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tonB/exbB/exbD homologues regulate polar motility

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The putative Poc complex controls two distinct *Pseudomonas aeruginosa* polar motility mechanisms

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Summary

Each *Pseudomonas aeruginosa* cell localizes two types of motility structures, a single flagellum and one or two clusters of type IV pili, to the cell poles. Previous studies suggested that these motility structures arrive at the pole through distinct mechanisms. Here we performed a swimming motility screen to identify polar flagellum localization factors and discovered three genes homologous to the TonB/ExbB/ExbD complex that have defects in both flagella-mediated swimming and pilus-mediated twitching motility. We found that deletion of *tonB3*, PA2983 or PA2982 led to non-polar localization of the flagellum and FlhF, which was thought to sit at the top of the flagellar localization hierarchy. Surprisingly, these mutants also exhibited pronounced changes in pilus formation or localization, indicating that these proteins may co-ordinate both the pilus and flagellum motility systems. Thus, we have renamed PA2983 and PA2982, *pocA* and *pocB*, respectively, for polar organelle co-ordinator to reflect this function. Our results suggest that TonB3, PocA and PocB may form a membrane-associated complex, which we term the Poc complex. These proteins do not exhibit polar localization themselves, but are required for increased expression of pilus genes upon surface association, indicating that they regulate motility structures through either localization or transcriptional mechanisms.

Introduction

The list of polarly localized bacterial factors continues to grow, but the mechanisms that regulate their production and localization remain poorly understood. For example, PopZ and HubP spatially co-ordinate specific subsets of polarly localized proteins in *Caulobacter crescentus* and *Vibrio cholerae*, but the mechanisms by which the production of polar structures is co-ordinated remains unknown (Bowman *et al*., 2010; Yamaichi *et al*., 2012). Furthermore, neither PopZ nor HubP are widely conserved such that the co-ordination of polar structures in most bacteria remains mysterious. To address this topic, we have focused on the motility structures of the Gram-negative bacterium *Pseudomonas aeruginosa. P. aeruginosa*, a clinically important opportunistic pathogen, produces a single polar flagellum and multiple polar type IV pili which contribute both to motility and to virulence (Amako and Umeda, 1982; Mattick, 2002), presenting excellent targets for understanding the

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mechanisms of polar protein localization. During the first stages of infection, the flagellum and pili are required for adherence to mammalian host cells (Hahn, 1997; Feldman *et al*., 1998). *P. aeruginosa* is predicted to bind to host mucin using surface structures, such as the flagellum, to remain in the lower respiratory tract (Scharfman *et al*., 2001). Once an infection has become established, chronic *P. aeruginosa* infection is maintained through strategies that increase resistance to antibiotic therapy, including formation of biofilms (Pier, 2002), a process that requires both flagella and type IV pili (O'Toole and Kolter, 1998).

Assembly of the *P. aeruginosa* flagellum results from a highly organized series of events, requiring approximately 50 genes. Flagellar gene transcription is divided into a hierarchy of four classes, each expressed in a specific temporal order (Chilcott and Hughes, 2000; Dasgupta *et al*., 2003). Although transcriptional regulation represents a major control mechanism for organizing flagellum assembly, it does not provide an explanation for the controlled placement of the flagellum at the cell pole. A putative GTP-binding protein FlhF is polarly localized and required for polar localization of the *Pseudomonas* flagellum (Pandza *et al*., 2000), such that FlhF has been considered to sit at the top of the flagellar localization genetic hierarchy. Misplacement of the flagellum to the lateral side of the cell, as is seen in a *flhF* mutant, results in a dramatic decrease in swimming motility despite a fully assembled, rotating flagellum (Pandza *et al*., 2000; Murray and Kazmierczak, 2006). FlhF has sequence similarity to the receptor protein of the bacterial signal recognition particle (SRP), a ribonucleoprotein complex that contains several proteins and the 4.5S RNA (Fekkes and Driessen, 1999). The factors that regulate FlhF localization remain unknown.

Type IV pili have a multitude of functions including twitching and gliding motility, adhesion, immune escape, DNA uptake, biofilm formation, microcolony formation, secretion, phage transduction and signal transduction. Pilus assembly and disassembly occurs rapidly using molecular motors dependent on ATP, and requires a dozen or more proteins (Mattick, 2002). Previously, we reported that *P. aeruginosa* type IV pilus formation is dependent on the actin homologue MreB, while flagellum production and FlhF localization are MreB-independent (Cowles and Gitai, 2010). Based on these results, we hypothesized that multiple, potentially independent, pathways are responsible for polar localization of *P. aeruginosa* pili and flagella: one pathway that requires MreB function and one pathway that is MreB-independent and involves FlhF.

To further elucidate the mechanisms of polar localization and identify additional proteins involved in this process, we identified three mutants with both swimming and twitching defects, suggesting that some proteins may be involved in regulating both flagella and pili. These genes, *tonB3*, PA2983 and PA2982 are homologous to the TonB–ExbB–ExbD complex and PA2983 and PA2982 associated in co-precipitation experiments, suggesting that, like their *Escherichia coli* homologues, they form a membrane-associated complex. We refer to this putative complex as the Poc (polar organelle co-ordinator) complex. We found that deletion of any one of these genes leads to random placement of the flagella, as well as defects in pili formation or localization, depending on the mutation. Furthermore, upregulation of pilus gene transcription upon surface association is absent in *tonB3* and PA2982 deletion mutants, indicating involvement in pilus gene transcriptional control.

These factors thus provide evidence that flagella and pili can be co-ordinated by a common set of proteins.

Results

Polar localization of flagella and type IV pili occurs independently

Typical *P. aeruginosa* cells contain a single flagellum and multiple type IV pili, both located at the pole. Our previous work suggests that each structure arrives at the pole through separate mechanisms. Pili can be located at the same pole as the flagellum, the opposite pole, or both poles. Furthermore, our previous discovery that pili production is upregulated upon surface association, while flagella are not, as well as the involvement of the actin homologue MreB specifically in pilus and not flagellum localization, suggests that these two structures are localized by separate mechanisms (Cowles and Gitai, 2010).

To test this hypothesis more directly, we used time-lapse microscopy to investigate whether localization of pilus and flagella structures is co-ordinated throughout the cell cycle using the GTPase FlhF and the chemotaxis histidine kinase CheA as markers for flagella localization and a previously characterized pilus retraction ATPase PilT as a marker for pilus localization (Cowles and Gitai, 2010). Previous work had shown that fluorescently labelled FlhF has a bipolar localization pattern with the focus of brightest intensity located at the flagellated pole, while CheA has a unipolar localization pattern at the flagellated pole (Guvener *et al*., 2006; Murray and Kazmierczak, 2006). We constructed a strain expressing arabinose-inducible C-terminal fusions of both FlhF (to cerulean, FlhF–CER) and CheA (to EGFP, CheA–EGFP), and compared FlhF–CER and CheA–EGFP localization to that of GFP–PilT. Here, we found that FlhF and CheA colocalized in immobile polar puncta throughout the cell cycle, with new puncta forming only at the new pole during cell division (Movies 1 and 2). In contrast, PilT puncta formation followed no predictable pattern, with puncta forming at either or both poles (Movie 3). Furthermore, we observed motile non-polar GFP–PilT puncta that seemed to move along the cell body until they reached a pole (Movie 3).

To examine the role of cell division and pole formation in pilus and flagella localization, we treated *P. aeruginosa* with cefsulodin to inhibit cell division and examined the localization patterns of FlhF–CER, CheA–EGFP and GFP–PilT in the resulting filamentous cells over time. FlhF–CER and CheA–EGFP began as bipolar puncta, but as the cell elongated, FlhF–CER and CheA–EGFP also became visible at the midpoint of the filament, and then at the quarters (Movies 4 and 5). In contrast, GFP–PilT formed a single unipolar focus that remained static throughout elongation of the filament. As the filament elongated, weak, seemingly mobile, foci simultaneously appeared at the midpoint and other pole (Movie 6). Furthermore, all three markers formed internal foci in filamented cells, splitting the filaments into equal compartments. This indicates that the curvature of the pole was not crucial for localization, and other mechanisms are therefore responsible for achieving polar localization of both flagella and pili.

Three hypothetical proteins are required for swimming motility and polar flagellum localization

Since flagella and pili localization occurred independently, we were interested in finding additional factors that contribute to localization of flagella or pili. To this end, we performed screens to find factors involved in their localization, beginning with flagella. *flhF* mutants that mislocalize flagella impair swimming such that we reasoned that additional regulators of flagella localization would also exhibit reduced swimming. We thus conducted a motility screen using the University of Washington *P. aeruginosa* PAO1 transposon mutant library (Jacobs *et al*., 2003). We screened 9440 mutants for swimming motility defects and found 241 mutants with reduced swimming compared with the wild-type parent. To differentiate mutants that had mislocalized flagella from those where no flagellum was produced, we used transmission electron microscopy (TEM) to determine the cellular location of the flagellum. The majority of mutants had insertions in known structural elements and did not produce a flagellum (data not shown). However, we also isolated mutants in two known regulators of flagellum number and localization, the anti-activator *fleN* and the GTPase *flhF*, respectively, confirming the validity of the screen.

