Supplemental Information for

Mechanism of type-III protein secretion: Regulation of FlhA conformation by a functionally critical charged-residue cluster

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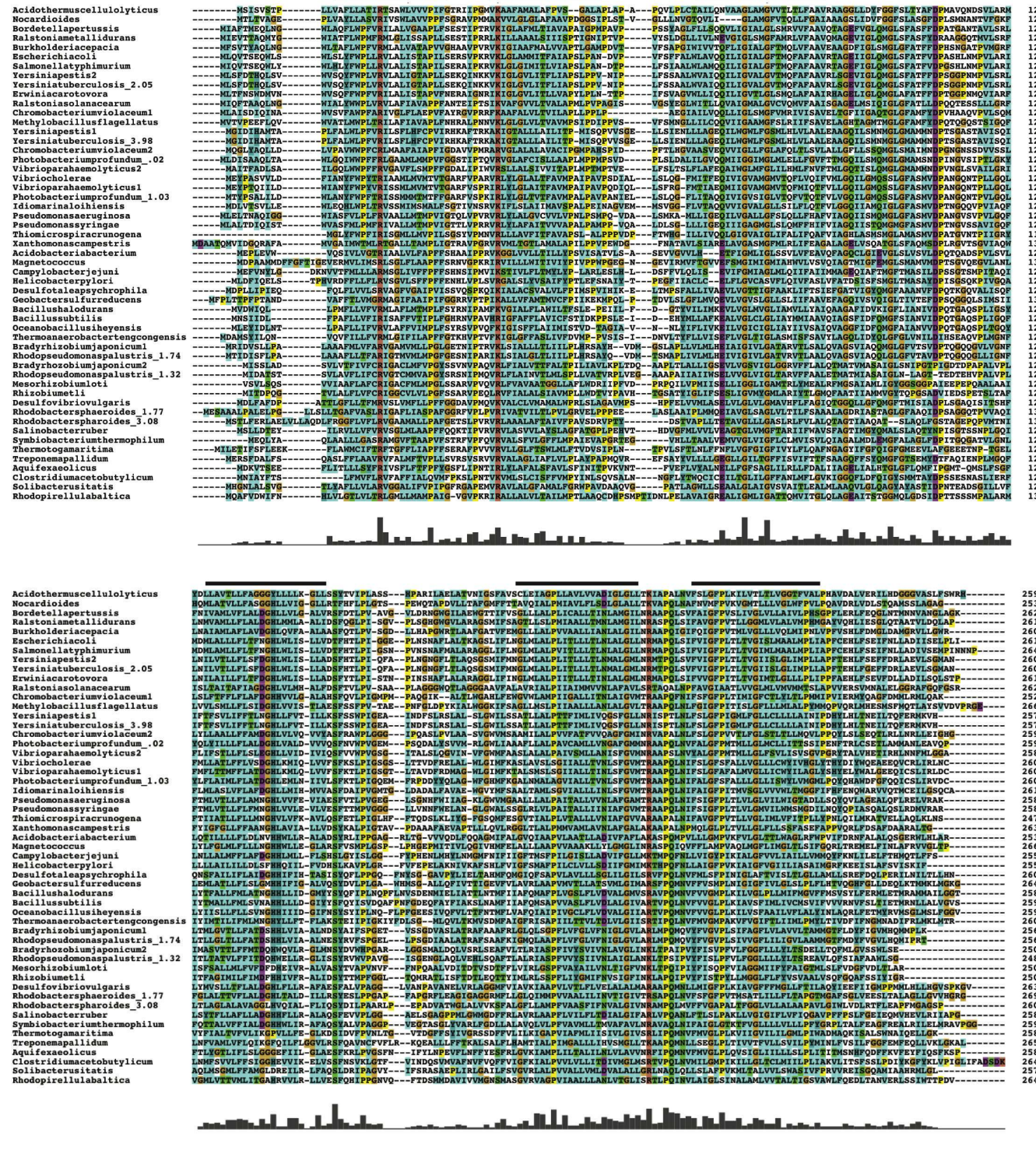
Figure S1. Sequence alignments of membrane proteins of the flagellar export apparatus. Lines above the alignments indicate the approximate positions of predicted trans-membrane segments. Asterisks indicate conserved proton-binding residues targeted for mutagenesis in the present study.

