Supplemental Information for

Type-III Secretion Pore Formed by Flagellar Protein FliP

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Figure S1. Patterns of sequence conservation in FliP. Conserved residues of interest and the conserved Met segment are indicated above the alignment. The region containing the signal sequence is not shown; TM = transmembrane segment.

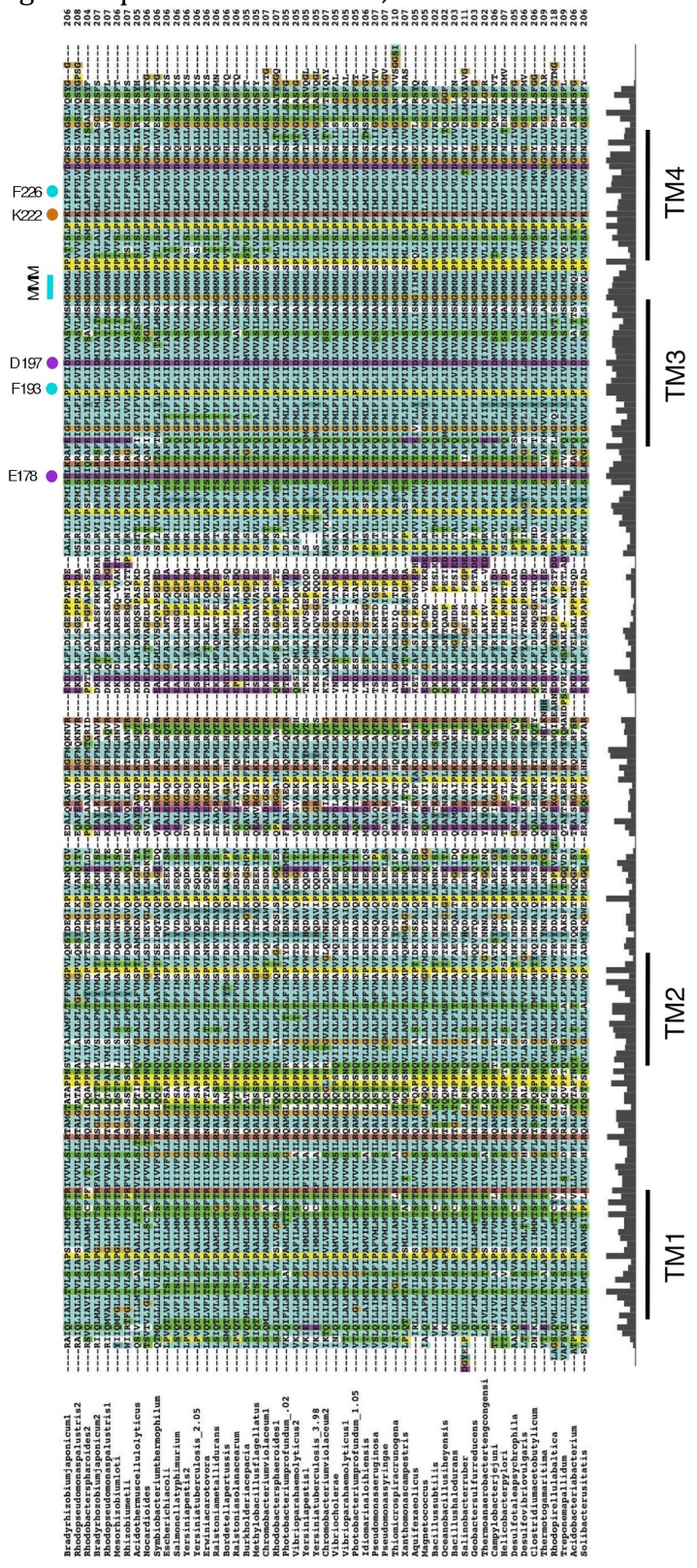


Figure S2. Normal functioning of HA-tagged FliP. The HA antigen is at the N-terminus of the mature protein (i.e., the protein with the signal sequence cleaved). Wild-type and HA-tagged FliP were expressed from plasmids in the Δ*fliP* strain, with induction by salicylate at the concentrations indicated. Plates contained tryptone broth and 0.26% agar. Plates were spotted with 3 μl of overnight cultures and incubated for 6 h at 32° C.