Using this approach, we identified three new factors involved in polar flagellum localization, PA0406, PA2983 and PA2982. PA0406 had previously been renamed *tonB3* (Huang *et al*., 2004) due to its similarity to *E. coli tonB*, a gene involved in energy transduction. PA2983 and PA2982 are predicted to encode hypothetical proteins, are co-transcribed (Fig. S1), and have sequence similarity to ExbB/MotA/TolQ and ExbD/MotB/TolR respectively. The homologues of the three proteins identified in our screen, namely TonB (PA0406 homologue), ExbB (PA2983 homologue) and ExbD (PA2982 homologue), represent a well-characterized complex in *E. coli* (Postle and Kadner, 2003). Available transposon insertions in the other TonB/ExbB/ExbD orthologues (*tonB2*, *exbB1*, *exbB2*, *exbD1* and *exbD2*) showed no differences in swimming or twitching motility from wild-type (data not shown). Interestingly, a survey of several bacterial genomes suggested that the presence of multiple TonB homologues generally correlates with the production of a single, polar flagellum while peritrichous bacteria tend to have only one TonB (Supplemental Table S1). Thus, it is possible that TonB systems control polar flagellum localization in other bacteria as well.

To confirm the causality of the transposon insertion swimming phenotypes, we constructed in-frame, non-polar deletions of each gene individually and demonstrated that the deletion mutants had swimming motility defects comparable to a *flhF* mutant in a standard plate assay (Fig. 1). We were unable to construct a PA2983–PA2982 double mutant, suggesting that deletion of both genes together was lethal. To visualize swimming motility on a single cell basis, we used tracking software to follow cells under the microscope. While wild-type cells swam in straight lines and quickly passed through the field of view, the majority of *tonB3*, PA2983 and PA2982 cells moved in corkscrew-like patterns with little progress made in any one direction (Movies 7–10). As a control, we observed a non-flagellated strain, *fliF*, and found that these cells did not move at all (Movie 11). To complement the swimming defects, we provided each gene to the respective deletion strain *in trans* and showed that swimming motility was fully restored (Fig. S2). Additionally, we monitored

bacterial growth in liquid media and found that all of the mutants grew like wild-type PAO1, indicating that swimming defects were not due to differences in growth (data not shown).

To determine if the swimming motility defects in the deletion mutants were also due to incorrect placement of the flagellum, we examined flagellum localization using TEM (Fig. 1). For these experiments, flagella were designated as non-polar if the base of the flagellum originated from the lateral side of the cell and were considered polar if the flagellum was located anywhere on the curved end of the cell. Using this definition, ~90% of wild-type cells had polar flagella, the remaining cells were non-flagellated, and no cells had non-polar flagella. In contrast, the deletion strains produced flagella at non-polar sites in approximately 50% of cells (Fig. 2A), which is the same proportion of cells with mislocalized flagella as seen in a *flhF* deletion (Fig. 2A). Under the growth conditions used in these experiments, an average *P. aeruginosa* cell measured 1.50 ± 0.18 μm long by 0.74 ± 0.07 μm wide (n = 30). We calculated surface areas by considering a cell to be a capsule-shaped object with a cylindrical body capped by two half spheres and determined that random placement of the flagellum would result in 50.6% of cells having the flagellum originate from the lateral sides of the cell and 49.4% of cells with a polar flagellum. Thus, deletion of *tonB3*, PA2983 or PA2982 results in an apparent random localization of the flagellum. Finally, we observed that *tonB3*, PA2983 and PA2982 mutant cells were qualitatively shorter than wild-type cells (Fig. 1). Quantification of cell size demonstrated that the mutant cells were ~15% shorter than wild-type (Supplemental Table S2).

tonB3, PA2983 and PA2982 affect the subcellular localization of known flagellar factors

Prior to this work, FlhF was the most upstream factor known to regulate flagellar localization. We therefore examined whether these proteins function upstream or downstream of FlhF. To assess the role of *tonB3*, PA2983 and PA2982 in FlhF localization, we constructed an arabinose-inducible C-terminal GFP fusion to FlhF. FlhF–GFP was bipolar in wild-type (Fig. 3A) and *flhF–gfp* complemented the swimming motility defect of a *flhF* deletion mutant (data not shown), indicating that the fusion was functional. When expressed in *tonB3*, PA2983 or PA2982 mutant backgrounds, the FlhF–GFP focus was located at non-polar sites (Fig. 3A and Fig. S3). Using an Alexa Fluor 488 carboxylic acid, succinimidyl ester to label the bacterial cell and flagellum, we found that the non-polar FlhF focus (FlhF–mCherry in this experiment) colocalized with the flagellum (Fig. 3B). Together these results demonstrate that *tonB3*, PA2983 and PA2982 are necessary for the proper polar localization of FlhF.

As an additional marker for flagellum protein localization, we created an arabinose-inducible C-terminal GFP fusion to CheA. CheA–GFP was unipolar with one to two foci present at a given pole (Fig. 3A) and *cheA–gfp* complemented the swimming defect of a *cheA* mutant (data not shown), showing that the fusion was functional. In *tonB3*, PA2983 or PA2982 mutants, CheA–GFP foci were non-polar (Fig. 3A and Fig. S3), similar to the pattern seen with FlhF–GFP. In the *flhF* mutant, CheA–GFP foci were likewise non-polar (Fig. 3A), suggesting that TonB3, PA2983 and PA2982 regulate localization of FlhF, which in turn regulates the localization of CheA. These results implicate TonB3, PA2983 and PA2982 as sitting at the top of the known flagellar localization hierarchy.

tonB3, PA2983 and PA2982 also regulate type IV pilus production

Given the expectation that flagella and pili would be regulated independently, the identification of *tonB3* as a flagellar regulator was surprising in light of a previous report that *tonB3* is required for pilus-driven *P. aeruginosa* twitching motility (Huang *et al*., 2004). We therefore investigated whether our newly discovered flagellar localization factors might also influence type IV pili. We directly imaged pili by TEM and found that deletion of *tonB3*, PA2982, PA2983 or *flhF* affects pilus production (Fig. 1). Nearly 100% of *tonB3*, PA2982 and *flhF* cells were non-piliated while the PA2983 mutant had a dramatic increase in the number of cells with non-polar pili (70% of cells compared with 3.5% in wild-type) (Fig. 2B). As type IV pili are required for twitching motility, we tested our mutants using a standard plate assay and found that the *tonB3*, PA2983 and PA2983 mutants were defective for twitching motility, similar to *pilA* mutants which lack the major pilin protein (Fig. 4).

The complete lack of population-level twitching motility was particularly surprising for the PA2983 mutant because unlike *tonB3* and PA2982 mutants, which exhibited almost no pili, most of the PA2983 mutant cells retained pili. We hypothesized that the lack of twitching motility in the PA2983 mutant could result from these remaining pili being non-functional (not pulling) or from these pili, which were largely non-polar, leading to opposing pulling forces on opposing sides of the cell, thereby resulting in a lack of productive movement in any one direction. To distinguish these two possibilities, we imaged the leading edge of twitching motility in a population of cells. In this assay, wild-type *P. aeruginosa* moved progressively across the field of view with individual cells moving in a jerking motion that indicated pilus activity (Movie 12). In contrast, non-piliated strains such as *pilA*, *tonB3* and PA2982 mutants did not move, either as a group or when examining the motion of single cells (Movies 13–15). PA2983 cells behaved like the non-piliated cells and did not move (Movie 16), indicating that the non-polar pili produced in this strain were not functional.

To better understand the mechanism by which *tonB3*, PA2983 and PA2982 affect pilus production, we examined the localization patterns of two polar pilus proteins, the pilus retraction ATPase PilT and the outer membrane secretin PilQ. Previously, we demonstrated that arabinose-inducible GFP–PilT and PilQ–mCherry fusions were functional and localized to the cell pole in the majority of wild-type PAO1 cells (Cowles and Gitai, 2010). When expressed in *tonB3*, PA2983 or PA2982 mutants, both GFP–PilT and PilQ–mCherry had a diffuse localization pattern in 100% of cells (Fig. 4). Because these genes are necessary for both flagellum and pilus regulation, we propose to rename PA2983 and PA2982 as *pocA* and *pocB*, respectively, for polar organelle co-ordinator.

PocA and PocB form a membrane-associated complex

The homology of PocA and PocB to ExbB and ExbD suggested that like their *E. coli* homologues, PocA and PocB may associate with one another to form a membrane-associated complex. ExbB and ExbD are transmembrane inner membrane proteins with the bulk of ExbB exposed to the cytoplasm and the bulk of ExbD exposed to the periplasm (Postle and Kadner, 2003). Together with TonB, the TonB–ExbB–ExbD complex utilizes the proton motive force across the inner membrane to provide energy for nutrient transport across the outer membrane (Postle and Larsen, 2007). To determine if TonB3 and the Poc

proteins are membrane associated, we created arabinose-inducible FLAG fusions to each protein. The arabinose-inducible constructs produced functional fusion proteins that complemented the swimming motility defects of the respective deletion mutants (Fig. S2). By separating soluble and insoluble fractions from clarified cell lysates, we determined that TonB3–iFLAG, PocA–FLAG and PocB–FLAG were located in the insoluble fractions (Fig. 5A), suggesting that, like their *E. coli* homologues, these proteins are membrane-associated. In addition, we examined the localization of each FLAG fusion in each of the deletion mutants and found that membrane association of each fusion protein is not dependent on the presence of the other proteins (Fig. 5A).