FliR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Pseudomonassyringae
Thiomicrospiracrunogena
Xanthomonascampestris
Acidobacteriabacterium
Magnetococcus
Campylobacterjejuni
Helicobacterpylori
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Geobactersulfurreducens
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Rhizobiumetli
Desulfovibriovulgaris
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Rhodobactersphaeroides_3.08
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Symbiobacteriumthermophilum
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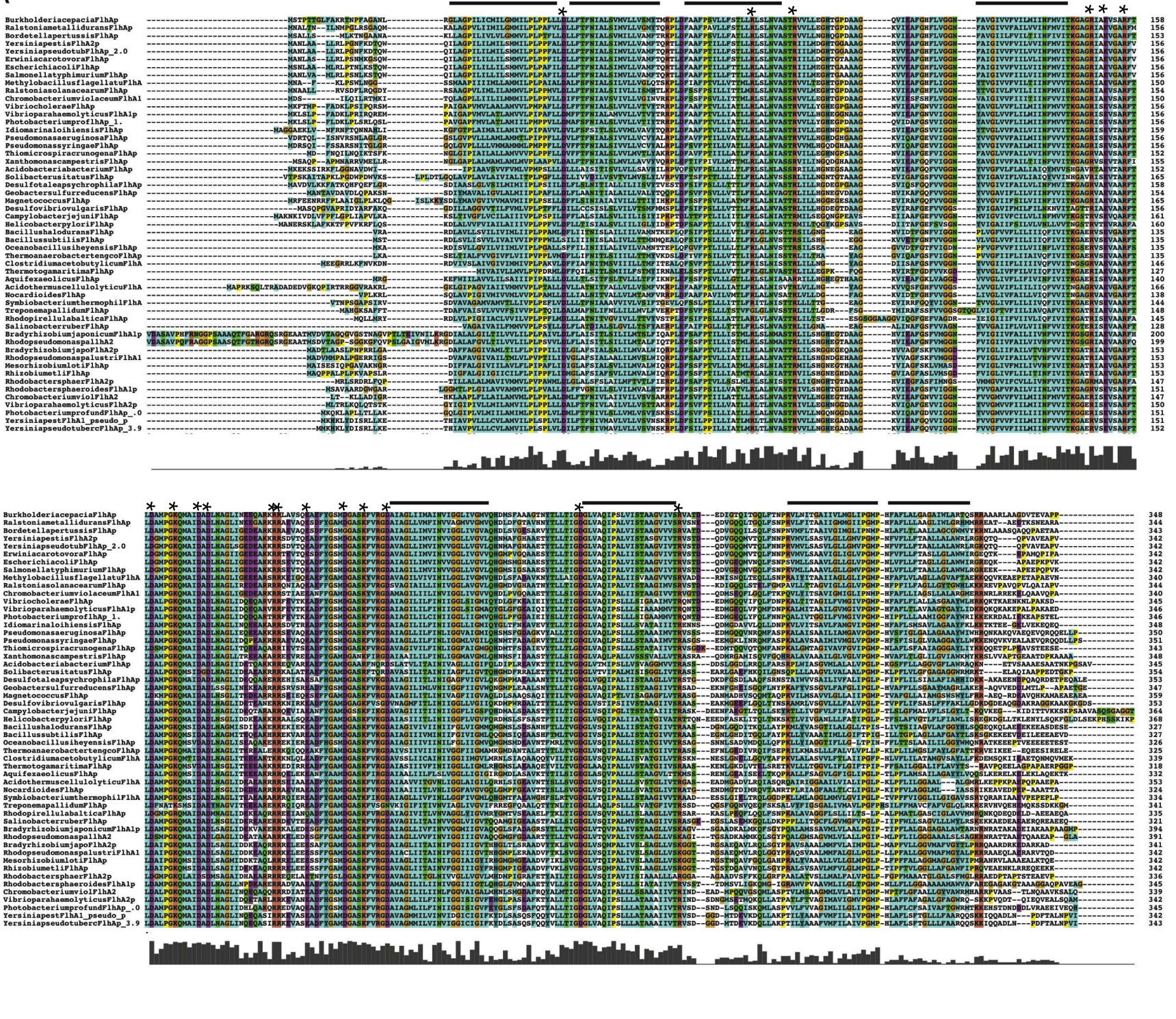
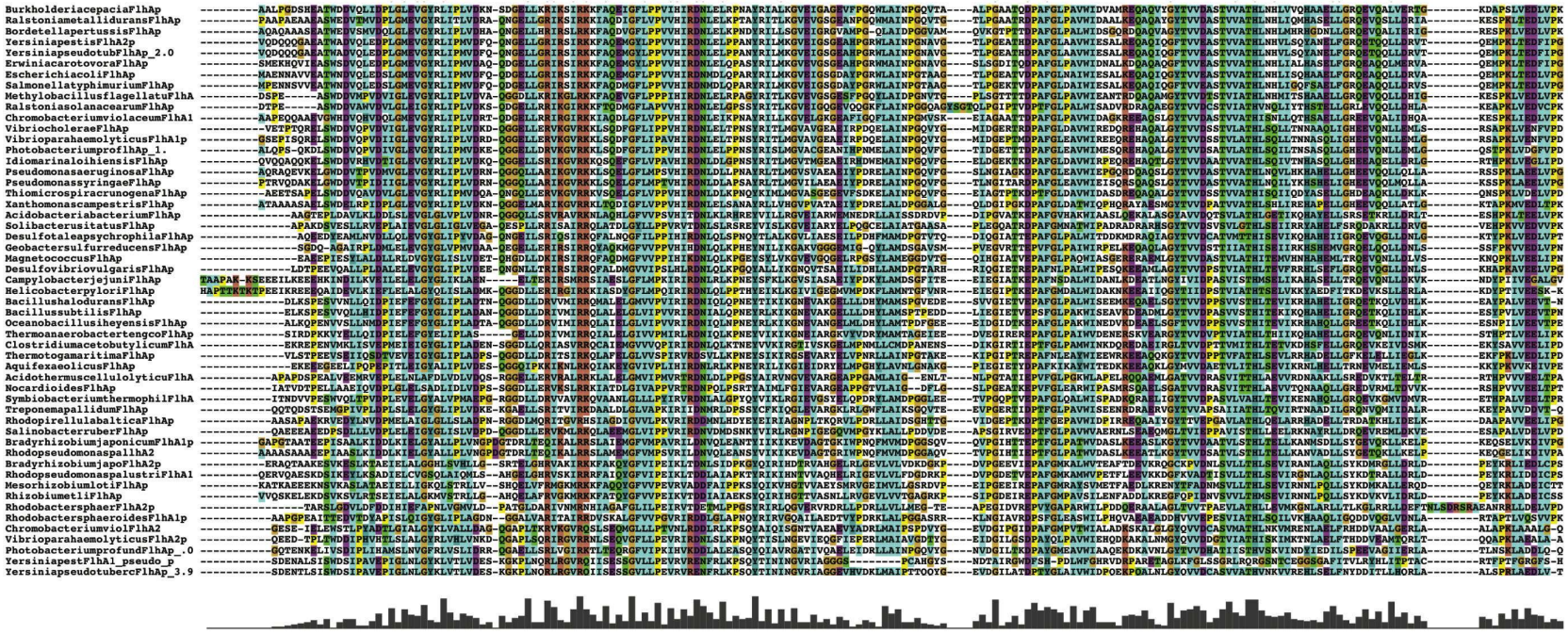
Treponemapallidum
Aquifexaeolicus
Clostridiumacetobutylicum
Solibacterusitatis
Rhodopirellulabaltica