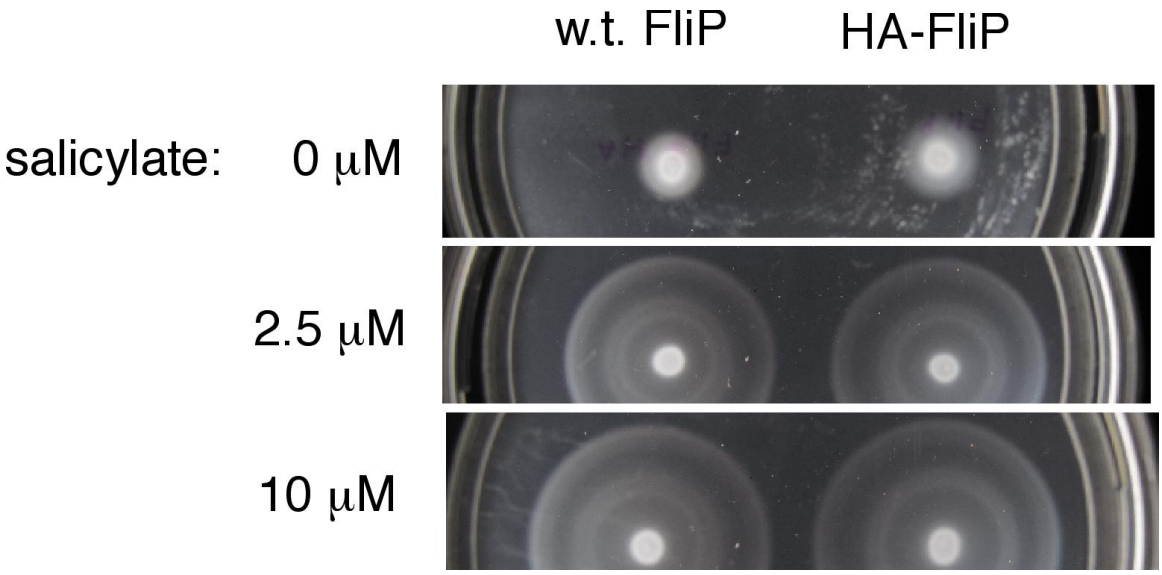


Figure S3. Function of FliP proteins with Cys replacements near the middles of the membrane segments. Positions of the Cys replacements are indicated at the top; each column corresponds to one of the four TM segments. Mutant proteins were expressed from plasmids in the Δ*fliP* strain, with induction by salicylate at the concentrations indicated. Plates contained tryptone broth and 0.26% agar. Plates were spotted with 3 µl of overnight cultures and incubated for 5h at 32° C. Function was similar to wild type for all of the Cys-mutant proteins except L225C, which also showed a sub-normal protein level on immunoblots (Figure 1, panel B).