To examine the potential protein–protein interactions between TonB3, PocA and PocB, we constructed C-terminal HIS-tagged versions of PocA and PocB for co-precipitation experiments. As with the FLAG fusions, PocA–HIS and PocB–HIS were able to complement the swimming motility defects of their respective deletion strains (Fig. S2), indicating that the fusions were functional. PocA–HIS pulled down PocB–FLAG in a co-precipitation assay using nickel affinity resin (Fig. 5B). We repeated the experiment using PocB–HIS and found that PocB–HIS precipitated PocA–FLAG, as well (Fig. 5B). Negative control strains containing PocA–HIS or PocB–HIS alone had no protein in the α-FLAG Western after precipitation (Fig. 5B). To determine if TonB3 is required for the PocA–PocB interaction, we performed the above co-precipitation experiment in the *tonB3* mutant background and found that PocA and PocB still interact in the absence of *tonB3* (Fig. S4). Co-precipitation experiments between PocA–HIS or PocB–HIS and TonB3–iFLAG were unable to pull down TonB3 (Fig. S5). Together, these results suggest that PocA and PocB interact while either TonB3 is not a part of the complex or its interaction with PocA and PocB is not detectable by this approach.

PocA and PocB are not polarly localized

We next sought to determine the mechanism by which TonB3 and the Poc proteins impact flagellar and pilus localization and production. These proteins are required for polar FlhF localization and the localization of polar proteins is often mediated by factors that are themselves found at the cell pole. We therefore investigated the localization of TonB3, PocA and PocB using immunofluorescence (IF) with α-FLAG antibodies. Consistent with their predicted membrane association, PocA–FLAG and PocB–FLAG localized to the cell periphery in liquid-grown cells (Fig. 5C) and surface-grown cells (data not shown). Additionally, we determined that the peripheral localization of PocA–FLAG and PocB–FLAG was unaffected by deletion of *tonB3*, *pocA* or *pocB* (Fig. 5C). Attempts to visualize TonB3 localization using IF for TonB3–iFLAG (Fig. 5C) or FlAsH reagent for a tetracysteine-tagged TonB3 (TonB3–iFlAsH) (data not shown) showed no fluorescence over background levels, suggesting that, despite arabinose induction, TonB3 is maintained at levels that remain below the limits of our detection.

tonB3 and pocB, but not pocA, are required for surface-regulated transcriptional upregulation of pilus genes

Since their diffuse localization suggests that PocA and PocB do not function as polar anchors, we tested the possibility that they function in regulating gene expression.

Previously, we demonstrated that the association of *P. aeruginosa* cells with solid surfaces promotes pilus production (Cowles and Gitai, 2010). For example, pilus gene expression, as monitored by *pilA* levels, is markedly increased during growth on surfaces with increasing stiffness. To determine if the Poc proteins are involved in this response to surfaces, we used quantitative PCR (qPCR) to examine *pilA* expression in the deletion mutants. For *tonB3* and *pocB*, we found that *pilA* levels were at wild-type levels during growth in liquid media but failed to increase in response to surface association (Fig. 6A), indicating that *tonB3* and *pocB* are important for pilus expression when cells are grown on a solid surface. In contrast, *pilA* expression was at wild-type levels in the *pocA* mutant under both conditions (Fig. 6A), suggesting that *pocA* is not necessary for *pilA* regulation. These results were consistent with our TEM data where we saw no pili in *tonB3* and *pocB* cells while *pocA* made non-polar pili (Fig. 2).

One possible mechanism for the environment-specific effects of *tonB3* and *pocB* deletion is that these factors are themselves regulated by surface association. We monitored *tonB3*, *pocA* and *pocB* transcription and found that *pocA* and *pocB* increased approximately two- to threefold during growth on a solid surface (Fig. 6B). Unlike *pocAB*, *tonB3* levels were the same in either growth condition (Fig. 6B). To determine if TonB3, PocA or PocB were altered in liquid versus surface growth conditions, we generated natively expressed FLAG-tagged versions of each protein and used Western blots to measure protein levels. C-terminal fusions to PocA (PocA–FLAG) or PocB (PocB–FLAG) complemented the swimming defects of *pocA* or *pocB* mutants, respectively, while TonB3 required an internal FLAG fusion (TonB3–iFLAG; between aa 160 and 161) in order to retain functionality (Fig. S2). As was seen with the transcriptional studies, PocA and PocB increased approximately two- to threefold upon surface association while TonB3 levels were statistically unaffected (Fig. 6C).

Because *tonB3* and *pocB* deletion disrupted regulation of *pilA* expression, we considered the possibility that these factors could be a sensor required for the response to surface association. To test this hypothesis, we performed microarrays comparing gene expression in cells grown in liquid medium to expression from cells at the leading edge of motility on the surface of agar plates. If deletion of *tonB3*, *pocA* or *pocB* prevented *P. aeruginosa* from differentiating liquid from surface growth, we predicted that the mutant expression profiles would be vastly different from wild-type. Wild-type *P. aeruginosa* PAO1 had 3247 genes that had more than a twofold change in response to surface association (Fig. 7A). By comparing the gene expression profile of wild-type PAO1 to the profiles of each mutant grown under the same growth conditions, we determined that, although there were some differences from wild-type, general transcription patterns were unaffected by the loss of *tonB3*, *pocA* or *pocB* (Fig. 7A), indicating that these factors do not seem to impact overall *P. aeruginosa* surface sensing.

Despite the relative similarities between the expression profiles of wild-type, *tonB3*, *pocA* and *pocB* strains, we observed that most pilus genes did not respond as strongly to surfaces in *tonB3* and *pocB*. To directly assess these differences, we performed additional microarrays directly comparing wild-type to mutant gene expression after both strains were grown on a solid surface. The data from these arrays indicate that, in general, pilus gene

transcripts are lower in the *tonB3* and *pocB* mutants (Fig. 7B), showing that, although dispensable for the overall response to surface association, *tonB3* and *pocB* are involved in the specific upregulation of pilus expression. Notably, known regulators of pilus biogenesis (i.e. PilR/S, FimS/AlgR, Vfr) were not statistically different in the mutant strains. Additionally, although present at lower levels, all known pilus biogenesis genes were transcribed in the mutant strains, indicating that pilus production was not disrupted do to the complete loss of any one transcript. Unlike *tonB3* and *pocB*, *pocA* had no significant effect on the levels of most pilus genes (Fig. 7C). This result may begin to explain why *tonB3* and *pocB* cells lack pili altogether while *pocA* cells still produce pili, albeit at non-polar locations.

Because the mutants still produced flagella, we predicted that flagellum gene expression would not be altered in these strains. Indeed, deletion of *tonB3*, *pocA* or *pocB* had no effect on expression of the flagellum subunit gene *fliC* under liquid or surface growth conditions (Fig. S6). To further investigate a possible transcriptional mechanism for *tonB3*, *pocA* and *pocB* regulation of flagellum localization, we analysed the microarray data from the comparisons of wild-type gene expression to the mutant expression profiles and saw that the vast majority of flagellum genes do not change upon loss of these factors (Fig. S6). Thus, across both pili and flagella the absence of an organelle correlates with reduced transcript levels while the mislocalization of an organelle (namely flagella in all three mutants and pili in *pocA*) does not.

Discussion

Prior to this work, results from several studies, including our own, suggested that independent processes regulate flagellum and pilus production. The four-tiered hierarchy for flagellum gene transcription (Dasgupta *et al*., 2003) doesn't immediately suggest a connection to pilus production and screens for swimming or twitching motility mutants seem to have focused on one form of motility or the other. Our previous results showing that MreB and surface association regulate pilus production but not flagellum formation (Cowles and Gitai, 2010) and that flagella and pili localize at independent subcellular sites and cell cycle time points also led us to believe that these two processes were regulated separately. However, our identification of three proteins that regulate both flagellum and pilus production suggest that these polar structures are actually coupled, with TonB3, PocA and PocB functioning near the top of each structure's regulatory hierarchy.

Multiple mechanisms contribute to flagella and pili localization

Previously, FlhF was thought to function at the top of the flagellar localization hierarchy. FlhF is mislocalized in the absence of *tonB3*, *pocA* or *pocB*, resulting in non-polar placement of the flagellum at the point where FlhF puncta has formed. This non-polar flagella placement phenocopies the *flhF* deletion mutant, suggesting that TonB3, PocA and PocB direct polar localization of flagella by directly or indirectly impacting FlhF polar localization. Furthermore, new FlhF puncta form only at the new pole of dividing cells, either following or just prior to division, indicating that regulation of FlhF, and therefore flagella localization, is linked to the cell cycle.

