Supplemental Material List

1. Table S1 – List comparing bacterial TonB systems and motility structures
2. Table S2 – Quantitation of *tonB, pocA,* and *pocB* mutant cell size
3. Table S3 – List of strains used in this study
4. Table S4 – List of primers used in this study
5. Supplemental Methods – materials and methods details for experiments presented as supplemental material
6. Figure S1 – Reverse transcriptase PCR used to experimentally demonstrate co-transcription of *pocA* and *pocB*. Relevance: Co-transcription was predicted from the genome sequence but not yet published with experimental evidence.
7. Figure S2 – Swimming motility complementation of deletion mutants with full length and FLAG- and HIS-tagged proteins. Relevance: Complementation of swimming motility defects demonstrates causality of deletion mutations and provides evidence that FLAG- and HIS-tagged proteins are functional.
8. Figure S3 – Quantitation of FlhF-GFP and CheA-GFP localization. Relevance: Quantitative assessment of fluorescent fusion localization patterns in the different mutant strains.
9. Figure S4 – Co-precipitation of PocA and PocB in *tonB3* mutant. Relevance: Demonstrates that *tonB3* is not required for the physical interaction between LomA and LomB.
10. Figure S5 – Co-precipication experiments with TonB3. Relevance: Illustrates that TonB3 cannot be precipitated by PocA or PocB under these conditions.
11. Figure S6 – Expression of flagellar genes in *poc* mutants. Relevance: Unlike pili production genes, flagellar genes are not affected by the loss of *tonB3, pocA,* or *pocB*.
12. Movies 1-4 – FlhF, CheA, and PilT localization during growth. Relevance: Demonstrates cellular localization patterns of flagellar and pilus proteins are different during growth.
13. Movies 5-9 – Single cell swimming of wild-type and mutant strains. Relevance: Representative examples of how flagellar mislocalization affects swimming motility in single cells.
14. Movies 10-14 – Twitching motility of wild-type and mutant strains. Relevance: Shows lack of twitching motility in Poc mutants, including *pocA* which makes non-polar pili.

Supplemental Table S1 – Bacterial TonB Systems and Motility Structures

Bacterium	TonB Systems	Flagellum localization	Type IV pili
Pseudomonas aeruginosa	3	Monotrichous	Yes
Escherichia coli	1	Peritrichous	Yes
Klebsiella pneumoniae	1	Peritrichous	No
Pseudomonas putida	3	Monotrichous	Yes
Salmonella enterica	1	Peritrichous	No
Vibrio cholerae	2	Monotrichous	Yes
Vibrio vulnificus	3	Monotrichous	Yes
Xanthomonas campestris	3	Monotrichous	Yes

Supplemental Table S2 – Quantification of cell size (in pixels)

Strain	**Cell Width**	**Cell length**
wildtype	1.30 ± 0.02	4.47 ± 0.17
tonB3	1.29 ± 0.01	3.75 ± 0.05
PA2983 (*pocA*)	1.31 ± 0.01	3.92 ± 0.09
PA2982 (*pocB*)	1.285 ± 0.002	3.80 ± 0.01

Supplemental Table S3 – Strains used in this study

Strain	**Genotype**	**Reference or source**
***Pseudomonas* strains**		
Pseudomonas aeruginosa PAO1	Wild-type *P. aeruginosa* strain ATCC 15692	C. Manoil, University of Washington, Seattle
ZG520	PAO1 Δ*tonB3*	This study
ZG521	PAO1 Δ*pocA*	This study
ZG522	PAO1 Δ*pocB*	This study
ZG523	PAO1 Δ*flhF*	This study
ZG524	PAO1 Δ*fliF*	This study
ZG525	PAO1 Δ*pilA*	This study
ZG506	PAO1 pJN105	Cowles and Gitai, 2010
ZG526	PAO1 Δ*tonB3* pJN105	This study
ZG527	PAO1 Δ*pocA* pJN105	This study
ZG528	PAO1 Δ*pocB* pJN105	This study
ZG529	PAO1 pJN(*tonB3*)	This study
ZG530	PAO1 pJN(*pocA*)	This study
ZG531	PAO1 pJN(*pocB*)	This study
ZG532	PAO1 Δ*tonB3* pJN(*tonB3*)	This study
ZG533	PAO1 Δ*pocA* pJN(*pocA*)	This study
ZG534	PAO1 Δ*pocB* pJN(*pocB*)	This study
ZG535	PAO1 pJN(*flhF-gfp*)	This study
ZG536	PAO1 Δ*tonB3* pJN(*flhF-gfp*)	This study
ZG537	PAO1 Δ*pocA* pJN(*flhF-gfp*)	This study
ZG538	PAO1 Δ*pocB* pJN(*flhF-gfp*)	This study
ZG539	PAO1 pJN(*cheA-gfp*)	This study
ZG540	PAO1 Δ*tonB3* pJN(*cheA-gfp*)	This study
ZG541	PAO1 Δ*pocA* pJN(*cheA-gfp*)	This study
ZG542	PAO1 Δ*pocB* pJN(*cheA-gfp*)	This study
ZG502	PAO1 *gfp-pilT*	Cowles and Gitai, 2010
ZG543	PAO1 Δ*tonB3 gfp-pilT*	This study
ZG544	PAO1 Δ*pocA gfp-pilT*	This study
ZG545	PAO1 Δ*pocB gfp-pilT*	This study
ZG547	PAO1 Δ*fliF gfp-pilT*	This study
ZG548	PAO1 Δ*pilA gfp-pilT*	This study
ZG549	PAO1 *pilQ-mCherry*	This study
ZG550	PAO1 Δ*tonB3 pilQ-mCherry*	This study
ZG551	PAO1 Δ*pocA pilQ-mCherry*	This study
ZG552	PAO1 Δ*pocB pilQ-mCherry*	This study
ZG554	PAO1 Δ*fliF pilQ-mCherry*	This study