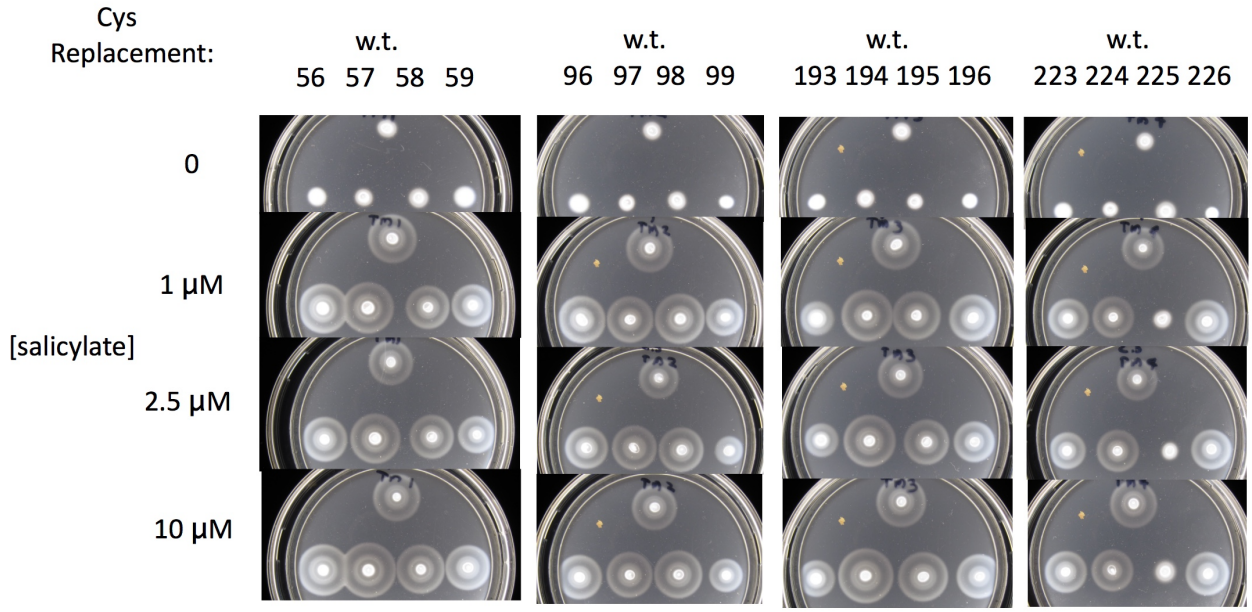


Figure S4. *(A)* Further increase in Cd^{2+} sensitization in the mutant with double Cys replacement F193C/F226C, relative to the F226C replacement alone. The experiment was carried out in the same way as those in Figure 3, panels A-C; growth was at 32° C. Open symbols, uninduced; filled symbols, cultures induced with 10 μM salicylate.

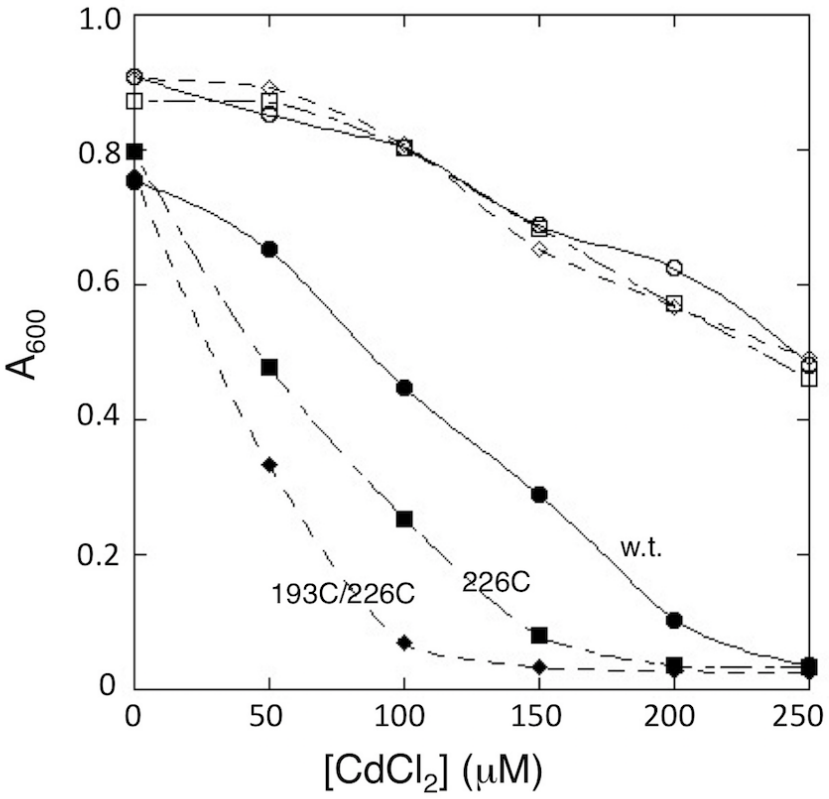


Figure S5. *(A and B)* Sensitization of cells to Mn^{2+} upon expression of FliP, and increased sensitization with F226C mutant FliP. Two independent experiments are shown. Data are time-courses of growth in absence or presence of Mn^{2+}, with or without induction by 1.5 μM salicylate. The control strain contained the parent vector pKG116. The horizontal axis is time in hours; $MnCl_2$ was added at hour 10.9 (panel A) or 11.4 (panel B), to 1.5 mM (A) or 1.0 mM (B). Growth was in TB medium at 37° C. *(C)* Absence of sensitization to ethanol by either wild-type or F226C FliP. Ethanol was added (to 2.8%) at time 10.5 h.