While TonB3, PocA and PocB affect flagellar localization, their effects on type IV pili are more complex. Deletion of *tonB3* or *pocB* resulted in the absence of pili formation, while *pocA* deletion led to the formation of mislocalized, non-functional pili. Thus, even within the hypothesized Poc complex, the individual genes may have distinct roles in regulating pili formation. One such mechanism appears to be transcriptional, since we did not observe the expected wild-type increase in pili gene transcription upon surface association in the absence of *tonB3* and *pocB*. However, the *pocA* deletion did not have transcriptional defects, but rather defects in pilus localization, suggesting a second mechanism regulating pili localization independent of transcription.

Membrane topology of the putative TonB3–PocA–PocB complex may contribute to function

How a single putative TonB3–PocA–PocB complex can use several different methods (i.e. localization versus transcript levels) to impact both flagella and pili remains to be determined. In fact, we cannot rule out the possibility that these three proteins do not form a single complex or that they may interact with other factors or in different combinations with each other for their distinct effects. We hypothesize that cellular location and membrane topology contribute to the functions of each individual factor. TonB3 and PocB homologues TonB and ExbD have single transmembrane regions with the majority of the protein in the periplasm, whereas the PocA homologue ExbB has three transmembrane regions with the majority forming a large cytoplasmic loop (Postle and Kadner, 2003). Interestingly, this spatial grouping within the putative complex members is consistent with differences in phenotype. While all three proteins regulate flagellum localization, the largely periplasmic TonB3 and PocB regulate pilus production transcriptionally, while the mainly cytoplasmic PocA regulates pili localization independent of transcription. Since the *pocA* mutant still makes pili, it may have an association with unique factors, potentially located in the cytoplasm, distinct from TonB3 and PocB. Additionally, downstream factors unique to either the flagella or pili may determine the functions of each individual complex. Alternatively, separate complexes may exist that affect the flagella and pili in different ways. It will be important to determine the identity of additional downstream factors, and to investigate whether this regulation occurs as part of a TonB3–PocA–PocB complex or in isolation.

PocAB impact polar localization of flagella without being polarly localized themselves

While the importance of *tonB3*, *pocA* and *pocB* for both *P. aeruginosa* polar motility structures is clear, the mechanism(s) by which these factors function remains unclear. Homologues of TonB3, PocA and PocB provide the energy for transporting large molecules across the outer membrane using the proton motive force (Postle and Larsen, 2007). It is possible that this energy transduction aspect is important for their function in localization; however, while *Pseudomonas* TonB1 and TonB2 have been implicated in iron and haem uptake (Zhao and Poole, 2000), a transport role for TonB3 has not been established. Furthermore, it is unclear why energy transduction would be needed for flagellum localization. Reports have indicated that membrane potential is important for cell division in *Bacillus subtilis*, and perturbations of the proton motive force modulate localization of several cell division proteins including MinD, FtsA and MreB (Strahl and Hamoen, 2010).

Since MreB is important for pili formation in *P. aeruginosa*, membrane potential could provide a mechanism whereby MreB localization affects pili localization in the *pocA* mutant. Additionally, we found that *tonB3*, *pocA* and *pocB* cells are somewhat shorter than wild-type, suggesting a possible connection to cell size maintenance during division or, at least, more pleotropic effects of these mutations. Furthermore, since flagella localization is tied to the cell cycle, perhaps it is also dependent on membrane potential. Additional studies are needed to determine if other proteins are mislocalized in the absence of *tonB3*, *pocA* and *pocB*.

Alternatively, it is possible that these proteins may have adopted an independent and previously unknown function in localization and formation of flagella and pili. For example, they could serve as anchors to bring additional factors to the poles. We have shown that PocA and PocB are membrane associated, but have no discrete localization within the membrane, so it remains unclear how a complex that is not localized could direct polar localization of additional proteins. We were unable to examine the localization of TonB3 or precipitate it with either PocA or PocB. This is consistent with studies with *E. coli* homologues, where TonB does not pull down without cross-linking (Ollis *et al*., 2009). Therefore it remains possible that TonB3 could be localized to the poles and that transient interactions with PocAB could direct polar localization of flagella.

TonB3 and PocB contribute to upregulation of pilus genes in response to surface association

One way in which non-localized proteins can regulate the assembly of a polar motility complex is by regulating motility protein production. Consistent with this model, we found that TonB3 and PocB affected pilus gene expression in an environment-specific manner. In particular, we previously described an increase in pilus gene transcription upon surface association and this surface-dependent pilus upregulation was less robust in *tonB3* and *pocB* mutants. These mutants did not affect the overall expression profile in response to surface growth but rather altered pilus transcript levels. Thus, TonB3 and PocB specifically function in pilus regulation as opposed to surface sensing itself. How surface recognition occurs and leads to TonB3- and PocB-dependent upregulation of pilus genes remains an open question. Multiple sensors may contribute to global expression profile changes in response to surface association, which would lead to little difference detected in single mutants. A screen for sensors using fusions to the pilus gene promoters could prove illuminating.

While transcriptional regulation can partially explain the absence of pili in *tonB3* and *pocB*, these factors also appear to have a transcription-independent role in subcellular localization. Specifically, *pocA* mutants disrupt pilus localization (but not assembly) without significantly perturbing pilus gene regulation, and *pocA*, *pocB* and *tonB3* all disrupt flagellar and FlhF localization without disrupting flagellar gene regulation. Future studies of the protein interaction partners of TonB3, PocA and PocB will help dissect if these proteins form one or multiple complexes and will provide insight into the mechanism(s) by which this predicted complex co-ordinates pili and flagella formation and directs proteins such as FlhF to the cell poles.

Experimental procedures

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 (C. Manoil, University of Washington) was used as wild-type for all experiments. All strains are listed in Supplemental Table S3. All bacterial cultures were grown in Luria–Bertani (LB) broth (Miller, 1972) at 37°C. Plasmids were conjugated from *E. coli* S17-1 (λ*pir*) or electroporated (25 μF; 200 Ω; 2.5 kV) into *P. aeruginosa* strains and were maintained with the following antibiotic concentrations: tetracycline (Tet), 25 μg ml^{-1} for *E. coli* and 100 μg ml^{-1} for *P. aeruginosa*; gentamicin (Gent), 15 μg ml^{-1} for *E. coli* and 30 μg ml^{-1} for *P. aeruginosa*.

Molecular biological methods

Standard molecular biological methods were used for this study (Sambrook *et al*., 1989). Restriction enzymes (NEB, Ipswich, MA), plasmid preparation, gel extraction and PCR purification kits (Qiagen, Valencia, CA) were all used according to manufacturer's recommendations. Constructs were confirmed by sequencing (Genewiz, South Plainfield, NJ). PCR amplification of DNA was performed using Platinum *Pfx* according to manufacturer's directions (Invitrogen, Carlsbad, CA). Primers (0.2 μM) used in this study (Integrated DNA Technologies, Coralville, IA) are described in Supplemental Table S4.

Motility assays

To assess gross motility, overnight cultures of wild-type *P. aeruginosa* were spotted onto 0.3% agar LB plates (for swimming motility) and stabbed into 1% agar LB plates (for twitching motility). Swimming motility was measured as the distance moved from the point of inoculation on the surface of the agar plate after 20 h. Twitching motility was observed by removing the agar and staining cells attached to the Petri dish with 1% crystal violet after 48 h.

Deletion construction

Non-polar, in-frame deletions of *tonB3*, *pocA* and *pocB* were made by PCR-amplifying regions upstream and downstream of each gene, performing sewing PCR to fuse those products together, cloning the final deletion fragment into pEX18Tc (Hoang *et al*., 1998), and using conjugation to transfer the deletion construct into wild-type PAO1. A 924 bp fragment of *tonB3* was deleted using 0406UpFOREcoRI and 0406UpREV to amplify the region upstream of *tonB3* and 0406DnFOR and 0406DnREVXbaI to amplify the region downstream. A 594 bp fragment of *pocA* was deleted using 298382UpFOREcoRI and 2983UpREV to amplify the region upstream of *pocA* and 2983DnFOR and 298382DnREVXbaI to amplify the region downstream. A 414 bp fragment of *pocB* was deleted using 298382UpFOREcoRI and 2982UpREV to amplify the region upstream of *pocB* and 2982DnFOR and 298382DnREVXbaI to amplify the region downstream. Selected exconjugants were grown overnight in LB broth, streaked to LB 5% sucrose plates for counterselection, and patched to LB ± Tet plates to confirm Tet sensitivity. PCR was used to identify deletion mutants.

Transmission electron microscopy

To prepare samples, overnight cultures were inoculated to 1% agar LB plates and incubated at 37°C for 24 h. Cells were collected from the leading edge of surface motility and gently resuspended in PBS. For TEM images and flagellum and pilus counts in a population, 100 cells from three independent replicates were collected and samples were processed as described (Cowles and Gitai, 2010) (Confocal and Electron Microscopy Core Facility Laboratory, Princeton University). Samples were blinded to assure objective scoring.

