Table S1: Bacterial Strains & Vectors

Strain	Description	Source/Reference
***E. coli* strains**		
DH5α	F-, φ80lacZ, M15, Δ(*lacZYA-argF*), U169, *recA1, endA1, hsdR17(rk-,mk+), phoAsupE44, thi-1, gyrA96, relA1*, λ-	Invitrogen
BTH101	F-, *cya-99, araD139, galE15, galK16, rpsL1 (StrR), hsdR2, mcrA1, mcrB1, relA1*	Euromedex
SM10	*thi-1, thr, leu, tonA, lacy, supE, recA*, RP4-2-Tcr::Mu, Km^r; mobilizes plasmids into *P. aeruginosa* via conjugation	Simon, et al. (1)
***P. aeruginosa* strains**		
PAK	Wild-type	J. Boyd
ΔpilM	Deletion of *pilM*	M. Ayers, et al. (2)
pilN::FRT	FRT scar at position 124 within *pilN*	M. Ayers, et al. (2)
pilO::FRT	FRT scar at position 328 within *pilO*	M. Ayers, et al. (2)
pilP::FRT	FRT scar at position 86 within *pilP*	M. Ayers, et al. (2)
pilT::FRT	FRT scar at position 540 within *pilT*	C. B. Whitchurch, et al. (3)
pilN::FRT/pilT::FRT	FRT scar at position 124 within *pilN* and FRT scar at position 540 within *pilT*	H. Takhar, et al.(4)
pilA::FRT	FRT scar at SphI site within *pilA*	J. V. Kus, et al. (5)
pilN ES132-133VA	*pilN ES132-133VA*	This study
pilN ES132-133VA/pilT::FRT	*pilN ES132-133VA* with FRT scar at position 540 within *pilT*	This study
pilN MR141-142KL	*pilN MR141-142KL*	This study
pilN MR141-142KL /pilT::FRT	*pilN MR141-142KL* with FRT scar at position 540 within *pilT*	This study
pilN M141K	*pilN M141K* single mutation of *pilN MR141-142KL*	This study
pilN R142L	*pilN R142L* single mutation of *pilN MR141-142KL*	This study

pilN EV157-158AD	*pilN EV157-158AD*	This study
pilN EV157-158AD /pilT::FRT	*pilN EV157-158AD* with FRT scar at position 540 within *pilT*	This study
pilN E157A	*pilN E157A* single mutation of *pilN EV157-158AD*	This study
pilN V158D	*pilN V158D* single mutation of *pilN EV157-158AD*	This study
pilN Triple	Triple mutation *pilN ESMREV132-133-141-142-157-158VAKLAD*	This study
pilN L81K	*pilN L81K*	This study
pilN L81A	*pilN L81A*	This study
pilN L82K	*pilN L82K*	This study
pilN LD64-65KK	*pilN LD64-65KK*	This study
pilN L64K	*pilN L64K* single mutation of *pilN LD64-65KK*	This study
pilN D65K	*pilN D65K* single mutation of *pilN LD64-65KK*	This study
pilN chimera	*pilN chimera* – residues 23-45 replaced with *pilO* residues 21-43	This study
pilO M92A	*pilO M92A*	This study
pilO M92K	*pilO M92K*	This study
pilO chimera	*pilO chimera* – residues 21-43 replaced with *pilN* residues 23-45	This study
Vectors	Description	Source/Reference
pEX18Gm	Suicide vector used for gene replacement, Gm^R	T. T. Hoang, et al. (6)
pFLP2	2.6-kb BamHI–SphI fragment from pALB2 ligated into the SmaI site, Ap^R	T. T. Hoang, et al. (6)
pKT25	Kn^R	G. Karimova, et al. (7)
pUT18C	Ap^R	G. Karimova, et al. (7)