ZG555	PAO1 Δ*pilA pilQ-mCherry*	This study
ZG556	PAO1 *tonB3-iFLAG*	This study
ZG557	PAO1 *pocA-cFLAG*	This study
ZG558	PAO1 *pocB-cFLAG*	This study
ZG559	PAO1 pJN(*tonB3-iFLAG*)	This study
ZG560	PAO1 Δ*tonB3* pJN(*tonB3-iFLAG*)	This study
ZG561	PAO1 Δ*pocA* pJN(*tonB3-iFLAG*)	This study
ZG562	PAO1 Δ*pocB* pJN(*tonB3-iFLAG*)	This study
ZG563	PAO1 pJN(*pocA-cFLAG*)	This study
ZG564	PAO1 Δ*tonB3* pJN(*pocA-cFLAG*)	This study
ZG565	PAO1 Δ*pocA* pJN(*pocA-cFLAG*)	This study
ZG566	PAO1 Δ*pocB* pJN(*pocA-cFLAG*)	This study
ZG567	PAO1 pJN(*pocB-cFLAG*)	This study
ZG568	PAO1 Δ*tonB3* pJN(*pocB-cFLAG*)	This study
ZG569	PAO1 Δ*pocA* pJN(*pocB-cFLAG*)	This study
ZG570	PAO1 Δ*pocB* pJN(*pocB-cFLAG*)	This study
ZG571	PAO1 *pocA-cHIS*	This study
ZG572	PAO1 *pocB-cHIS*	This study
ZG573	PAO1 pJN(*pocA-cFLAG*) *pocB-cHIS*	This study
ZG574	PAO1 pJN(*pocB-cFLAG*) *pocA-cHIS*	This study
ZG575	PAO1 Δ*tonB3 pocA-cHIS*	This study
ZG576	PAO1 Δ*tonB3 pocA-cHIS* pJN(*pocB-cFLAG*)	This study
ZG577	PAO1 pJN(*tonB3-iFLAG*) *pocmA-cHIS*	This study
ZG578	PAO1 pJN(*tonB3-iFLAG*) *pocB-cHIS*	This study
***E. coli* strains**		
ZG514	S17-1 λpir pEX18Tc (Δ*tonB3*)	This study
ZG515	S17-1 λpir pEX18Tc (Δ*pocA*)	This study
ZG516	S17-1 λpir pEX18Tc (Δ*pocB*)	This study
ZG517	S17-1 λpir pEX18Tc (Δ*flhF*)	This study
ZG518	S17-1 λpir pEX18Tc (Δ*fliF*)	This study
ZG519	S17-1 λpir pEX18Tc (Δ*pilA*)	This study

Supplemental Table S4 – Primers used in this study

Primer	Sequence	Function
0406UpFOREcoRI	AACGAATTCGTCTATTCGACCTACCTGCTGG	Deletion of *tonB3*
0406UpREV	GCTGGACAGGCGGAAAAGTCTATGACG	Deletion of *tonB3*
0406DnFOR	CTTTTCCGCCTGTCCAGCAAGTAGCGC	Deletion of *tonB3*
0406DnREVXbaI	NNNTCTAGATTCGTCGTGGGTAAAGACCGG	Deletion of *tonB3*
298382UpFOREcoRI	NNNGAATTCATCGAACTGCTGGTAGTGCCC	Deletion of *pocA* or *pocB*
2983UpREV	TTCCTCGACGGCTTGAACCAGTTCCCA	Deletion of *pocA*
2983DnFOR	GTTCAAGCCGTCGAGGAAGGCAAAGCG	Deletion of *pocA*
2982UpREV	ACATCAGGGCCGTCTGCGGCGGAATTT	Deletion of *pocB*
2982DnFOR	CGCAGACGGCCCTGATGTCGTTCTCCG	Deletion of *pocB*
298382DnREVXbaI	NNNTCTAGACCAGGAGATGCTCGAGTCGGG	Deletion of *pocA* or *pocB*
FlhFGWUp	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGAGGGGTTCGGGCAATG	Fusions to *flhF*
FlhFGWDn	GGGGACCACTTTGTACAAGAAAGCTGGGTACGCCGGTTGCTGGTAGAG	Fusions to *flhF*
CheAGWUp	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCTGGGAGCAGCCGAATG	Fusions to *cheA*
CheAGWDn	GGGGACCACTTTGTACAAGAAAGCTGGGTAGATGCGCCGTGCGTAACG	Fusions to *cheA*
GWrfpFORSpe	NNNACTAGTACAAGTTTGTACAAAAAAGCTG	Vector construction
GWrfpREVSpe	NNNACTAGTTTACTTGTACAGCTCGTCC	Vector construction
AraCAmp	CCGCCATTCAGAGAAGAAACC	Confirmation of fusions
FlhFreverseMluI	GGGGACGCGTGCCGGCACGCCGCGCC	*flhF*-CERC fusion
FlhFforwardSpeI	GGGGACTAGTCTGAGGGGTTCGGGCAATG	*flhF*-CERC fusion
CERCreverseEcoRISacI	GCCGGAGCTCAAAAGAATTCTTACTTGTACAGCTCGTC	*flhF*-CERC fusion
CheAforwardEcoRI	GGGGGAATTCCTCTGGGAGCAGCCGAATG	*cheA*-GFP fusion
CheAreverseMluI	GGGGACGCGTGATGCGCCGTGCGTAA	*cheA*-GFP fusion
EGFPreverseXbaISacI	GCCGGAGCTCAAAATCTAGATTACTTGTACAGCTCGTC	*cheA*-GFP fusion