Construction of fluorescent fusions

The Gateway® Technology (Invitrogen, Carlsbad, CA) was utilized to construct C-terminal fluorescent fusions to *flhF* and *cheA* as described (Cowles and Gitai, 2010). Briefly, *flhF* and *cheA* genes were PCR-amplified using primers FlhFGWUp, FlhFGWDn, CheAGWUp and CheAGWDn, respectively, and cloned into pDONR223. *flhF* and *cheA* were then transferred into the destination vectors pJN(Gateway–GFP) and pJN(Gateway–mCherry) for *flhF* and pJN(Gateway–GFP) for *cheA*. pJN(Gateway–GFP) and pJN(Gateway–mCherry) were constructed by PCR-amplifying GFP or mCherry in conjunction with the Gateway® cassette using the primers GWr-fpFORSpe and GWrfpREVSpe and cloning the products into pJN105 (Newman and Fuqua, 1999; Baynham *et al*., 2006). The expression vectors pJN(*flhF–gfp*), pJN(*flhF–mCherry*) and pJN(*cheA–gfp*) were transformed into wild-type, *tonB3*, *pocA* and *pocB* mutants. Plasmid maintenance was confirmed using primers AraCAmp and GWrfpREVSpe. pSW(*gfp–pilT*) and pSW(*pilQ–mCherry*) fusions were created previously (Cowles and Gitai, 2010) and conjugated into the *tonB3*, *pocA*, *pocB*, *flhF*, *fliF* and *pilA* deletion strains.

FlhF-CERC was created by PCR-amplifying *flhF* from genomic DNA with FlhFfowardSpeI and FlhFreverseMluI primers and digesting the product with SpeI and MluI. CERC was removed from pVCERC-5 (Thanbichler *et al*., 2007) with MluI and SacI and ligated to *flhF*. The resulting fusion was used as template for PCR using FlhFforwardSpeI and CER-CreverseEcoRISacI primers. *flhF*-CERC was then cloned into pSW196 (Baynham *et al*., 2006) using SpeI and SacI enzymes.

CheA–GFP was created by PCR-amplifying *cheA* from genomic DNA with CheAfowardEcoRI and CheAreverseMluI primers and digesting the product with EcoRI and MluI. EGFP was removed from pVGFP5 (Thanbichler *et al*., 2007) with MluI and SacI and ligated to *cheA*. The resulting fusion was used as template for PCR using CheAforwardEcoRI and EGFPreverseXbaISacI primers. *cheA*-CERC was then cloned into pSC8(*flhF-CERC*) using EcoRI and SacI enzymes.

Microscopy of fluorescent fusions

Pseudomonas aeruginosa carrying pJN(*flhF–gfp*), pJN(*flhF–mCherry*), pJN(*cheA–gfp*), pSW(*gfp–pilT*) and pSW(*pilQ–mCherry*) were grown overnight in LB broth with the appropriate antibiotic, subcultured 1:100 into fresh LB broth containing 0.02% arabinose, and grown for 3 h at 37°C. To collect images, bacterial cells were placed on pads made from 1% agarose in PBS and visualized using a 100× 1.4NA objective on a Nikon 90i microscope equipped with a Rolera XR camera and NIS Elements software. For studies examining

colocalization of FlhF–mCherry and the flagellum, overnight cultures were subcultured with arabinose as described above and grown for 3 h at 37°C. Two hundred and fifty microlitres of cells were pelleted at 2000 *g* for 5 min, and the pellets were resuspended gently in 250 µl of DB buffer [(5 mM KH_2PO_4, 5 mM Na_2HPO_4, adjusted to pH 6.5), 1 mM $CaCl_2$, 2 mM $MgCl_2$]. Cells were washed in DB buffer before being resuspended in 20 µl DB buffer. One microlitre of the Alexa Fluor 488 carboxylic acid, succinimidyl ester dye (10 mg ml^{-1} in DMSO) (Invitrogen, Carlsbad, CA) was added and samples were incubated with shaking (~ 100 r.p.m.) for 1 h at room temperature in the dark. Cells were pelleted, washed twice with 250 µl of DB buffer, and resuspended in a final volume of 250 µl DB buffer. Images were collected as described above. For cefsulodin experiments, strains were prepared as described above (grown overnight, subcultured 1:100 with 0.02% arabinose and incubated for 3 h to log phase) and spotted to agarose pads containing 30 µg ml^{-1} cefsulodin. Time-lapse images were collected every 15 min for 8 h.

Real-time PCR

Total RNA from wild-type *P. aeruginosa* PAO1 was isolated from cells grown in LB broth to an OD_{600} of 5.0 (liquid-grown cells) and from cells collected at the leading edge of motility from the surface of LB agar plates after 24 h (surface-grown cells) with three replicates per growth condition for each strain examined. RNA was processed as described (Cowles and Gitai, 2010). Reactions for real-time PCR were performed in duplicate in 10 µl reactions with SYBR® Green PCR Master mix (Applied Biosystems, Foster City, CA), cDNA template, and appropriate primers as described (Cowles and Gitai, 2010). Transcript levels of *pilA, fliC, tonB3, pocA* and *pocB* were measured with primers listed in Supplemental Table S4. Cycle threshold results for each sample were normalized according to *rpoD* levels (amplified with RpoDForRT and RpoDRevRT) and then converted to relative values factoring in a twofold change in product amount per cycle.

Western blot analysis

Whole-cell lysates from *P. aeruginosa* strains were isolated from cells grown in LB broth to an OD_{600} of 5.0 (liquid-grown cells) and from cells collected at the leading edge of motility from the surface of LB agar plates (surface-grown cells) for 24 h with three replicates per growth condition. Cell samples were processed for immunoblot quantification as described (Cowles and Gitai, 2010). Samples were separated on 15% SDS/PAGE gels and transferred to nitrocellulose membranes. For immunoblot analysis, membranes were blocked with 5% milk (w/v) in TBS-T, then washed and immunodetected in TBS-T. Primary anti-FLAG antiserum (Invitrogen, Carlsbad, CA) was used at 1:2000 and secondary anti-rabbit-HRP IgG conjugate (GE Healthcare, NA934V) was used at 1:8000. ECL Western Blotting Reagent (GE Healthcare, RPN2106) and film were used for signal detection; ImageJ software was used to quantify signal intensity (Abramoff *et al*., 2004).

Microarrays

Total RNA from wild-type, *tonB3, pocA* and *pocB* was isolated from cells grown in LB broth to an OD_{600} of 5.0 (liquid-grown cells) and from cells collected at the leading edge of motility from the surface of LB agar plates after 24 h (surface-grown cells) with three

replicates per growth condition for each strain examined. RNA was processed using the Trizol extraction method, cDNA synthesis and labelling, and microarray hybridization and analysis as described (Rutherford *et al*., 2011). Microarrays were designed using Agilent software in an 8× 15 grid format. Each array contained two 60-mer probes per open reading frame in the *P. aeruginosa* PAO1 genome (Agilent custom array 028678). Data were extracted with Agilent Feature Extractor and analysed on the Princeton University Microarray Database (PUMAdb, http://puma.princeton.edu) based on Gollub *et al*. (2003).

Construction of FLAG and HIS proteins

C-terminal FLAG-tagged PocA and PocB were made by PCR-amplifying *pocA* using primers 2983cFLAGUpEcoRI with 2983cFLAGDnXbaI (for inducible expression) or 2983cFLAGUpEcoRI2 with 2983cFLAGDnSpeI (for native expression) and *pocB* using primers 2982cFLAGUpEcoRI with 2982FLAGDnXbaI (for inducible expression) or 2983cFLAGUpEcoRI2 with 2982cFLAGDnSpeI (for native expression). Resulting PCR products were cloned into pJN105 for arabinose-inducible fusions or miniCTX1 (Hoang *et al*., 2000) for natively expressed fusions. C-terminal, arabinose-inducible HIS-tagged PocA and PocB were similarly constructed by PCR-amplifying *pocA* (2983cFLAGUpEcoRI with 2983cHISDnSpeI) and *pocB* (2982cFLAGUpEcoRI with 2982cHISDnSpeI) and cloning the products into pSW196 (Baynham *et al*., 2006). An arabinose-inducible FLAG tag was inserted into *tonB3* by PCR-amplifying upstream and downstream halves of *tonB3* using 0406UpFLAGEcoRI2 with 0406UpFLAG (519 bp) and 0406DnFLAG with 0406UpFLAGXbaI2 (504 bp) primers respectively. The two resulting products were sewn together using 0406UpFLAGEcoRI2 and 0406UpFLAGXbaI2 primers and cloned into pJN105. Similarly, a natively expressed internal TonB3–FLAG fusion was constructed using 0406UpFLAGEcoRI2 with 0406UpFLAG and 0406DnFLAG with 0406DnFLAGSpeI and cloning the resulting product into miniCTX1. Sequence-confirmed vectors were transformed into PAO1 strains of interest. Plasmid maintenance or integration at the attP site was confirmed by PCR.