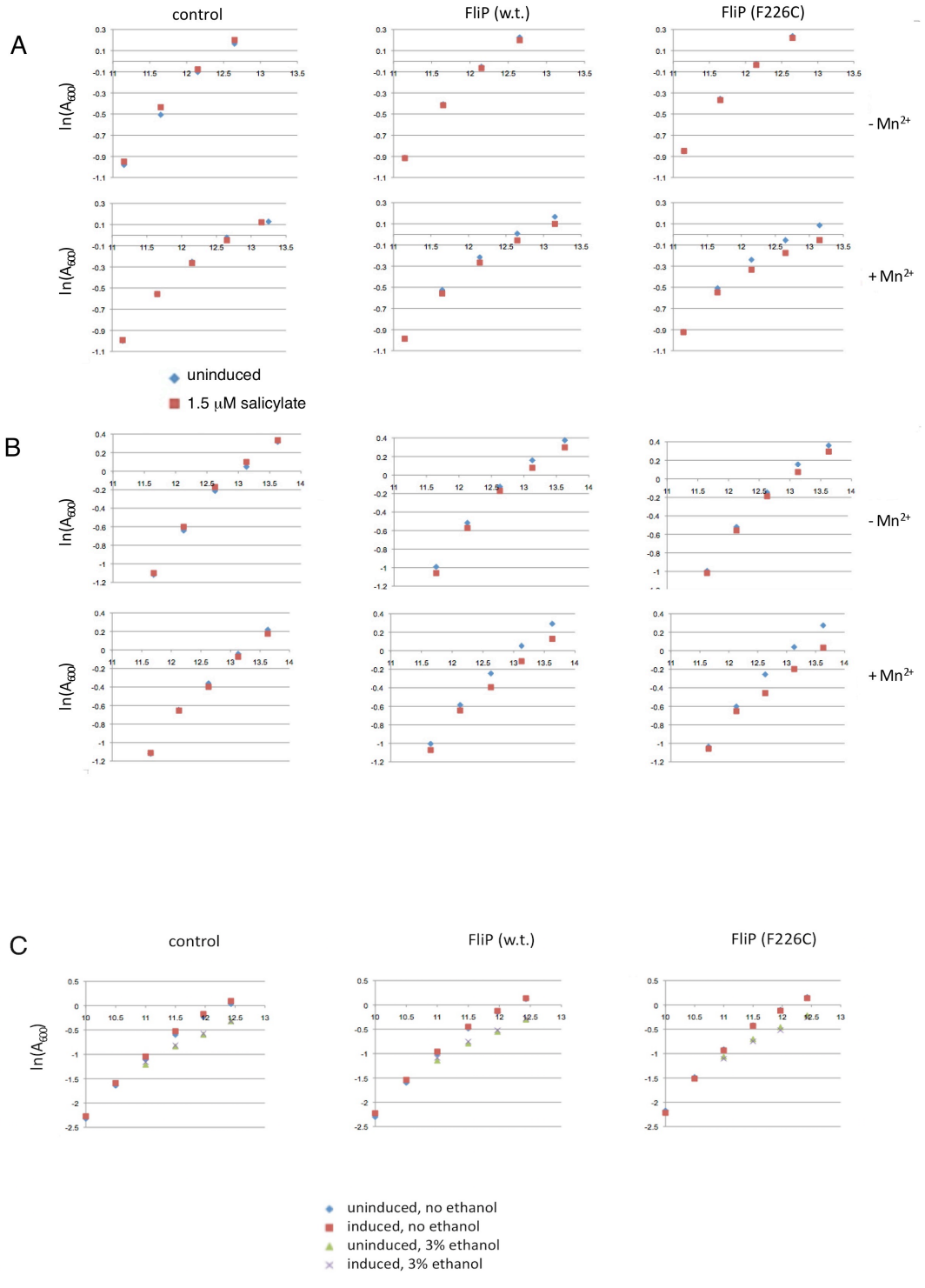
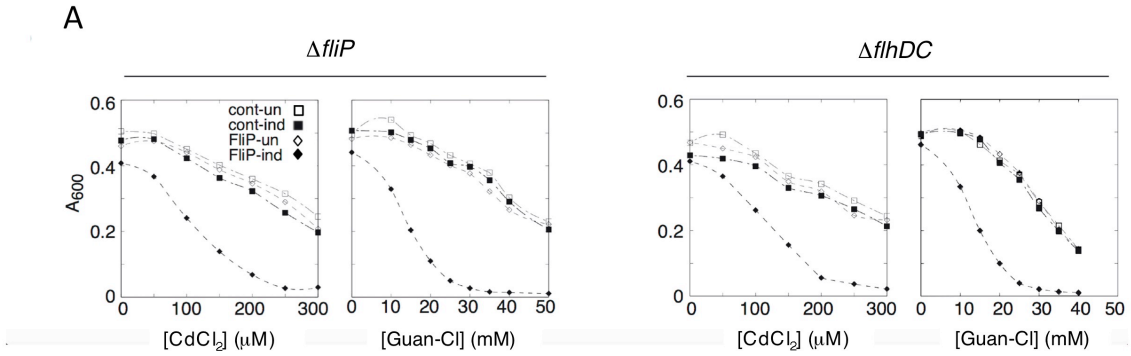