pUT18C::*pilNΔ44*	Ap^R	Howell Lab
pKT25::*pilNΔ44*	Kn^R	Howell Lab
pUT18C::*pilOΔ51*	Ap^R	Howell Lab
pKT25::*pilOΔ51*	Kn^R	Howell Lab
pUT18C::*pilN*	Ap^R	This study
pKT25::*pilN*	Kn^R	This study
pUT18C::*pilO*	Ap^R	This study
pKT25:: *pilO*	Kn^R	This study
pUT18C::*pilN ES132-133VA*	*pilN ES132-133VA*, Ap^R	This study
pUT18C::*pilN MR141-142KL*	*pilN MR141-142KL*, Ap^R	This study
pUT18C::*pilN EV157-158AD*	*pilN EV157-158AD*, Ap^R	This study
pUT18C::*pilN Triple*	*pilN ESMREV132-133-141-142-157-158VAKLAD*, Ap^R	This study
pUT18C::*pilN L81K*	*pilN L81K*, Ap^R	This study
pUT18C::*pilN L81A*	*pilN L81A*, Ap^R	This study
pUT18C::*pilN L82K*	*pilN L82K*, Ap^R	This study
pUT18C::*pilN LD64-65KK*	*pilN LD64-65KK*, Ap^R	This study
pUT18C::*pilN chimera*	*pilN chimera* – residues 23-45 replaced with *pilO* residues 21-43 , Ap^R	This study
pKT25::*pilO M92A*	*pilO M92A*, Kn^R	This study
pKT25::*pilO M92K*	*pilO M92K*, Kn^R	This study
pKT25::*pilO chimera*	*pilO chimera* – residues 21-43 replaced with *pilN* residues 23-45 , Kn^R	This study
pkT25::*pilM*	Kn^R	This study
pEX18Gm::*pilMNO - pilN ES132-133VA*	Suicide vector containing PAK *pilMNO* with *pilN ES132-133VA*, Gm^R	This study
pEX18Gm::*pilMNO - pilN MR141-142KL*	Suicide vector containing PAK *pilMNO* with *pilN MR141-142KL*, Gm^R	This study

pEX18Gm::*pilMNO - pilN M141K*	Suicide vector containing PAK *pilMNO* with *pilN M141K* single mutation of *pilN MR141-142KL*, Gm^R	This study
pEX18Gm::*pilMNO - pilN R142L*	Suicide vector containing PAK *pilMNO* with *pilN R142L* single mutation of *pilN MR141-142KL*, Gm^R	This study
pEX18Gm::*pilMNO - pilN EV157-158AD*	Suicide vector containing PAK *pilMNO* with *pilN EV157-158AD*, Gm^R	This study
pEX18Gm::*pilMNO - pilN E157A*	Suicide vector containing PAK *pilMNO* with *pilN E157A* single mutation of *pilN EV157-158AD*, Gm^R	This study
pEX18Gm::*pilMNO - pilN V158D*	Suicide vector containing PAK *pilMNO* with *pilN V158D* single mutation of *pilN EV157-158AD*, Gm^R	This study
pEX18Gm::*pilMNO - pilN Triple*	Suicide vector containing PAK *pilMNO* with *pilN ESMREV132-133-141-142-157-158VAKLAD*, Gm^R	This study
pEX18Gm::*pilMNO - pilN L81K*	Suicide vector containing PAK *pilMNO* with *pilN L81K*, Gm^R	This study
pEX18Gm::*pilMNO - pilN L81A*	Suicide vector containing PAK *pilMNO* with *pilN L81A*, Gm^R	This study
pEX18Gm::*pilMNO - pilN L82K*	Suicide vector containing PAK *pilMNO* with *pilN L82K*, Gm^R	This study
pEX18Gm::*pilMNO - pilN LD64-65KK*	Suicide vector containing PAK *pilMNO* with *pilN LD64-65KK*, Gm^R	This study
pEX18Gm::*pilMNO – pilN L64K*	Suicide vector containing PAK *pilMNO* with *pilN L64K* single mutation of *pilN LD64-65KK*, Gm^R	This study
pEX18Gm::*pilMNO – pilN D65K*	Suicide vector containing PAK *pilMNO* with *pilN D65K* single mutation of *pilN LD64-65KK*, Gm^R	This study
pEX18Gm::*pilMNO – pilN chimera*	Suicide vector containing PAK *pilMNO* with *pilN* chimera - residues 23-45 replaced with *pilO* residues 21-43, Gm^R	This study
pEX18Gm::*pilNOP - pilO M92A*	Suicide vector containing PAK *pilNOP* with *pilO M92A*, Gm^R	This study
pEX18Gm::*pilNOP - pilO M92K*	Suicide vector containing PAK *pilNOP* with *pilO M92K*, Gm^R	This study

pEX18Gm::*pilNOP - pilO chimera*	Suicide vector containing PAK *pilNOP* with *pilO* chimera - residues 21-43 replaced with *pilN* residues 23-45, Gm^R	This study
pEX18Ap::*pilT::Gm::FRT*	Suicide vector containing *pilT* disrupted with FRT-flanked gentamicin cassette at position 540, Ap^R and Gm^R	M. L. Asikyan et al. (8)