PA0406ForRT	CTTCACCCTGTTCATCGC	qRT-PCR
PA0406RevRT	TGATTTCCAGGGTCTTGC	qRT-PCR
PA2983ForRT	ATCAAGGGCAAGCAACTGAG	qRT-PCR
PA2983RevRT	TGCACTCCTTCATGATCTCG	qRT-PCR
PA2982ForRT	CTGCTGTTCTTCGTGGTCAG	qRT-PCR
PA2982RevRT	CCGAGAGTTCCAGTTGCTTC	qRT-PCR
pilAForRT	CGCTGAAGACCACTGTTGAA	qRT-PCR
pilARevRT	GTTTCGGTCGCAGTAGAAGC	qRT-PCR
fliCForRT	GACCTCCACTCAGGATCTGG	qRT-PCR
fliCRevRT	CGGAGGTGATGGTCAGTACA	qRT-PCR
rpoDForRT	CTCAGCGGCTATATCGATCC	qRT-PCR
rpoDRevRT	CTCGTCGTCCTTCTCTTTCG	qRT-PCR
LomAUp	TGGGAACTGGTTCAAGCC	RT-PCR for *pocAB*
LomADn	CAGCTCGTGGATGACACC	RT-PCR for *pocAB*
LomAMid	AGGCATTGATCACCACCG	RT-PCR for *pocAB*
LomBUp	GCTCAACATCGAGCTGCC	RT-PCR for *pocAB*
LomBDn	CTGGTGGGTGGACTTGGC	RT-PCR for *pocAB*
LomBMid	CTGACCACGAAGAACAGC	RT-PCR for *pocAB*
0406UpFLAGEcoRI2	NNNGAATTCTGTCCAAGGCGCGGAGTG	Complementation and inducible FLAG fusion
0406UpFLAG	CTTGTCGTCGTCGTCCTTGTAGTCGGTCTTGCTCGGTGCCTT	Internal FLAG fusion
0406DnFLAGXbaI2	NNNTCTAGACTACTTGCTGGACAGCCG	Complementation and inducible FLAG fusion
0406DnFLAG	GACTACAAGGACGACGACGACAAGCAGGCACAGAAGGCCGAG	Internal FLAG fusion
0406UpFLAGEcoRI	NNNGAATTCCAGCCAGCAGATCATGGC	Native FLAG fusion

0406DnFLAGSpeI	NNNACTAGTAGCCAAGGCTGATCAGGC	Native FLAG fusion
2983cFLAGUpEcoRI	NNNGAATTCCGGGGATATGCCACTGTG	Complementation and HIS and FLAG fusions
2983DnREVXbaI	NNNTCTAGATCACGCTTTGCCTTCCTCGAC	Complementation
2983cFLAGDnXbaI	NNNTCTAGATCACTTGTCGTCGTCGTCCTTGTAGTCCGCTTTGCCTTCCTCGAC	Inducible FLAG fusion
2983cFLAGUpEcoRI2	NNNGAATTCGGTTTTCGAACAGTGGGC	Native FLAG fusion
2983cFLAGDnSpeI	NNNACTAGTTCACTTGTCGTCGTCGTCCTTGTAGTCCGCTTTGCCTTCCTCGAC	Native FLAG fusion
2982FOREcoRI	NNNGAATTCCGAGGAAGGCAAAGCGTG	Complementation and HIS and FLAG fusions
2982DnREVXbaI	NNNTCTAGATCAGGGCTTGGCCGCCGT	Complementation and inducible FLAG fusion
2982cFLAGDnSpeI	NNNACTAGTTCACTTGTCGTCGTCGTCCTTGTAGTCGGGCTTGGCCGCCGTGTT	Native FLAG fusion
2983cHISDnSpeI	NNNACTAGTTCAGTGGTGGTGGTGGTGGTGCGCTTTGCCTTCCTCGAC	HIS fusion
2982cHISDnSpeI	NNNACTAGTTCAGTGGTGGTGGTGGTGGTGGGGCTTGGCCGCCGTGTT	HIS fusion

Underlined sequence indicates restriction enzyme site.

Supplemental Methods

Co-transcription of pocAB

Total RNA was isolated and processed as described in Materials and Methods. PCR reactions were performed using RNA (+/- reverse transcriptase (RT)) or DNA as template and the following primers to amplify products from internal regions of *pocA* (LomAUp and LomADn for 306bp), *pocB* (LomBUp and LomBDn for 220bp), and across the *pocAB* intergenic region (LomAMid and LomBMid for 282bp)

Complementation of deletion mutants

To complement the swimming motility defects, *tonB3*, *pocA*, and *pocB* were cloned with their native RBS into pJN105 and transformed into wild-type PAO1 or the respective deletion mutant. The 960 bp *tonB3* was PCR amplified with 0406UpFLAGEcoRI2 and 0406DnFLAGXbaI2, the 635 bp *pocA* was amplified with 2983cFLAGUpEcoRI and 2983DnREVXbaI, and the 441 bp *pocB* was amplified with 2982ForEcoRI and 2982DnREVXbaI. Swimming motility was assessed by spotting wild-type and mutant strains with pJN105 or the complementing pJN (gene) construct onto LB Gm plates with 0.3% agar and incubating at 37°C for 20 h.