FlhA



FlhA (cont.)


Burkholderiacepaciaflhap
Ralstoniametallidurans Flhap
Bordetellapertussis Flhap
Yersiniapestis Flha2p
Yersiniapseudotub Flhap_2.0
Erwiniacarotovora Flhap
Escherichiacoli Flhap
Salmonellatyphimurium Flhap
Methylobacillusflagellatu Flha
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Chromobacteriumviolaceum Flha1
Vibriocholerae Flhap
Vibrioparahaemolyticus Flha1p
Photobacteriumprof lhap_1.
Idiomarinaloihiensis Flhap
Pseudomonasaeruginosa Flhap
Pseudomonassyringae Flhap
Thiomicrospiracrunogena Flhap
Xanthomonascampestris Flhap
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Solibacterusitatus Flhap
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Bacillussubtilis Flhap
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Thermoanaerobactertengcong Flhap
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Rhodopirellulabaltica Flhap
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Rhizobiumetli Flhap
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Rhodobactersphaeroides Flha1p
Chromobacteriumviol Flha2
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FlhB

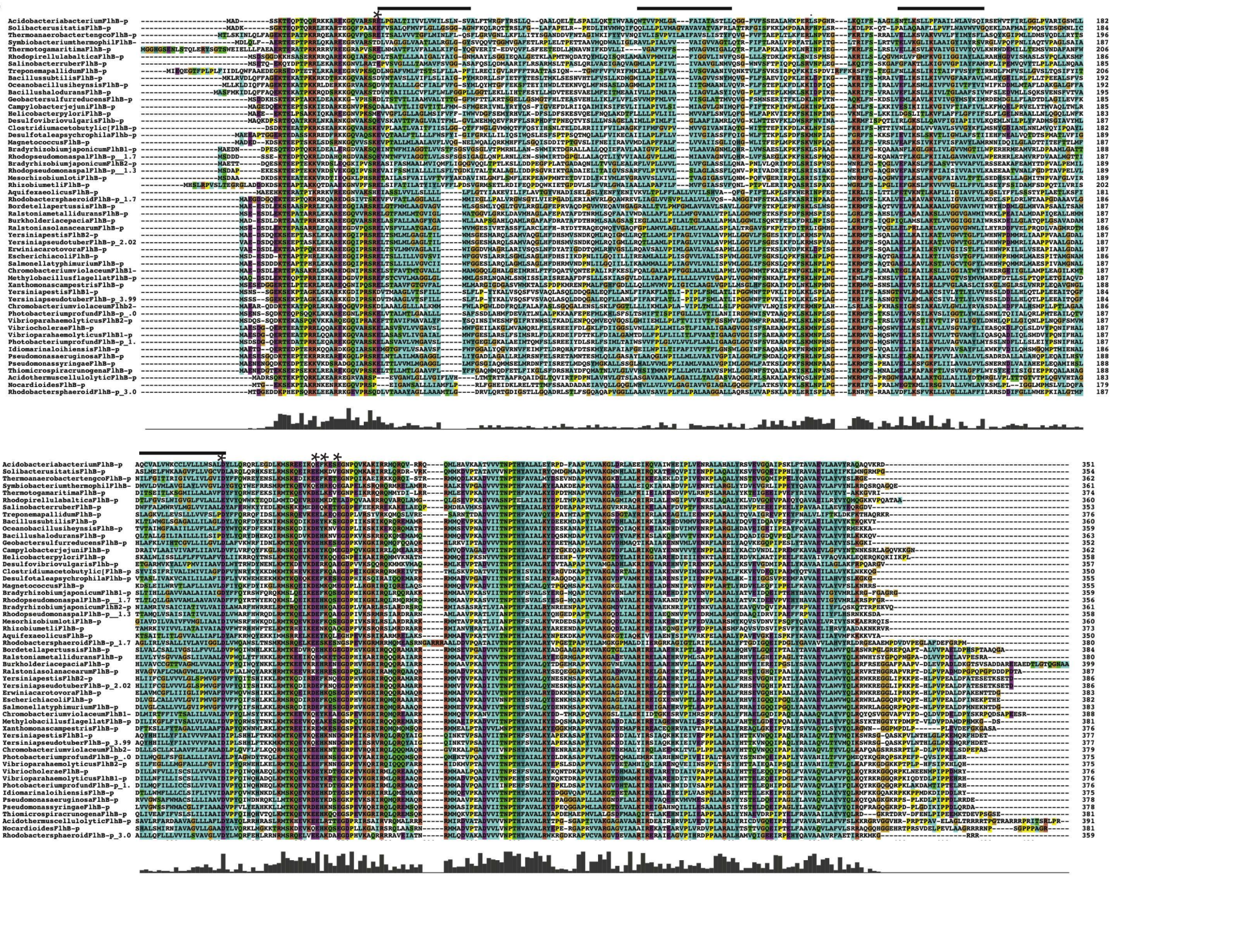


Figure S2. *Top:* FliP and FliQ mutations causing strong motility defects do not display dominant-negative effects. FliP mutations D197G, E178A, and K222A allow weak function; the other FliP mutations and the FliQ mutations give immotile phenotype. Wild-type (LT2) cells were transformed with salicylate-inducible plasmids expressing the indicated proteins. Induction with salicylate was at the levels indicated. Plates were incubated at 32° C for 5.5 h. *Bottom:* Relatively mild dominant-negative effects of five motility-abolishing FlhA mutations (Contrast with the strong dominance of R147A, R154A, and D158A mutations shown in Figure 2.)