Table S2: Oligonucleotide Primer Sequences

Name	Oligonucleotide Sequence (5' – 3')
PilOΔ51F	TATATAACTCTAGAGATGAGTGACATGCAGGCTCAGCTCGAAC
PilOΔ51R	TATATATAGAATTCTTCATTTCTTCAGCCCCTTGTCGTTGTAGC
PilNΔ44F	TATATAACTCTAGAGATGGCGGCCATCGAGAAC
PilNΔ44R	TATATATAGAATTCTTCATTTCTTGGCTCCTTGCGC
Bth PilOF	TATATAACTCTAGAGATGAGTCTGGCCAGTTCCCTGGAAAGTC
Bth PilOR	TATATATAGAATTCTTCATTTCTTCAGCCCCTTGTCGTTGTAGC
Bth PilNF	TATATAACTCTAGAGATGGCACGGATCAACCTTCTACCCTGG
Bth PilNR	TATATATGGAATTCGTCATTTCTTGGCTCCTTGCGCAACCCC
Bth PilMF	TAT ATA TAT CTA GAG GTG CTA GGG CTC ATA AAG AAG AAA G
Bth PilMR	TAT TAT AAG AAT TCG TCA GTC GAA ACT CCT CAA CGC C
PilN ES132-133VA F	CCGGCGCGGCCGTGGCCAACAACCGCGTTTCC
PilN ES132-133VA R	GGAAACGCGGTTGTTGGCCACGGCCGCGCCGG
PilN MR141-142KL F	CCGCGTTTCCAATCTCAAGCTCAACATGGACGCGTCCGAGTGGC
PilN MR141-142KL R	GCCACTCGGACGCGTCCATGTTGAGCTTGAGATTGGAAACGCGG
PilN M141K F	CGCGTTTCCAATCTCAAGCGCAACATGGACGCG
PilN M141K R	CGCGTCCATGTTGCGCTTGAGATTGGAAACGCG
PilN R142L F	CGGTTTCCAATCTCATGCTCAACATGGACGCGGTCCG
PilN R142L R	CGGACCGCGTCCATGTTGAGCATGAGATTGGAAACCG
PilN EV157-158AD F	CCGCCCCGACCCTGAACGCGGACAAGGCGGTGACCC

PilN EV157-158AD R	GGGTCACCGCCTTGTCCGCGTTCAGGGTCGGGGCGG
PilN E157A F	CGACCCTGAACGCGGTCAAGGCGGTG
PilN E157A R	CACCGCCTTGACCGCGTTCAGGGTCG
PilN V158D F	CGACCCTGAACGAGGATAAGGCGGTGACCCA
PilN V158D R	TGGGTCACCGCCTTATCCTCGTTCAGGGTCG
PilN L81K F	GCGAACTGAAGTCGCGGCGCCAGCAAAAGCTCGAGCGGATGAAGAT
PilN L81K R	GATCTTCATCCGCTCGAGCTTTTGCTGGCGCCGCGACTTCAGTTCGC
PilN L81A F	CGCCAGCAATTGGCCGAGCGGATGAAG
PilN L81A R	CTTCATCCGCTCGGCCAATTGCTGGCG
PilN L82K F	GCGAACTGAAGTCGCGGCGCCAGCAATTGAAGGAGCGGATGAAGAT
PilN L82K R	GATCTTCATCCGCTCCTTCAATTGCTGGCGCCGCGACTTCAGTTCGC
PilN LD64-65KK F	GCGCAAGGAAATCGTCGTAAAGAAGGCCCGGATCAAGGAAATCAGC
PilN LD64-65KK R	CGCTGATTTCCTTGATCCGGGCCTTCTTTACGACGATTTCCTTGCGC
PilN L64K F	CGCAAGGAAATCGTCGTAAAAGACGCCCGGATCAAG
PilN L64K R	CTTGATCCGGGCGTCTTTTACGACGATTTCCTTGCG
PilN D65K F	AAGGAAATCGTCGTACTCAAAGCCCGGATCAAGGAA
PilN D65K R	TTCCTTGATCCGGGCTTTGAGTACGACGATTTCCTT
PilO M92A F	GCCTACAAGGCACAGATGAAGGAGGCGGAAGAGTCCTTTGGCGCC
PilO M92A R	GGCGCCAAAGGACTCTTCCGCCTCCTTCATCTGTGCCTTGTAGGC
PilO M92K F	GCCTACAAGGCACAGATGAAGGAGAAGGAAGAGTCCTTTGGCGCC
PilO M92K R	GGCGCCAAAGGACTCTTCCTTCTCCTTCATCTGTGCCTTGTAGGC
PilM F	TATATATATGTGCTAGGGCTCATAAAGAAG
PilP R	TATATATGGAATTCGTCAGGAGCGTTCCTTGAGAGTCAG