Figure S6. *(A)* FliP-induced sensitization to Cd^{2+} and guanidinium is not related to the presence of the HA tag, in either the Δ*fliP* or Δ*flhDC* backgrounds. The experiments shown used native (untagged) FliP instead of the HA-tagged version shown in most of the Figures. *(B)* Complementation of a Δ*fliP* mutant with either wild-type or F226C FliP requires an induction level (~ 1 μM salicylate) comparable to that causing strong sensitization to Cd^{2+} or Mn^{2+} (1.5 μM; Figure 3, panel D; Figure S5).



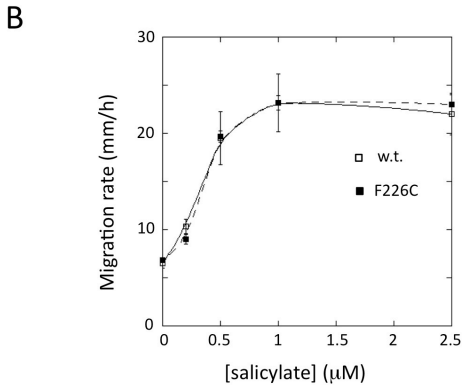


Figure S7. Nearly normal function of the FliP protein deleted for one of the conserved Met residues in the loop between TM3 and TM4. While labeled ΔMet_{210}, the mutation could be regarded as a deletion of any of the conserved Met residues 209-211. The FliP proteins were expressed from plasmids, induced with 2.5 μM salicylate.

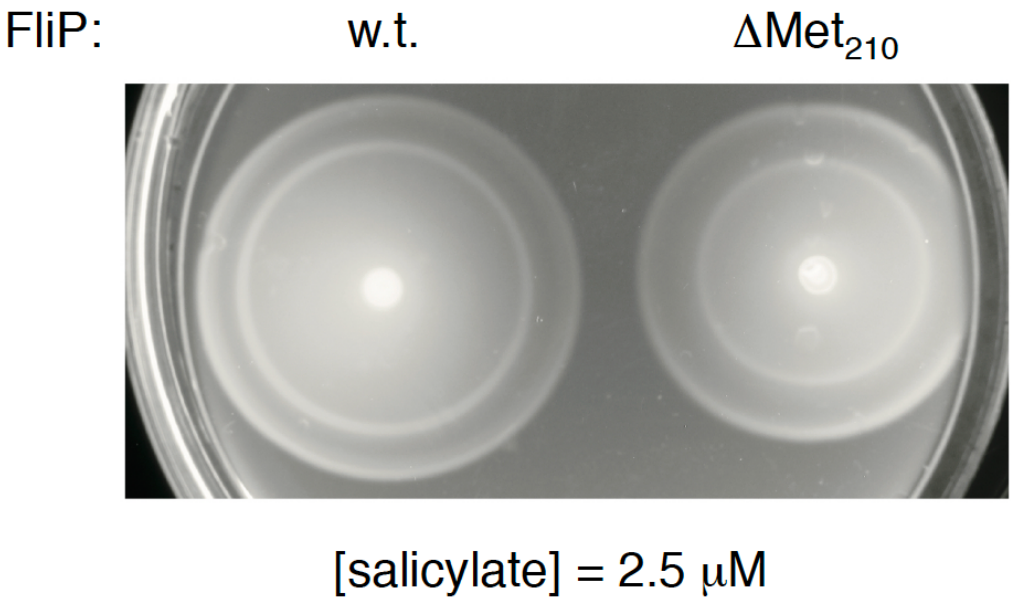


Figure S8. Sensitization to Cd^{2+} and guanidinium is similar with wild-type and ΔMet FliP. Proteins were expressed from plasmids in the Δ*fliP* background, with induction by 2.5 μM salicylate. The similar sensitizations seen with these agents contrasts with the case of choline (Figure 4), where sensitization was much greater with the ΔMet mutant protein.