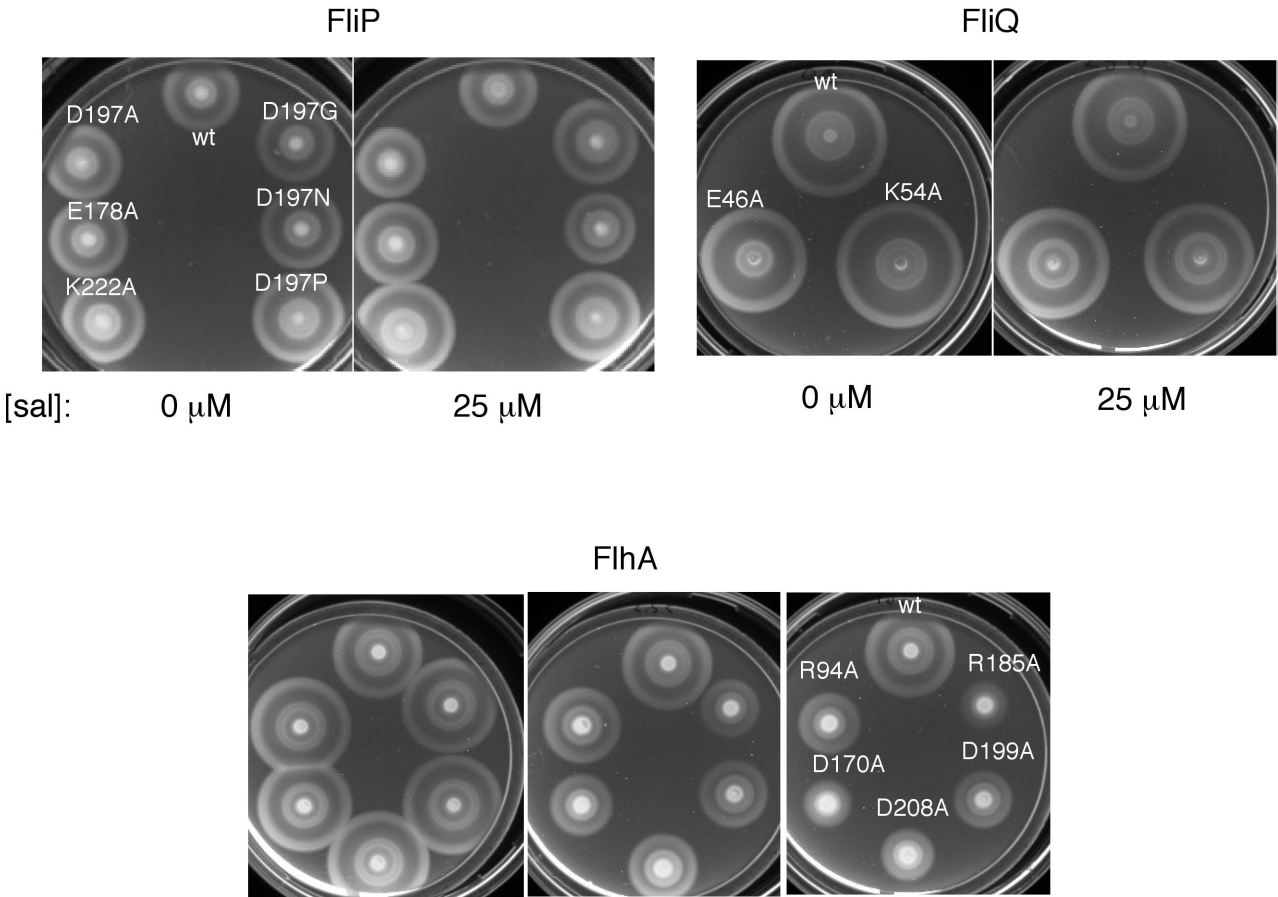


Figure S3. Enhanced motility of the FlhB D208A mutant upon moderate overexpression of FlhA. The background was TH12644, transformed with a derivative of pMS123 carrying the D208A mutation, together with the IPTG-regulated FlhA-expressing plasmid pEK1007. The plate