SUPPLEMENTARY FIGURES AND LEGENDS

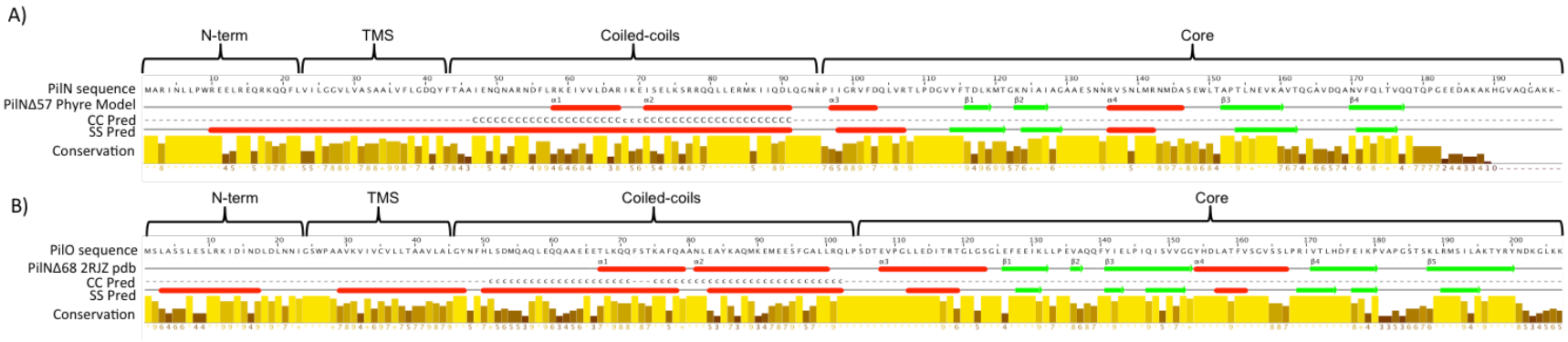

Fig. S1. Amino acid sequences and secondary structures of PilN and PilO. The sequence alignments for **(A)** PilN and **(B)** PilO are shown. The predicted cytoplasmic N-termini (N-term), the transmembrane segments (TMS), the coiled-coils (CC) and the core regions used in this study are indicated above with open brackets. Sequence conservation of the PilN and PilO families from a subset of Pseudomonads (*P. aeruginosa* PAK, *P. fulva* 12-X, *P. stutzeri* A1501, *P. syringae* pv. phaseolicola 1448A, & *P. protegens* CHA0) is indicated by the bars. High conservation of the residues is indicated by a high bar and a bright yellow color, whereas low conservation is displayed as a low bar and a dark brown color. The α-helices (red rectangles) and β-strands (green arrows) present in the PilOΔ68 structure (PDB 2RJZ (9)), the PilNΔ57 Phyre[2] homology model, and in the secondary-structure predictions (SS pred) are shown. The predicted CC regions (CC pred) are indicated with “c”, with the size indicating the confidence of these predictions with “C” and “c” representing stronger and weaker predictions, respectively. The sequences were gathered from the Pseudomonas Genome Database (10) and this figure was prepared using Jalview 2.8.2 (11, 12).