Isolation of soluble and insoluble fractions

Samples were prepared by diluting overnight cultures 1:500 in fresh medium and incubation at 37°C to OD_{600} = 0.5. Cells were pelleted at 5000 r.p.m. for 10 min at 4°C and resuspended in lysis buffer (50 mM PO_4, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$), normalizing to OD_{600} = 20. Cells were lysed using a French pressure cell (at 20 000 psi) followed by centrifugation at 2500 r.p.m. to remove unbroken cells. Soluble and insoluble fractions were isolated by ultracentrifugation (100 000 r.p.m.) for 30 min. FLAG-tagged protein levels were measured by Western blot as described above.

Immunofluorescence

Overnight cultures were diluted into fresh medium and grown to OD_{600} = 0.5. Cells were mixed gently with 5× fix solution [12.5% paraformaldehyde, 150 mM $NaPO_4$ (pH 7.5)] and incubated at room temperature for 15 min. Samples were then incubated on ice for 20 min before being washed three times in PBS and resuspended in 75 µl GTE [50 mM glucose, 10 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 7.5)] containing lysozyme (10 µg ml^{-1}). Fixed

cells were spotted to poly-Lysine-coated slides, dried, and immersed in cold methanol for 5 min and cold acetone for 5 min. Samples were rehydrated and blocked in 2% BSA in PBS and incubated with anti-FLAG antibody (Invitrogen, Carlsbad, CA) diluted 1:250 in PBS with 2% BSA for 1 h. Slides were then washed 10 times with PBS and incubated for 1 h in the dark with goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen) diluted 1:200. Cells were washed 10 times with PBS and visualized by microscopy as described above.

Co-precipitation of PocA and PocB

To investigate protein–protein interactions between TonB3, PocA and PocB, overnight cultures carrying pSW (PocA–HIS) or pSW (PocB–HIS) alone, pJN (TonB3–iFLAG), pJN (PocA–FLAG) or pJN (PocB–FLAG) alone, or a combination of HIS and FLAG constructs were subcultured 1:500 into LB gentamicin and incubated at 37°C to OD_{600} = 0.5. Cells were pelleted at 5000 r.p.m. for 10 min at 4°C and resuspended in lysis buffer (50 mM PO_4, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$), normalizing to OD_{600} = 20. Clarified lysates were made using a French pressure cell (at 20 000 psi) followed by centrifugation at 2500 r.p.m. to remove unbroken cells. Lysates were combined with 1% Thesit (Sigma, St. Louis, MO), 10 mM imidazole and 100 µl nickel resin (Qiagen, Valencia, CA) and incubated at 4°C for 90 min with gentle shaking. Resin was pelleted, washed in lysis buffer + Thesit and imidazole, and incubated at room temperature for 5 min with gentle shaking. This wash step was repeated three times with samples collected for immunoblots after each one. The final resin pellet was resuspended in 2× SDS-PAGE loading dye. Western blots to detect FLAG protein were performed as described above.

Statistical analysis

P-values were calculated using a Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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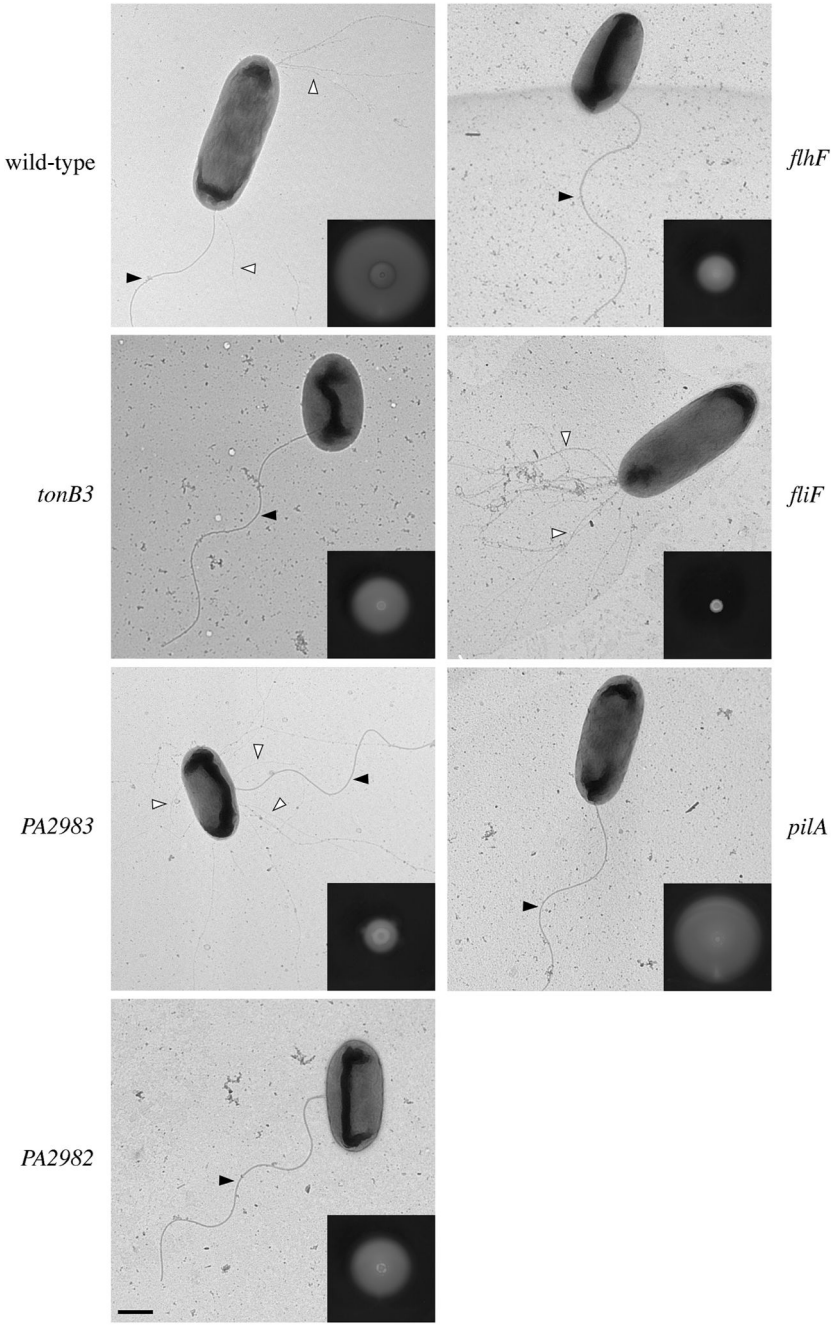

Fig. 1.

Swimming motility and flagella and type IV pili localization are disrupted in *tonB3*, PA2983 (*pocA*), PA2982 (*pocB*) and *flhF* mutants. Transmission electron microscopy (TEM) images of wild-type and mutant *P. aeruginosa* strains. *fliF* and *pilA* strains were used as controls that lacked flagella and pili respectively. Closed arrowheads denote flagella and open arrowheads indicate representative pili. Scale bar, 500 nm. (Inset) Representative images of swimming motility on 0.3% agar plates.