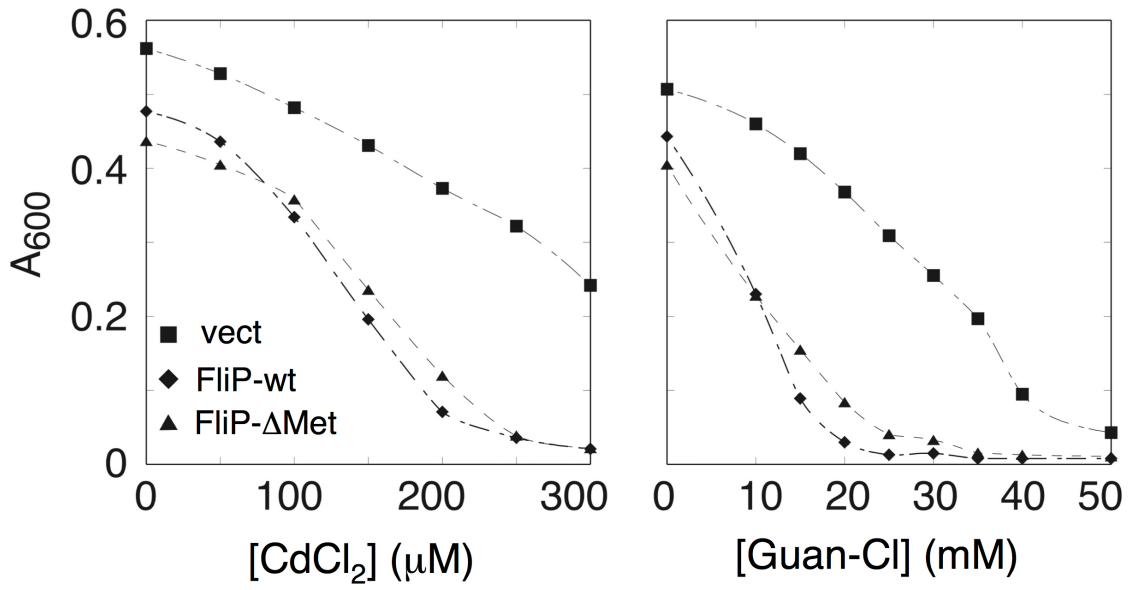


Figure S9 (following page). *(A)* Cellular and secreted levels of wild-type (WT) FliK and FliK-TOP7 expressed from the chromosomal P*araB* promoter by addition of 0.1% arabinose. FliK-TOP7 accumulated in the cytoplasm and prevented export of WT FliK. *(B)* Relative motility of the WT, a strain expressing FliK-TOP7 from the chromosomal P*araB* promoter, and a non-flagellated control (Δ*fliHIJ*) in the presence or absence of inducer. The diameter of the motility halo after 4.5 h incubation at 37 ºC was measured and is reported relative to the WT control on the same motility place. *(C)* Representative motility halos of the three strains. *(D)* Lack of significant rescue by the FliK-Top7 blocking construct when the growth-retarding agent is Cd^{2+}. The FliK-Top7 construct was present in the chromosomal *ara* locus, and where indicated (downward-pointing open triangles) was induced with 0.01% arabinose. Where indicated, wild-type FliP or the ΔMet variant were induced with 10 μM salicylate. Sensitization to Cd^{2+} requires only FliP, to which the FliK-Top7 protein should not be targeted. The absence of an effect here contrasts with the case of choline (Figure 5), where conductance depends on the full export apparatus, which can be blocked by FliK-Top7.