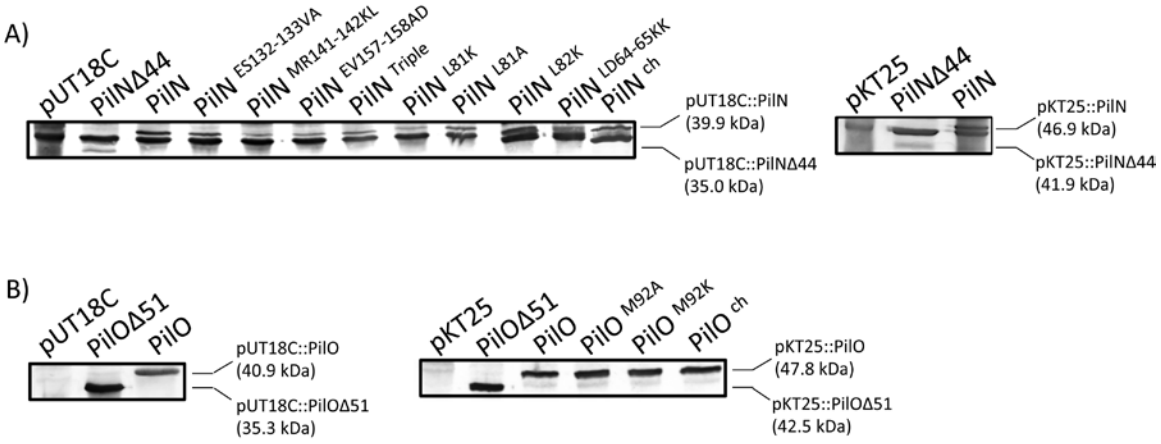

Fig. S2. All BTH fusion constructs are stable. Fusion constructs were tested for expression and stability via Western blotting using protein specific antisera for **(A)** PilN mutants and the PilN chimera (PilN ch), or **(B)** PilO mutants and the PilO chimera (PilO ch) in each vector.