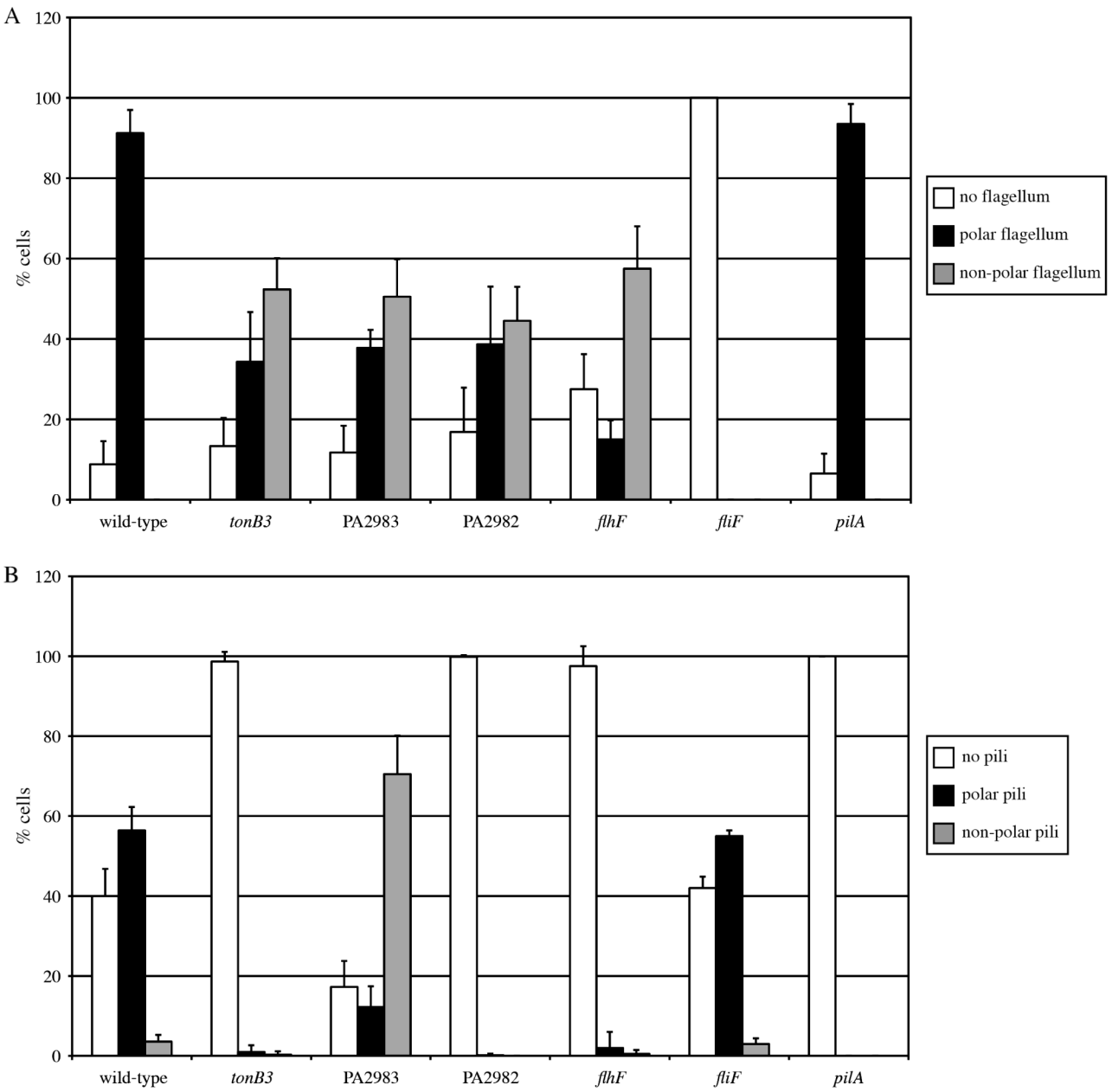

Fig. 2.

Quantification of flagella and type IV pili localization by TEM. The distribution of flagella (A) and type IV pili (B) collected from populations at the leading edge of motility on the surface of agar plates. *fliF* and *pilA* strains were used as controls that lacked flagella and pili respectively. Error bars represent the standard deviation (n = 3 replicates of 100 cells each).

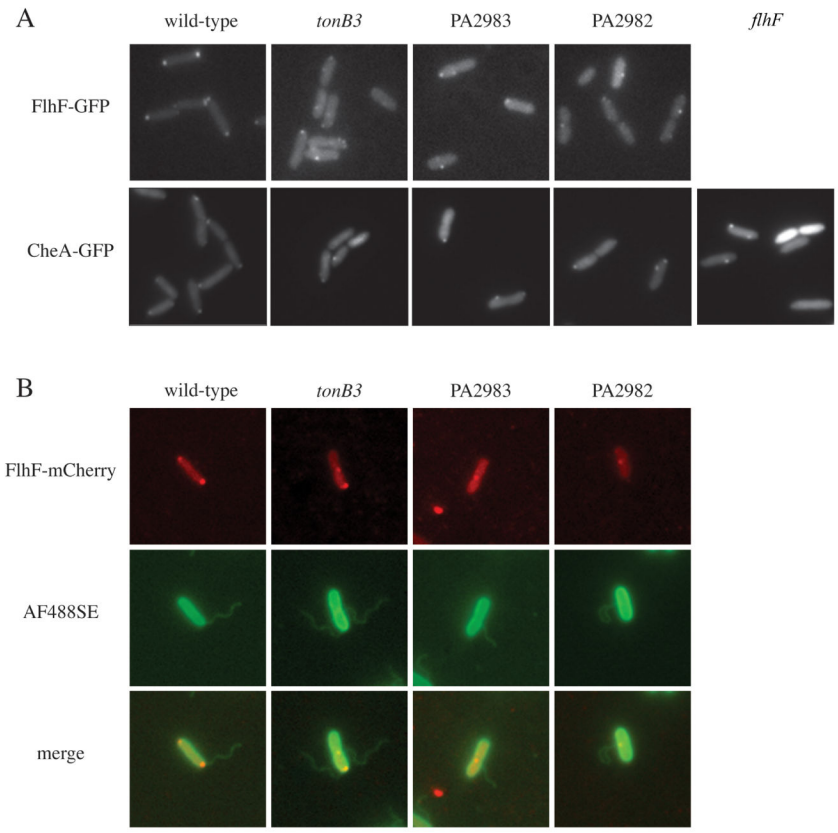

Fig. 3. *tonB3*, *pocA* and *pocB* mutations disrupt localization of FlhF–GFP and CheA–GFP fluorescent fusions

A. FlhF–GFP (top) and CheA–GFP (bottom) localization in wild-type, *tonB3*, PA2983 (*pocA*), PA2982 (*pocB*) and *flhF* strains.

B. Colocalization (bottom) of FlhF–mCherry (top) and Alexa Fluor 488-labelled flagella (middle) in wild-type and mutant strains.

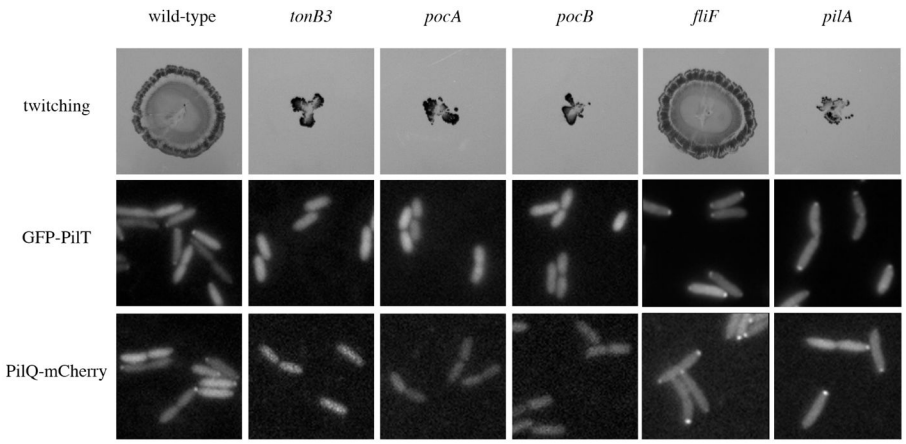

Fig. 4.

Twitching motility and GFP–PilT and PilQ–mCherry localization are impaired in *tonB3*, PA2983 (*pocA*) and PA2982 (*pocB*) mutants. (Top) Twitching motility of wild-type PAO1 and mutant strains. *fliF* and *pilA* strains were used as controls that lacked flagella and pili respectively. Localization of GFP–PilT (middle) and PilQ–mCherry (bottom) in cells taken from the leading edge of motility on the surface of agar plates.

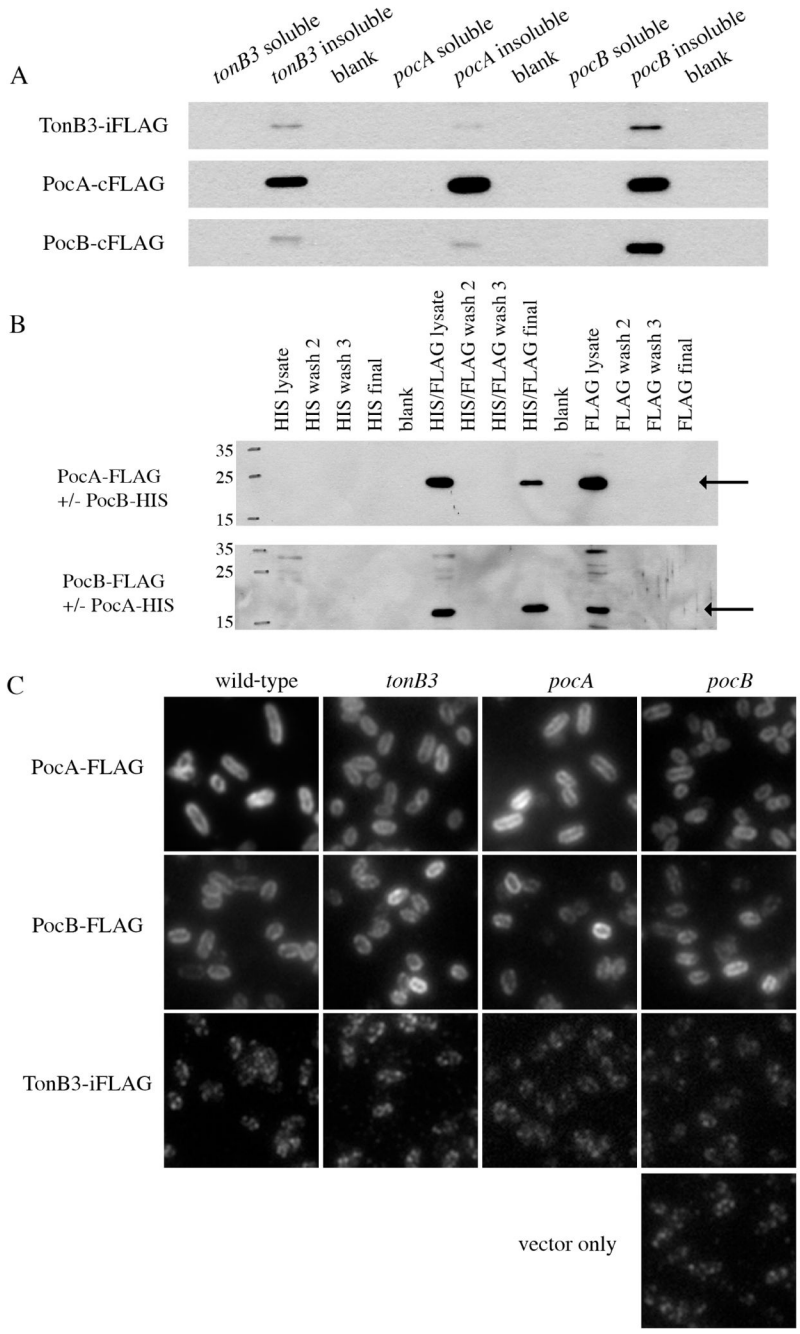

Fig. 5. PocA and PocB form a membrane-associated protein complex

A. Western blot examining the localization of TonB3–iFLAG, PocA–cFLAG and PocB–cFLAG fusion proteins to soluble or insoluble fractions from clarified cell lysates in *tonB3*, *pocA* or *pocB* mutant strains.