contained chloramphenicol and ampicillin (50 μg/m each), 2.5 μM sodium salicylate to induce the mutant FlhB, and 50 μM IPTG to induce FlhA. The plate was spotted with 3 μl drops of overnight cultures and incubated for 6 h at 32° C.

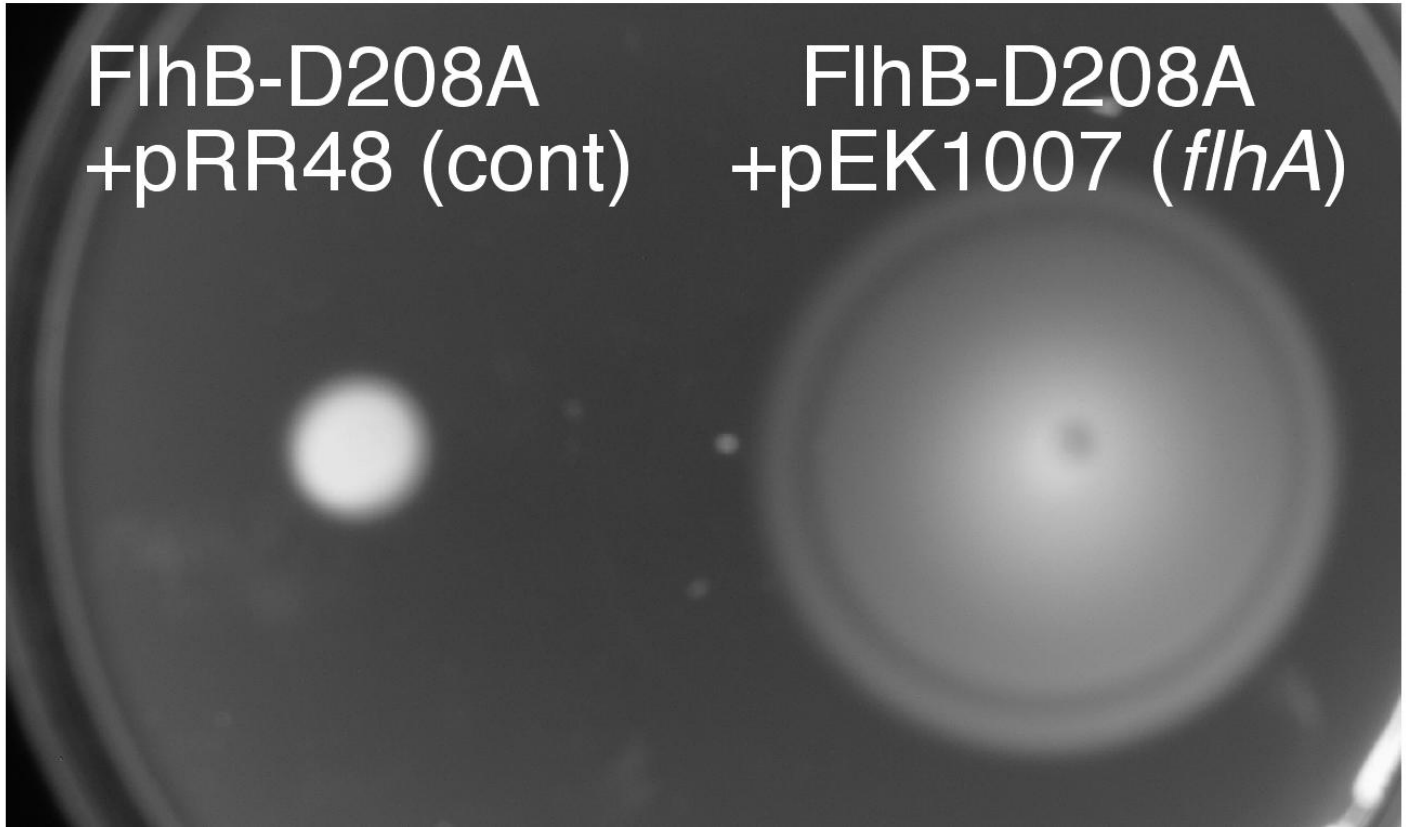
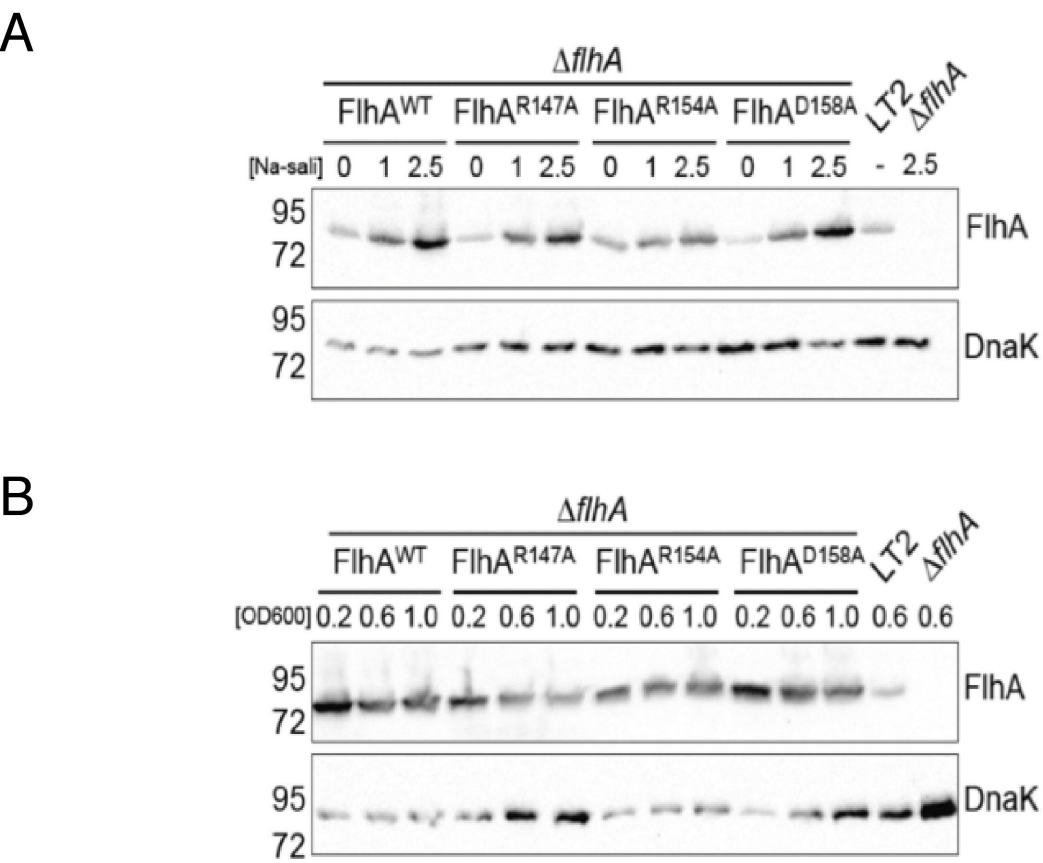