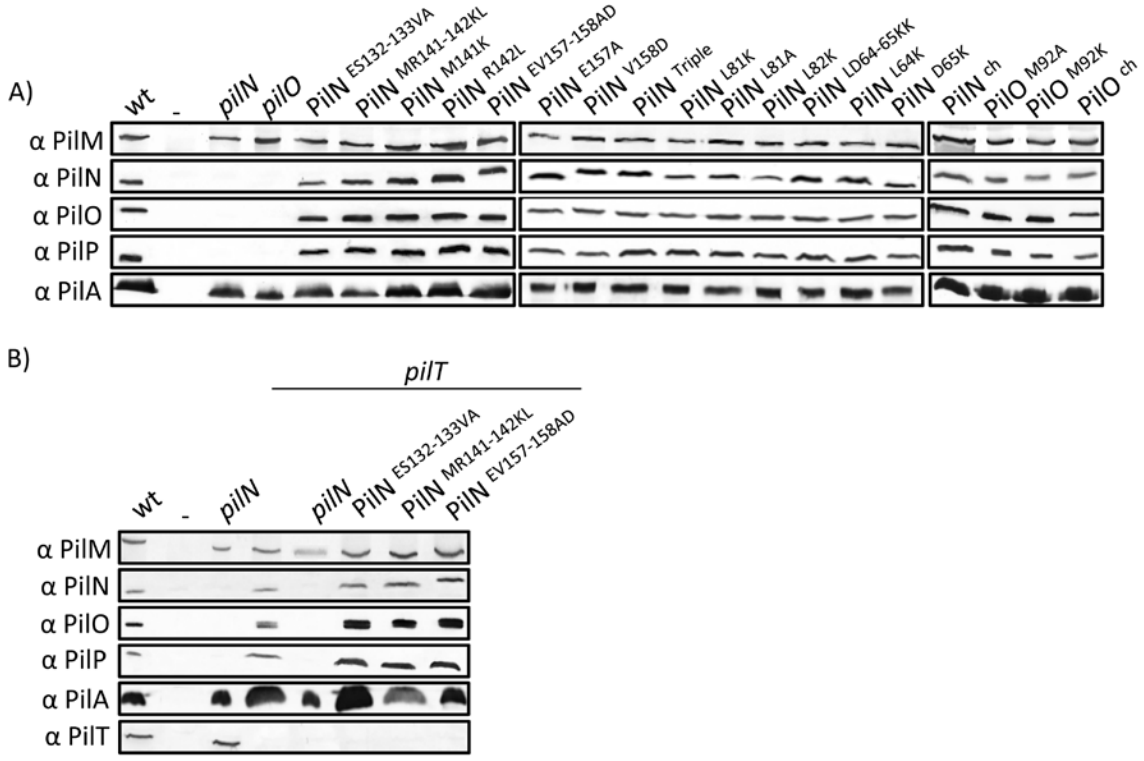

Fig. S3. Intracellular levels of alignment subcomplex proteins and PilA are unaffected by PilN and PilO chromosomal mutations. (A) All PilN and PilO mutations were introduced into the chromosome of *P. aeruginosa* and PilMNOPA proteins were tested for stability via Western blotting using protein specific antisera as designated on left. **(B)** PilN mutants ES132-133VA, MR141-142KL, and EV157-158AD were created in a retraction deficient background (*pilT*) and tested for stability via Western blotting using protein specific antisera for PilMNOPAT as designated on left.

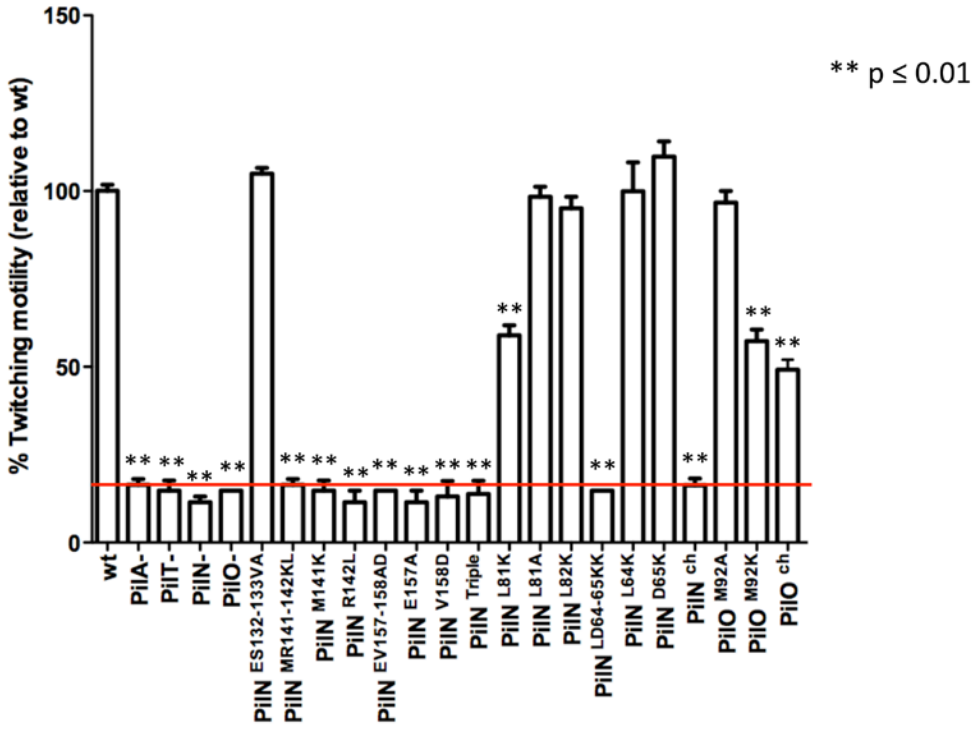

Fig. S4. Twitching motility of PilN and PilO mutants compared to wild type. Single colonies of each mutant were stab inoculated on a 1% LB agar plate and incubated for 36 h at 37 °C. The agar was removed and the plates were stained with a 1% crystal violet solution to visualize. Twitching motility zones were repeated in triplicate and measured using ImageJ software, and reported as the percent relative to wild-type (wt). Experiments were preformed in triplicate (N=3). Bars represent the means ± standard error. ** p ≤ 0.01.

