch, M. QsrR-dependent regulation of natural competence and type VI secretion in Vibrio cholerae. Nucleic Acids Res. 46, 10619–10634 (2018).
76. Blair, K. M., Turner, L., Winkelman, J. T., Berg, H. C. & Kearns, D. B. A molecular clutch disables flagella in the Bacillus subtilis biofilm. Science 320, 1636–1638 (2008).
77. Kearse, M. et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649 (2012).
78. Keymer, D. P., Miller, M. C., Schoolnik, G. K. & Boehm, A. B. Genotypic diversity of coastal Vibrio cholerae strains is linked to environmental factors. Appl. Environ. Microbiol. 73, 3705–3714 (2007).
79. Purdy, A., Rohwer, F., Edwards, R., Azam, F. & Bartlett, D. H. A glimpse into the expanded genome content of Vibrio cholerae through identification of genes present in environmental strains. J. Bacteriol. 187, 2992–3001 (2005).

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Author contributions
Conception, design and analysis were carried out by D.W.A. and M.B. and M.B. performed the research. D.W.A and M.B. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding authors upon request.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | All population level experiments were performed independently three times, as per our standard laboratory practise. No statistical methods were used to predetermine sample size. For the analysis of single cell characteristics e.g. piliation %, pilus length etc. the number of cells counted is stated in the methods. |
| Data exclusions | No data were excluded from the analysis. |
| Replication | Experiments were performed at least three independent times (biological repeats). All replication attempts were successful. |
| Randomization | To generate the data sets used for quantification of pilus characteristics in ‘snapshot’ and time-lapse microscopy, fields were chosen at random using phase-contrast imaging (i.e. without visualising the pill). |
| Blinding | Blinding was not performed and is not relevant in this study as we are working with well-defined bacterial strains. Furthermore, all experiments were repeated at least three independent times, with similar results. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| | Unique biological materials |
| | Antibodies |
| | Eukaryotic cell lines |
| | Palaeontology |
| | Animals and other organisms |
| | Human research participants |

Methods

| n/a | Involved in the study |
| --- | --- |
| | ChIP-seq |
| | Flow cytometry |
| | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All strains and plasmids described in this manuscript are available upon request.

Antibodies

Antibodies used

Primary anti-PilA antibodies were commercially raised in rabbits against synthetic peptides of A1552 PilA (Eurogentec, Belgium; #1510525). Other antibodies used were Direct-Blot™ HRP anti-E. coli RNA Sigma 70 (BioLegend; Cat# 663205) and anti-Rabbit IgG-HRP (Sigma; Cat#A9169).

Validation

Anti-PilA: tested against V. cholerae pilA knock-out strain (control included in Western blot); other antibodies were commercially available and served as loading control or secondary antibody.