Figure S4. *(A)* Comparison of levels of w.t. and mutant FlhA proteins in mid-logarithmic phase cells, with expression induced at various levels by sodium salicylate. Cultures were grown in LB at 37° C. FlhA was detected using rabbit polyclonal antibody, a gift of T. Minamino. *(B)* Expression of wild-type and mutant FlhA proteins at various phases of growth. *(C)* Absence of any growth defect upon expression of the mutant FlhA proteins. In each panel, one of the mutant variants is compared with the wild type (the same for all panels). *unind*, not induced; *ind*, induced with 10 μM sodium salicylate.



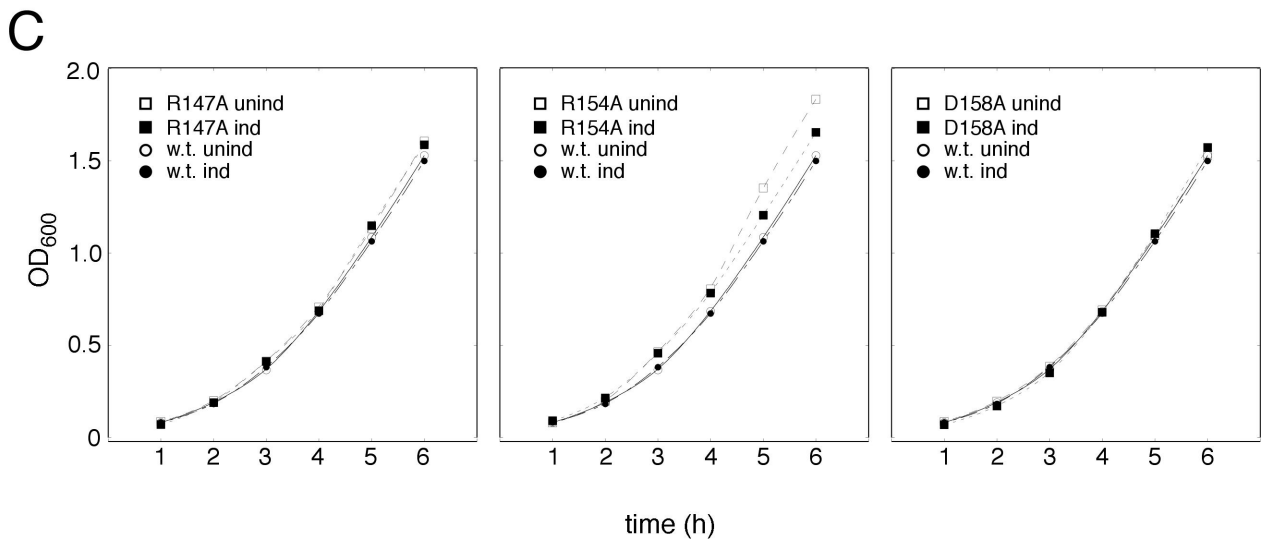
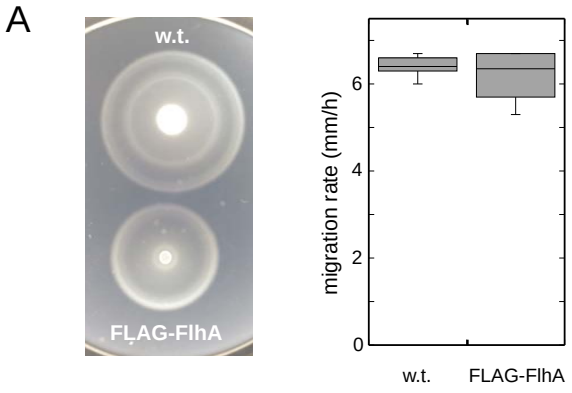
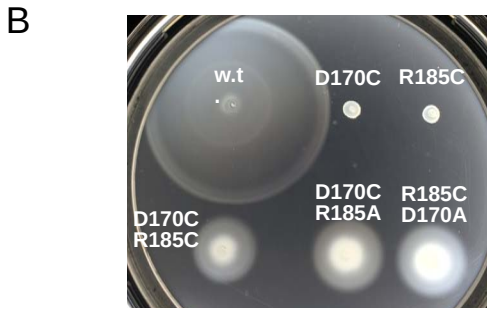