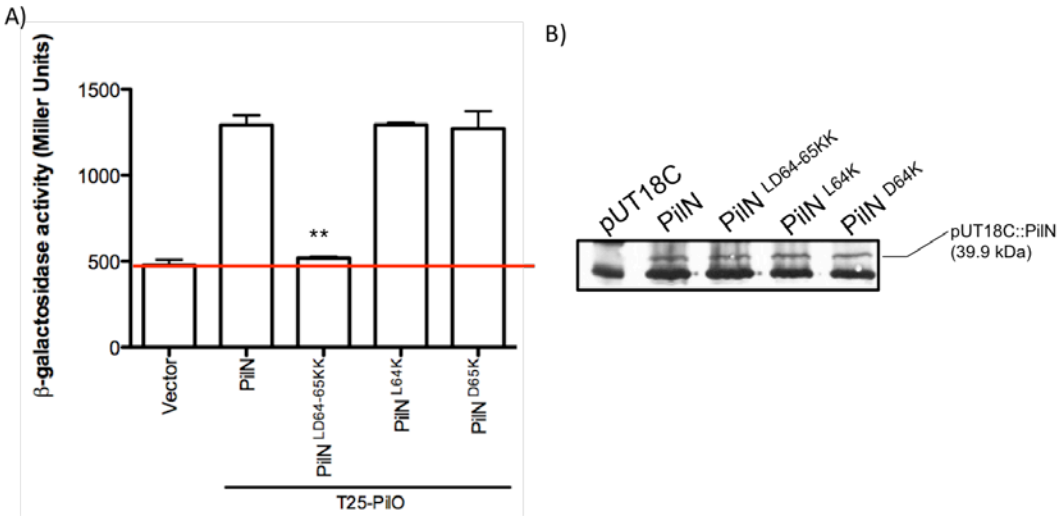

Figure S5: Interactions of PilN coiled-coiled L64 and point mutants with PilO. (A) PilN$^{LD64-65KK}$ single mutations (PilN^{L64K} and PilN^{D65K}) restore the interaction between PilN and PilO in the BTH assay, ** $p \leq 0.01$ compared to PilNO positive control. **(B)** PilN point mutants are expressed at the same level as the unmodified fusion protein as detected by WB using anti-PilN antisera.

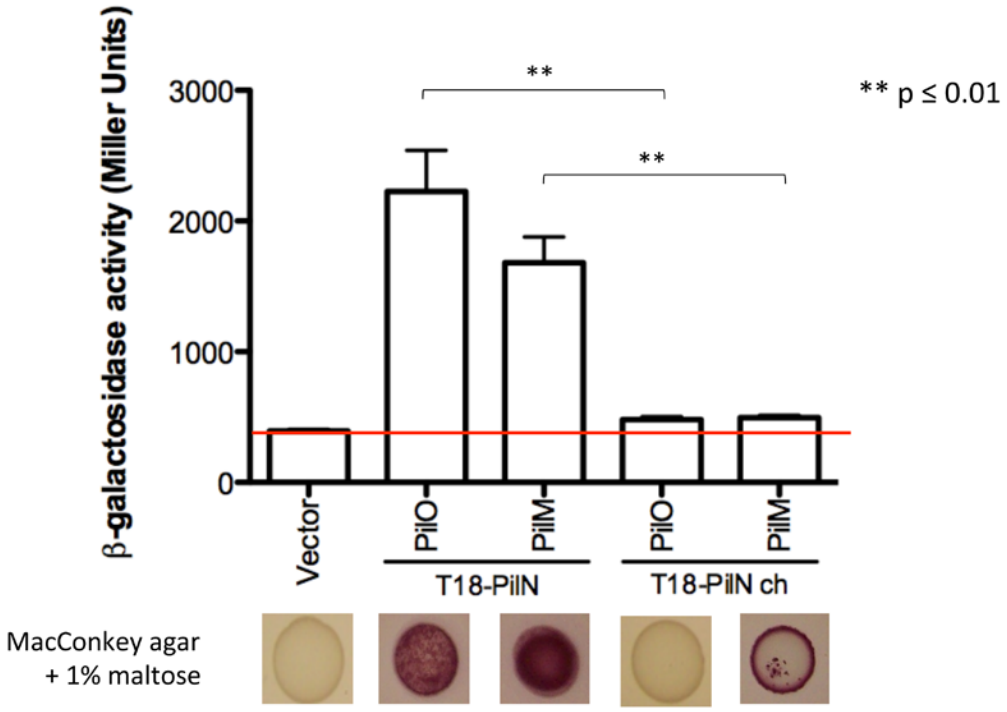

Fig. S6. Interaction between PilM and PilN in the BTH system is altered when the TMS of PilN is replaced with that of PilO. Full-length PilN and PilN chimera (PilN ch) fused at the N termini to the T18 fragment, were tested for interaction with full-length PilM and PilO fused at the N termini to the T25 fragment of adenylate cyclase from *Bordetella pertussis* using the BTH assay. Experiments performed in triplicate (N=3). Bars represent the means ± standard error. ** p ≤ 0.01.

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