Microscopy and motility

To microscopically observe the swimming motility of individual cells, overnight cultures were subcultured 1:100 in LB broth, incubated at 37ºC for 3h, placed on pads made from 1% agarose in PBS, and visualized by microscopy. Time lapse images were taken with no delay for 15 seconds to create movies. To observe twitching motility, 1mm^3 cubes of agar were removed from 1% agar LB plates and inverted on slides. Overnight cultures of wild-type and mutant PAO1

strains were spotted on the agar surface and twitching at the leading edge of motility was monitored by microscopy. When cells were spotted onto the agar pads, the arrangement of cells at the leading edge of motility varied from spot to spot. Any apparent differences seen between strains in terms of cell arrangement at the beginning of the time lapse movies (i.e. populations with flat edges versus individual or groups of cells) were not strain specific, but instead were happenstance based on the way the spotted culture dried on the pad.

Quantification of FlhF-GFP and CheA-GFP localization and cell size

For quantitation of fluorescence, cells expressing FlhF-GFP or CheA-GFP fusions were imaged using NIS Elements software with a Nikon Ti-E microscope equipped with a 100X 1.4NA objective and an Andor Clara camera. Images were analyzed using our own software written in Matlab 2013a. Binary masks of individual cells were constructed using the Canny edge detection algorithm to detect cell edges in phase contrast images. The position of each cell centroid was determined from the binary mask. The pixel with the greatest intensity in the fluorescence channel for each cell was identified and the 5x5 pixel region surrounding it was isolated and fit to the form $I = ae^{-\left(\frac{x-b}{c}\right)^2}$, where *a* was taken to be the intensity amplitude. Pixel regions with intensity amplitudes that were 10% greater than the average fluorescence within the cell were considered to be localized foci and those below this value were considered to be diffuse. The distance from the cell centroid was computed for all localized foci. Foci that were greater than 0.35 unit cell lengths from the cell centroid were considered to be polar while those less than this value were considered to be non-polar.

The width and length of individual cells were computed from binary masks constructed using the Canny edge detection algorithm using our own software written in Matlab 2013a. The length of the minor and major axes were computed from the second central moments of the binary masks. The minor or major axis length was taken to be the width or length of the cell, respectively. At least 200 cells were analyzed in each experiment. Data values in Table S2 are the average of three independent experiments and errors indicate standard deviation.

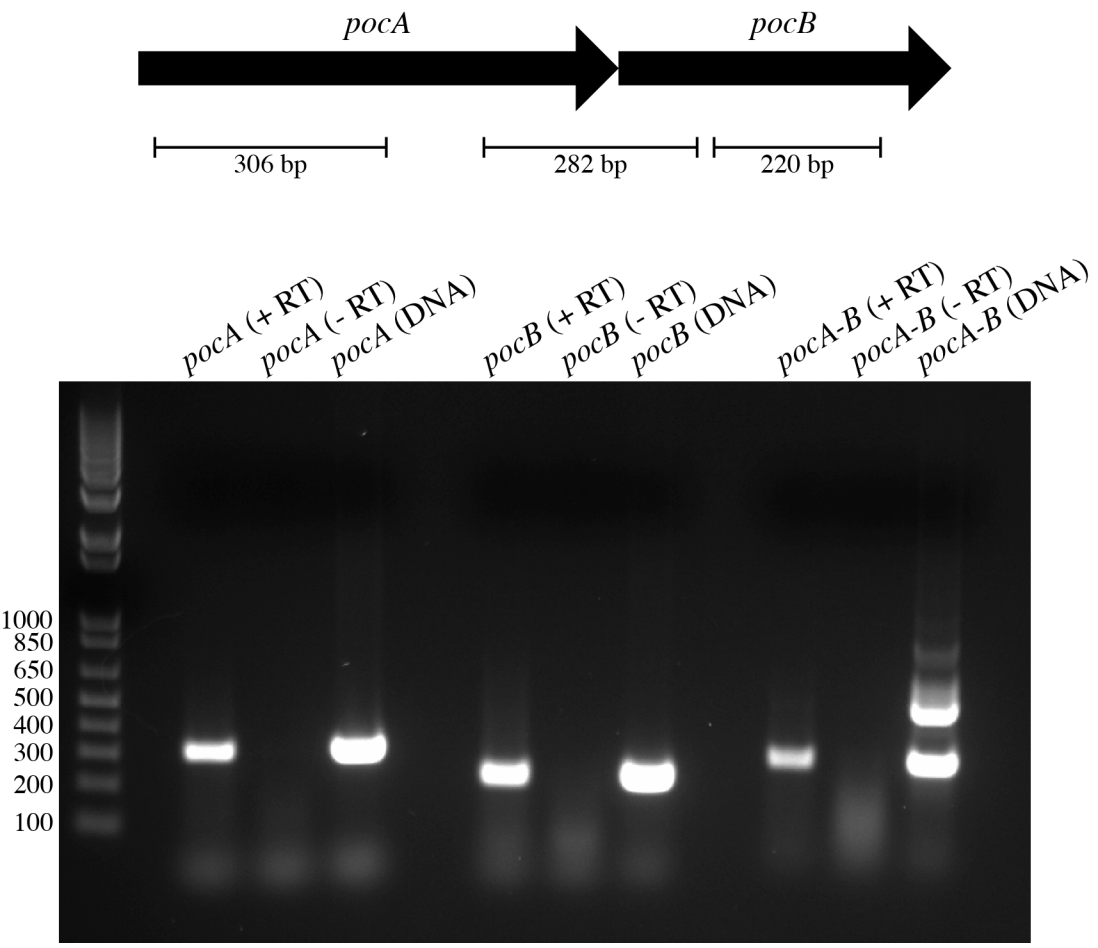


Figure S1. RT-PCR to examine the operon structure of *pocAB*. PCR reactions were performed using RNA (+/- reverse transcriptase (RT)) or DNA as template to amplify products from internal regions of *pocA* (306bp) and *pocB* (220bp) and across the *pocA-B* intergenic region (282bp).