B. Co-precipitation of PocA (22.9 kDa) and PocB (15.7 kDa) using HIS and FLAG tagged proteins. HIS or FLAG labels indicate negative control strains carrying only one fusion protein. HIS/FLAG denotes the strain carrying both PocA–FLAG with PocB–HIS (top) or PocB–FLAG with PocA–HIS (bottom).

C. Immunofluorescence investigating subcellular localization of TonB3–iFLAG, PocA–cFLAG and PocB–cFLAG in wild-type and mutant strains grown to stationary phase in liquid medium. The ‘vector only’ panel is a representative image of background localization patterns in all strains.

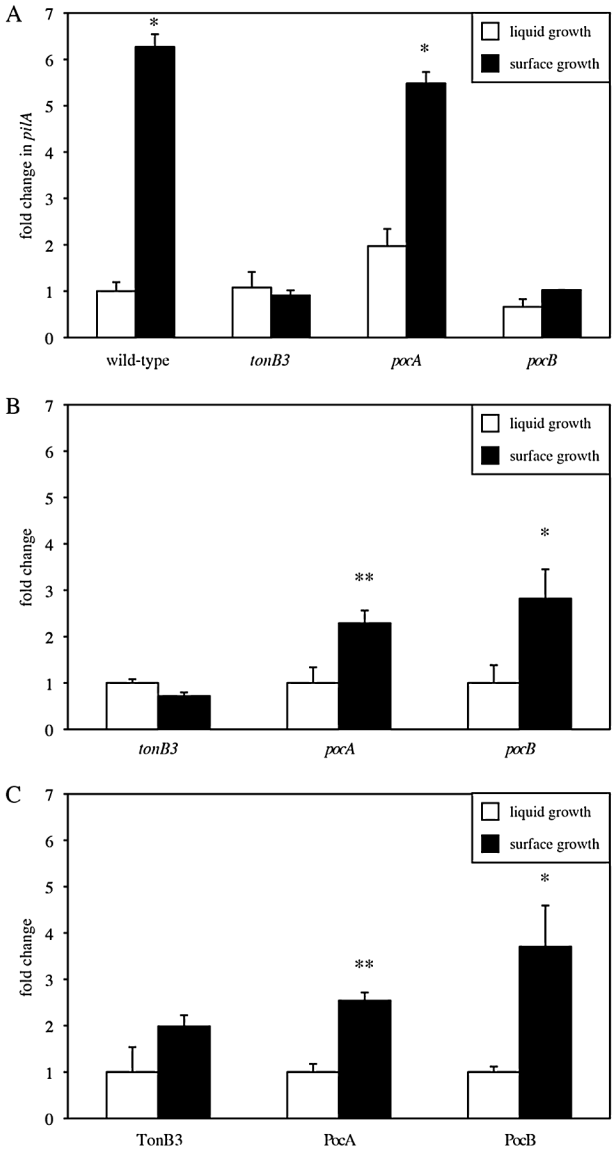

Fig. 6. Comparing expression of *pilA* and localization factors in liquid-grown and surface-grown cells

A. Relative levels of *pilA* in wild-type and mutants cells grown to stationary phase in liquid medium (white bars) and in cells taken from the leading edge of motility on agar plates (black bars).

B and C. Relative levels of *tonB3* and *poc* gene (B) and protein (C) expression in wild-type PAO1 grown in liquid (white bars) and surface (black) conditions.

Error bars represent the standard deviation ($n = 3$) and asterisks denote significant differences from liquid-grown cells (one asterisk indicates $P < 0.05$ and two asterisks indicate $P < 0.01$).

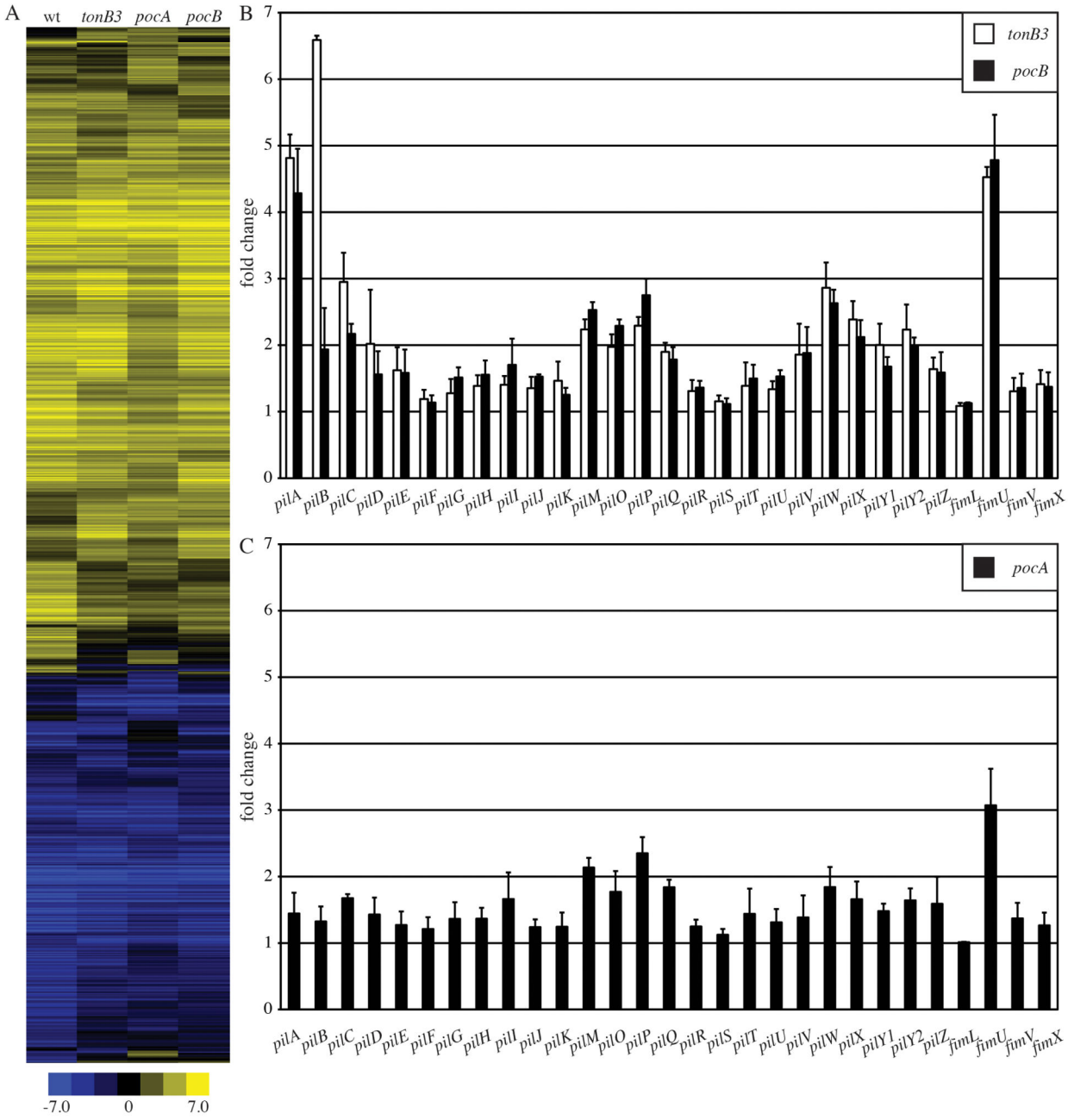

Fig. 7. *tonB3* and *pocB* but not *pocA* affect pilus gene expression in surface-grown cells

A. Microarray heat map of surface-grown cells in the wild-type and mutant backgrounds (3247 genes with >2-fold change). Yellow indicates increased expression in surface-grown cells compared with liquid-grown cells while blue indicates decreased levels in surface-grown cells.

B and C. Pilus gene expression from the microarray analysis in *tonB3* (B, white bars), *pocB* (B, black bars) and *pocA* (C) mutants. Fold change denotes expression levels in wild-type PAO1/expression levels in each mutant. Thus, a value of 1 indicates equal expression

between strains while values >1 indicate higher expression in wild-type compared with the mutant. Error bars indicate standard error (n = 4).