Figure S5. Properties of FlhA cysteine replacements; crosslinking by bis-maleimidohexane.. *(A)* Complementation of the Δ*flhA* strain by a plasmid expressing N-terminally FLAG-tagged FlhA. *Left*: Overnight cultures (LB, 32° C) of the indicated strains were spotted on a soft-agar (0.27%) tryptone plate and incubated for 8 h at 32° C. The plate contained 10 μM IPTG. The reduced swarm size relative to w.t. is caused by a ~2h delay in the onset of motility in cells with the FLAG-tagged protein. *Right:* Migration rates of cells containing wild-type of FLAG-tagged FlhA. Box plots are for six determinations. Once movement is underway, migration rate with FLAG-tagged FlhA is similar to wild-type. *(B)* Effects of Cys and Ala replacements of D170 and R185. Overnight cultures of the indicated mutant strains were spotted on the plate and incubated at 32° C for 8 h. *(C)* Inter-molecular crosslinking of FlhA positions 170 and 185 by the bifunctional reagent bis-maleimidohexane. Proteins were resolved on a 4%-20% gradient gel.





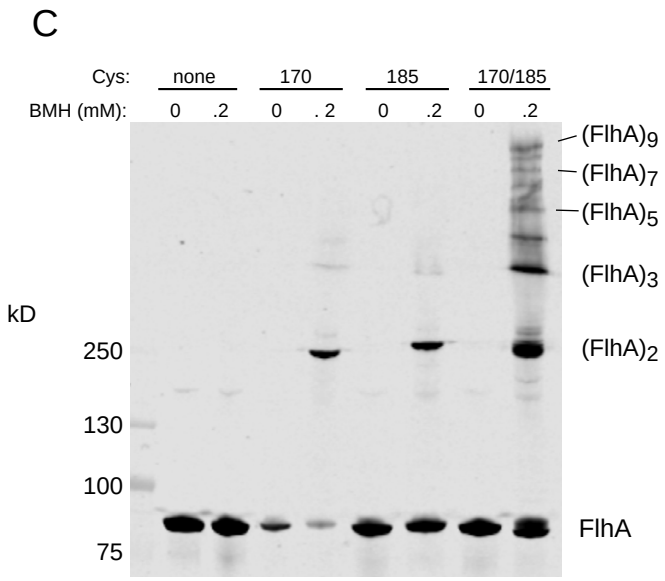


Figure S6. Additional crosslinking and proteolysis experiments. (A) Crosslinking experiment in a Δ*flhDC* strain, which expresses no flagellar genes from the chromosome. The Δ*flhA* strain is shown for comparison. Cys residues were present at positions 170 and 185 in all lanes. The experiment used a 7.5% acrylamide gel with a 1:70 bis:acrylamide ratio.
(B) Effect of detergent (0.05% Triton X-100). The Cys170/Cys185 FlhA protein was expressed in the Δ*flhA* background.Triton was added either after crosslinking (a), or before (b), as indicated. Although yield of the highest-MW products is decreased by Triton, product as large as heptamer is still observed (and dimer and trimer intensity is increased). The experiment used a 4%-20% gradient gel.
(C) Limited-proteolysis experiment like that in Figure 7 except carried out in the Δ*flhDC* strain.