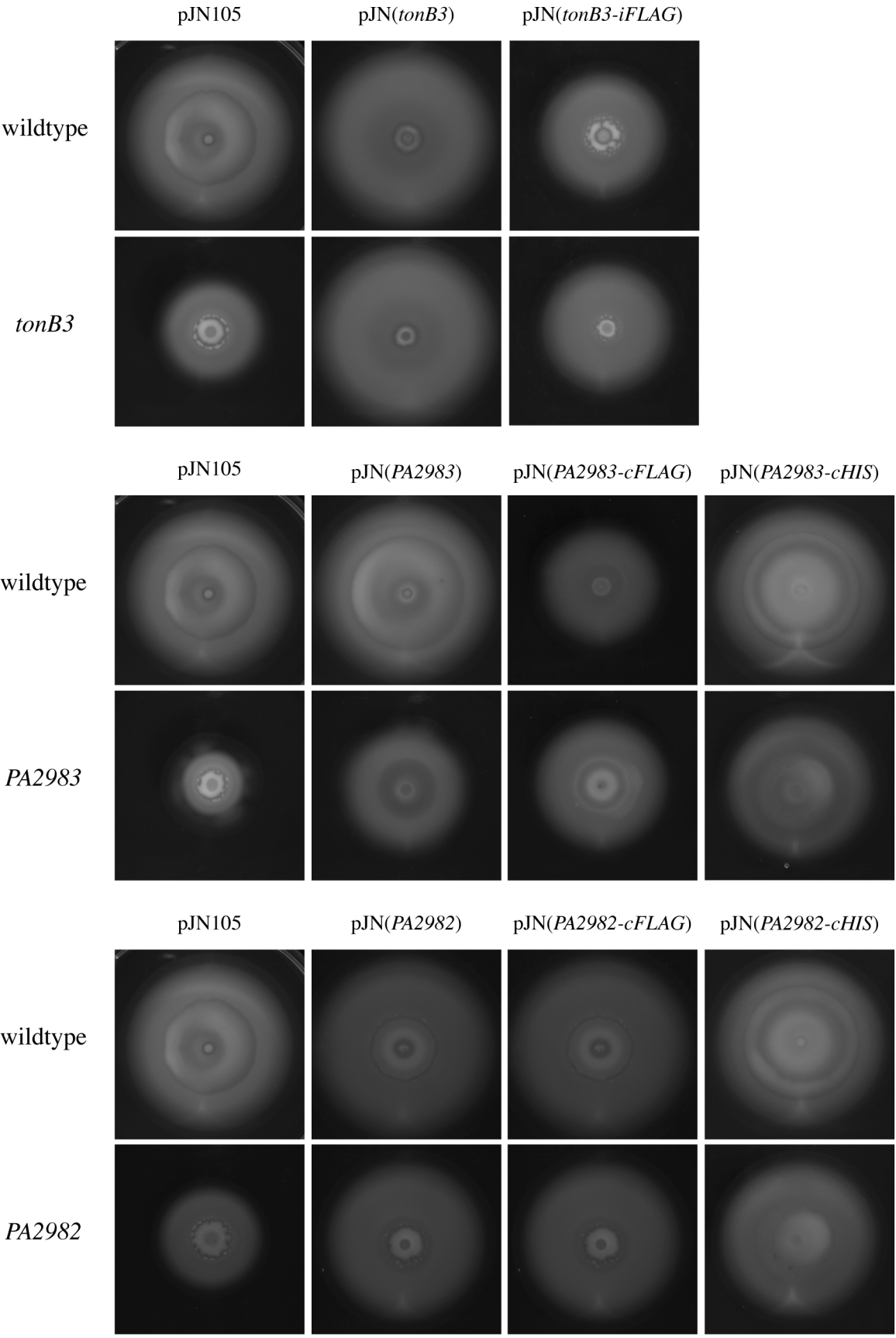


Figure S2. Complementation of *tonB3, PA2983*, and *PA2982* mutant swimming motility defects. Top (wildtype) and bottom (mutant) panels are representative images of strains carrying empty vector (pJN105), full length, FLAG-tagged, or HIS-tagged constructs.

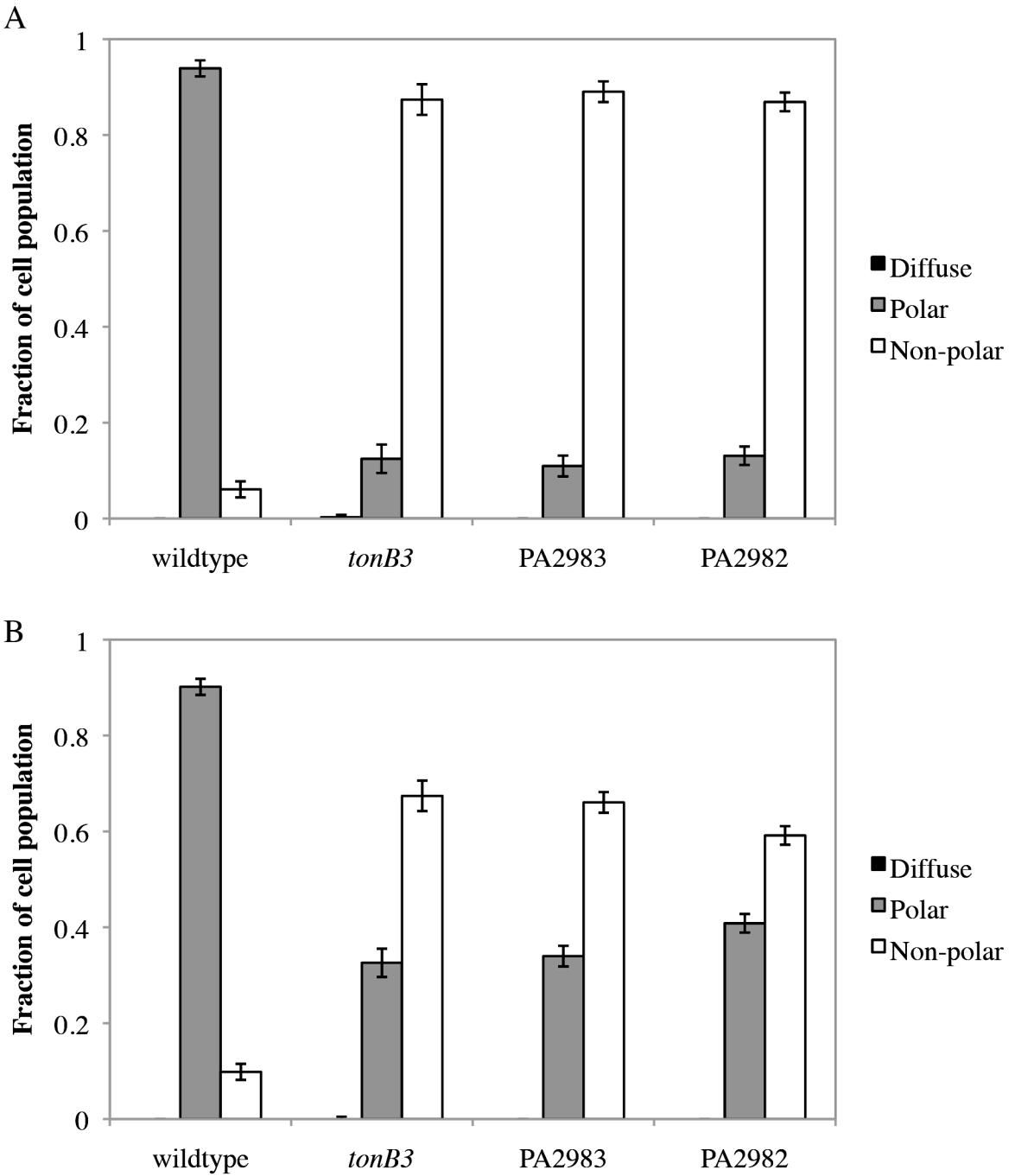


Figure S3. Quantitation of FlhF-GFP and CheA-GFP localization in *tonB3*, PA2983, or PA2982 mutants. FlhF-GFP (A) and CheA-GFP (B) fluorescence patterns were determined to be diffuse, localized at the cell pole or localized in a non-polar region in the cell. Details of fluorescence pattern determination are described in the Supplemental Experimental Procedures section. Bars are the average of three independent experiments and error bars indicate standard deviation. At least 200 cells were analyzed in each experiment.