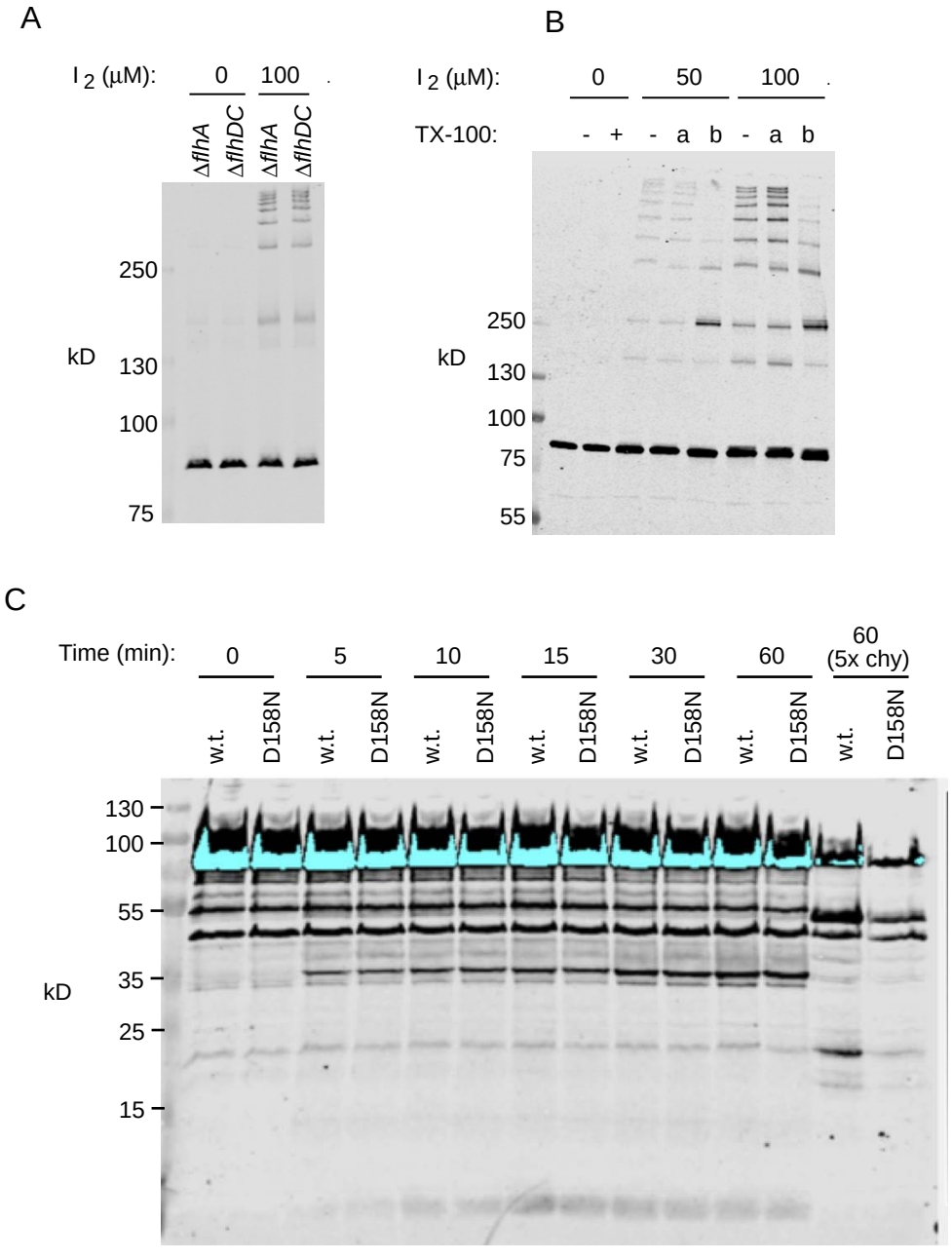


TABLE S1. Comparison of trans-membrane segment predictions for proteins of the flagellar export apparatus

	Prediction Method					
	MEMSATSVM	**HMMTOP**	**SOSUI**	**TMHMM**	**TMpred**	**TopPRED**
FlhA						
TM segment						
1	23-40	22-41	19-41	21-43	21-44	21-41
2	44-64	46-64	44-66	47-64	46-65	45-65
3	74-89	73-97	72-94	76-98	68-93	72-92
4	118-141	118-142	122-144	118-140	123-140	122-142
5	210-229	210-229	208-230	213-235	209-229	209-229
6	245-260	250-269	248-270	250-272	244-266	251-271
7	291-307	292-316	298-320	293-315	310-327	289-309
8	311-326					
(8a)						473-493
FlhB						
TM segment						
1	31-49	33-50	33-55	33-50	33-51	31-51
(2)			72-94			
2	81-111	91-115	99-121	93-115	96-117	88-108
3	144-174	146-162	144-166	146-168	149-168	144-164
4	181-211	189-213	185-207	189-211	189-207	187-207
(5)						331-351
FliP						
TM segment						
1	43-73	47-71	48-70	47-69	45-64	42-62
2	89-104	88-105	87-109	88-105	88-105	86-106
3	182-212	186-210	184-206	185-207	193-213	189-209
4	216-236	215-239	213-235	214-236	223-241	212-232
FliQ						
TM segment						
1	13-41	17-38	17-39	20-42	16-38	19-39
2	51-78	55-74	56-78	52-74	55-74	47-67
FliR						
TM segment						
1	9-32	9-33	12-34	15-34	13-30	15-35
2	42-57	44-60	43-65	41-58	44-61	41-61
3	70-100	65-88	70-92	73-95	71-94	65-85
(4)					98-116	
4	126-155	128-151	132-154	132-154	122-151	121-141
5	178-207	178-202	172-194	179-201	175-193	184-204
6	211-241	213-237	224-246	214-236	213-237	211-231