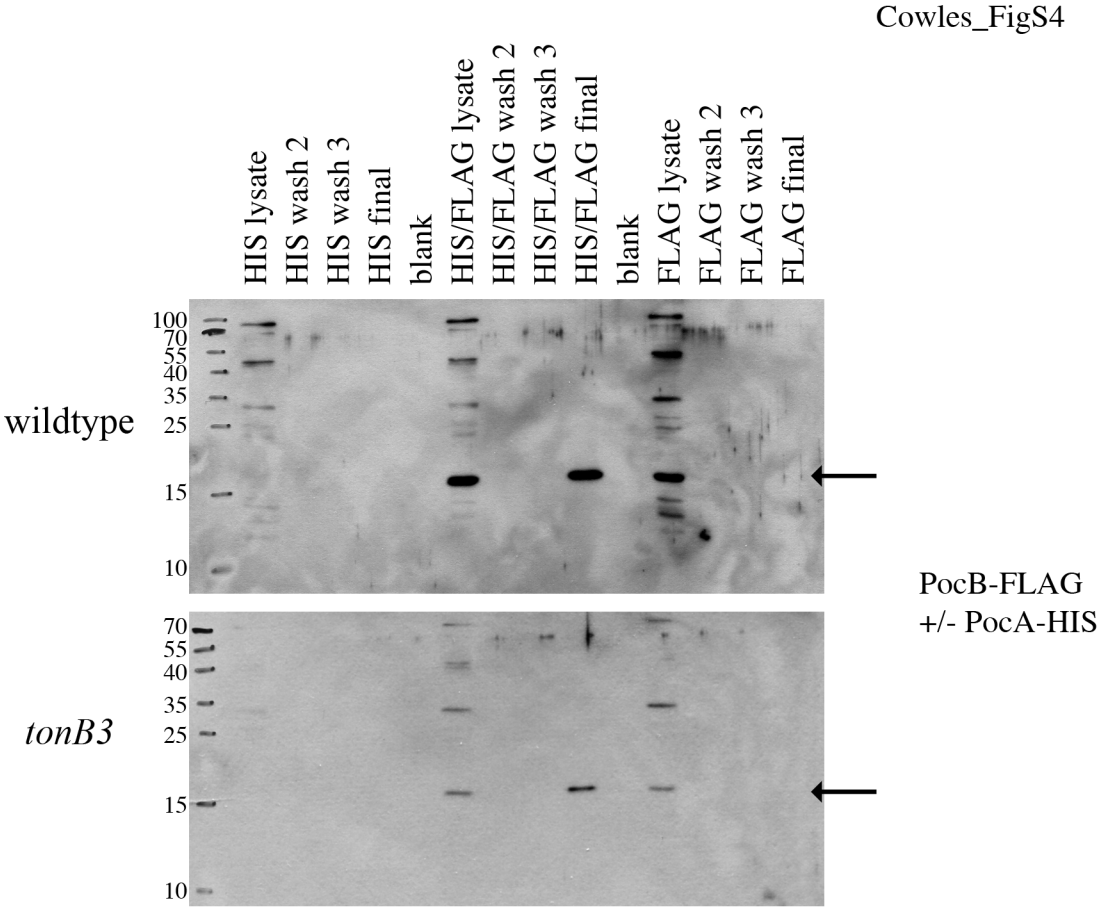


Figure S4. Western blots using anti-FLAG antibodies to detect PocB-FLAG during co-precipitation of PocA (22.9 kDa) and PocB (15.7 kDa) using HIS and FLAG tagged proteins.
HIS or FLAG labels indicate negative control strains carrying only one fusion protein.
HIS/FLAG denotes the strain carrying both PocB-FLAG with PocA-HIS in wildtype (top) or *tonB3* mutant (bottom). Arrows indicate PocB-FLAG.

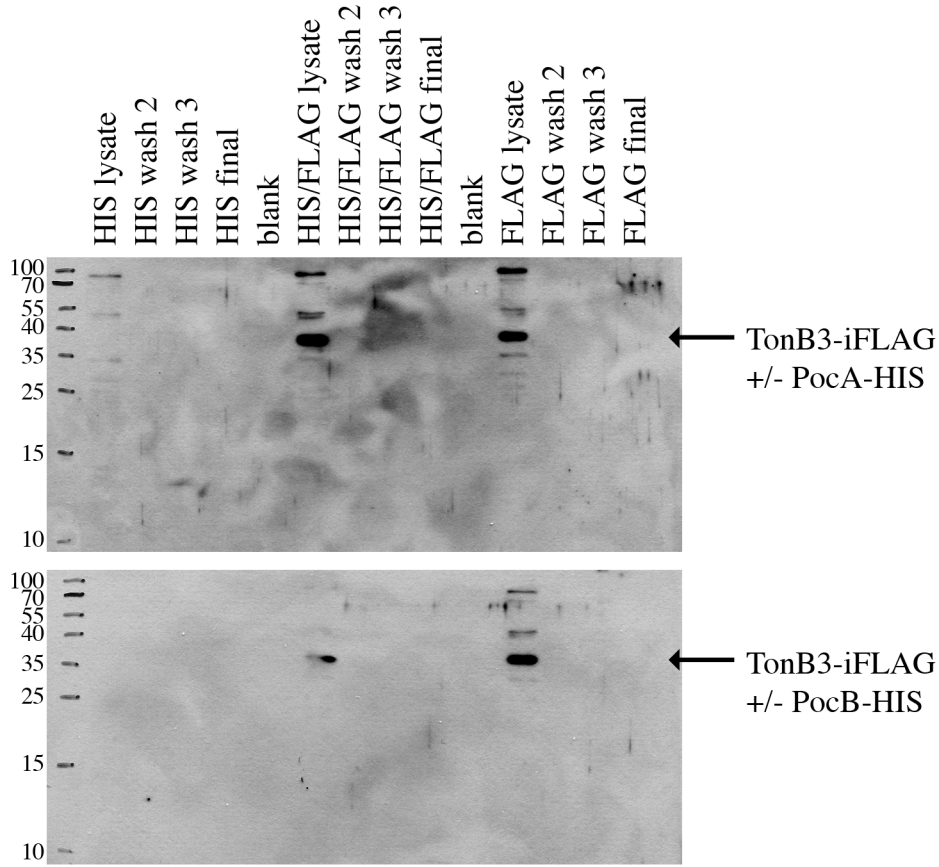
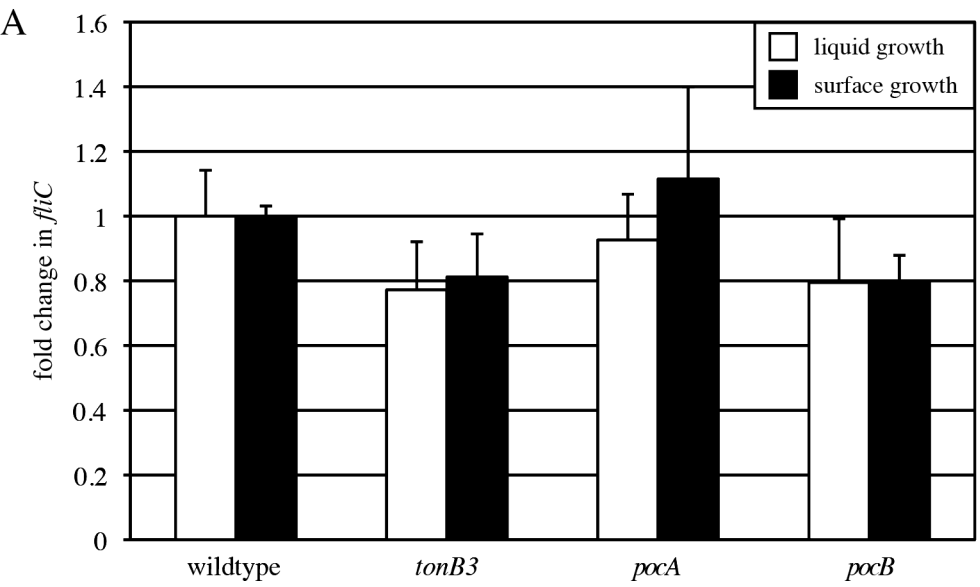


Figure S5. TonB3 does not co-precipitate with PocA or PocB. Western blots using anti-FLAG antibodies to detect internally FLAG-tagged TonB3 (36.3 kDa) after incubation with Ni resin to precipitate C-terminally HIS-tagged PocA (top) or PocB (bottom). Lysates, two wash fractions, and the final precipitated sample are shown for strains carrying either the HIS or FLAG tagged protein or both (HIS/FLAG).



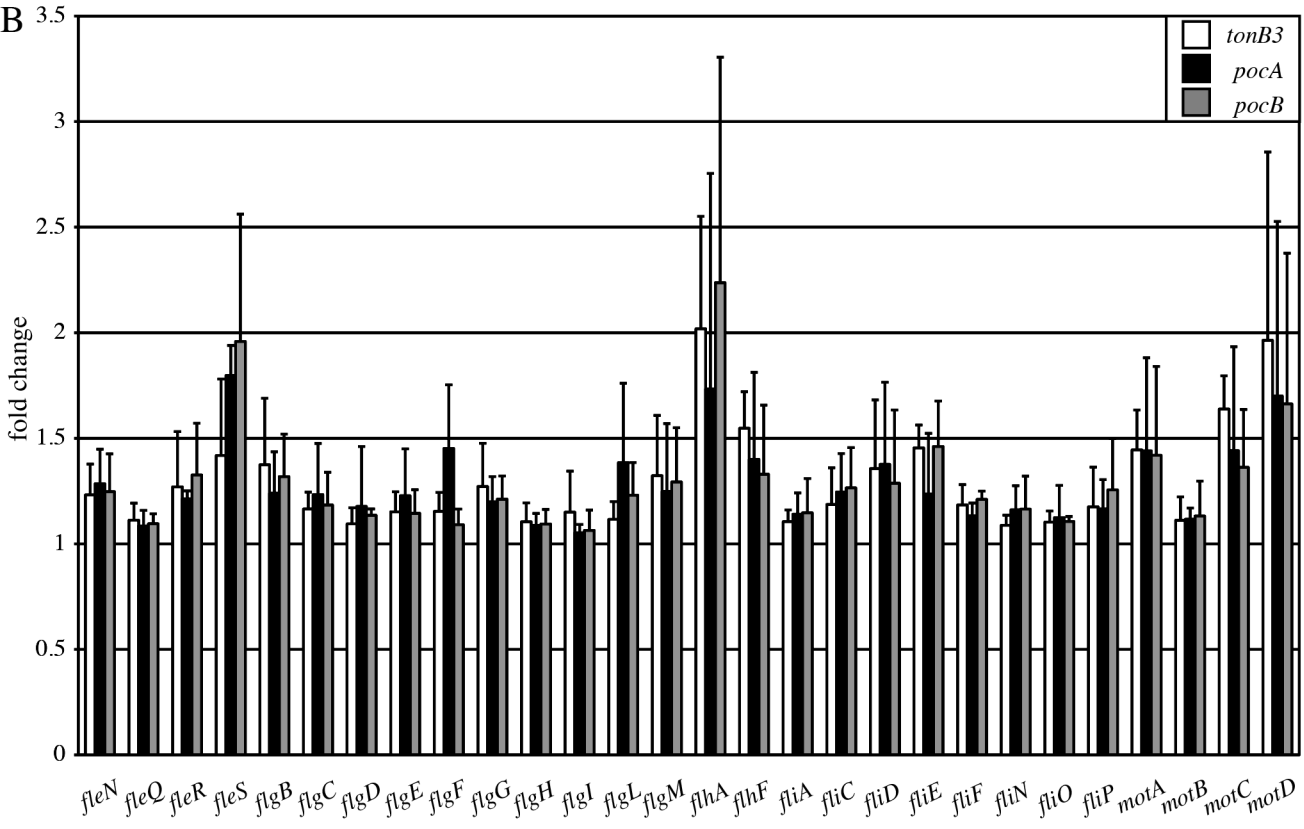


Figure S6. Flagellar gene expression is generally unaffected by mutations in *tonB3*, *pocA*, or *pocB*. (A) Fold changes in *fliC* expression in wild-type and mutant cells grown in stationary phase in liquid medium (white bars) and in cells taken from the leading edge of motility on agar plates (black bars). (B) Flagellar gene expression from the microarray analysis in *tonB3* (white bars), *pocA* (black bars) and *pocB* (gray bars) mutants. Fold change denotes expression levels in wildtype PAO1 / expression levels in each mutant. Thus, a value of 1 indicates equal expression between strains while values >1 indicate higher expression in wildtype compared to the mutant. Error bars indicate standard error (n=4).