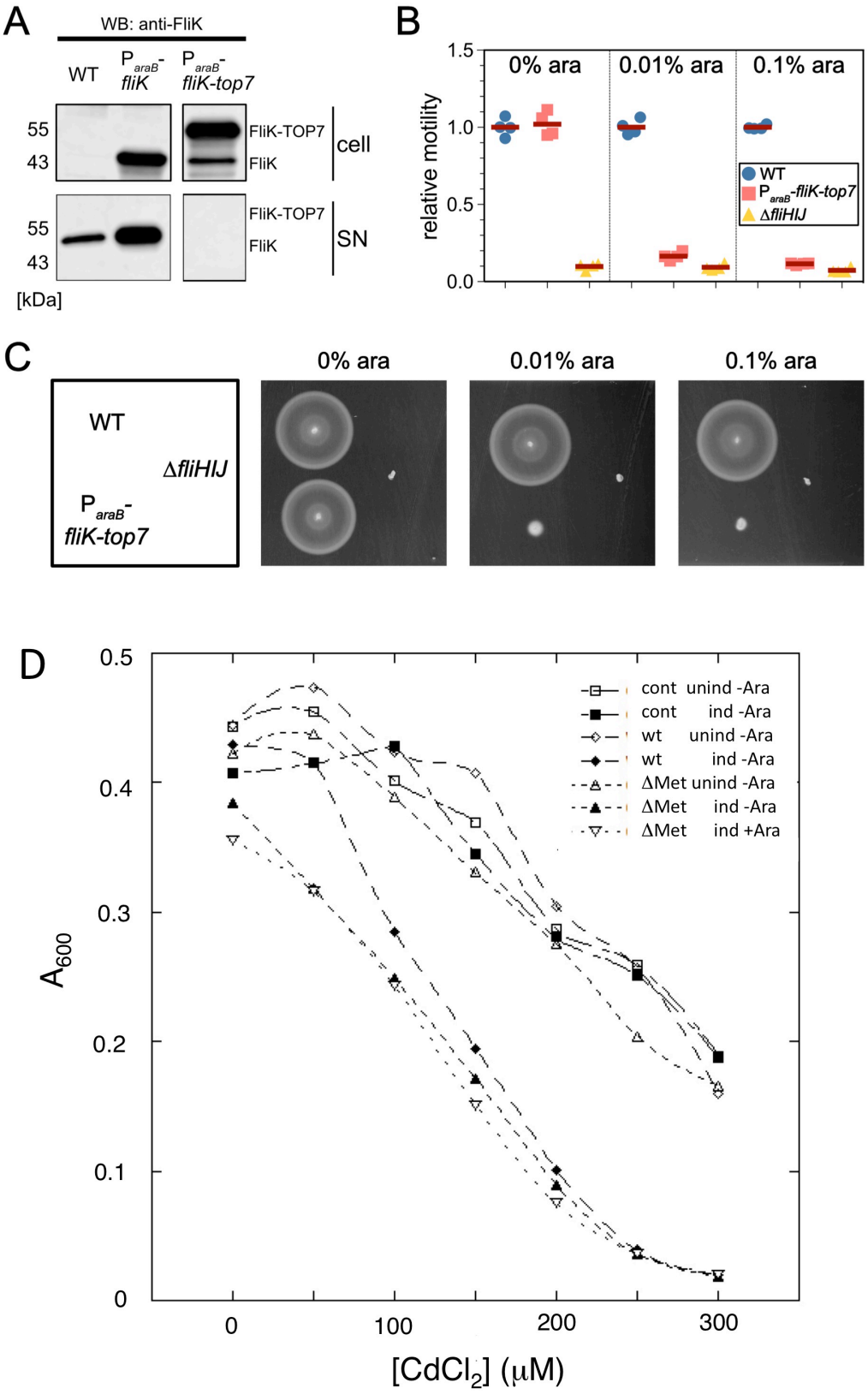

A
WB: anti-FliK
WT
P araB- fliK
P araB- fliK-top7
55
43
FliK-TOP7
FliK
cell
55
43
FliK-TOP7
FliK
SN
[kDa]
B
0% ara
0.01% ara
0.1% ara
relative motility
1.5
1.0
0.5
0.0
WT
P araB-fliK-top7
ΔfliHIJ
C
0% ara
0.01% ara
0.1% ara
WT
ΔfliHIJ
P araB- fliK-top7
D
cont unind -Ara
cont ind -Ara
wt unind -Ara
wt ind -Ara
ΔMet unind -Ara
ΔMet ind -Ara
ΔMet ind +Ara
A600
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
[CdCl2] (μM)

Figure S10. Effects of replacing conserved Met residues in FliP (positions 209-211) with residues of increased (Phe) or decreased (Ala) bulk. *(A)* Motility in soft-agar plates. Function is nearly eliminated in the triple-Phe mutants and is significantly impaired in the triple-Ala mutant. *(B)* Stability of the mutant FliP proteins. 3xFLAG-tagged FliP was detected by immunoblotting; FlhA was detected using anti-FlhA antibody. Total protein was detected using trichloroethanol staining. *(C)* Increased conductance by the triple-Ala mutant, but not the triple-Phe mutant, relative to the wild type. The experiment used guanidinium chloride.

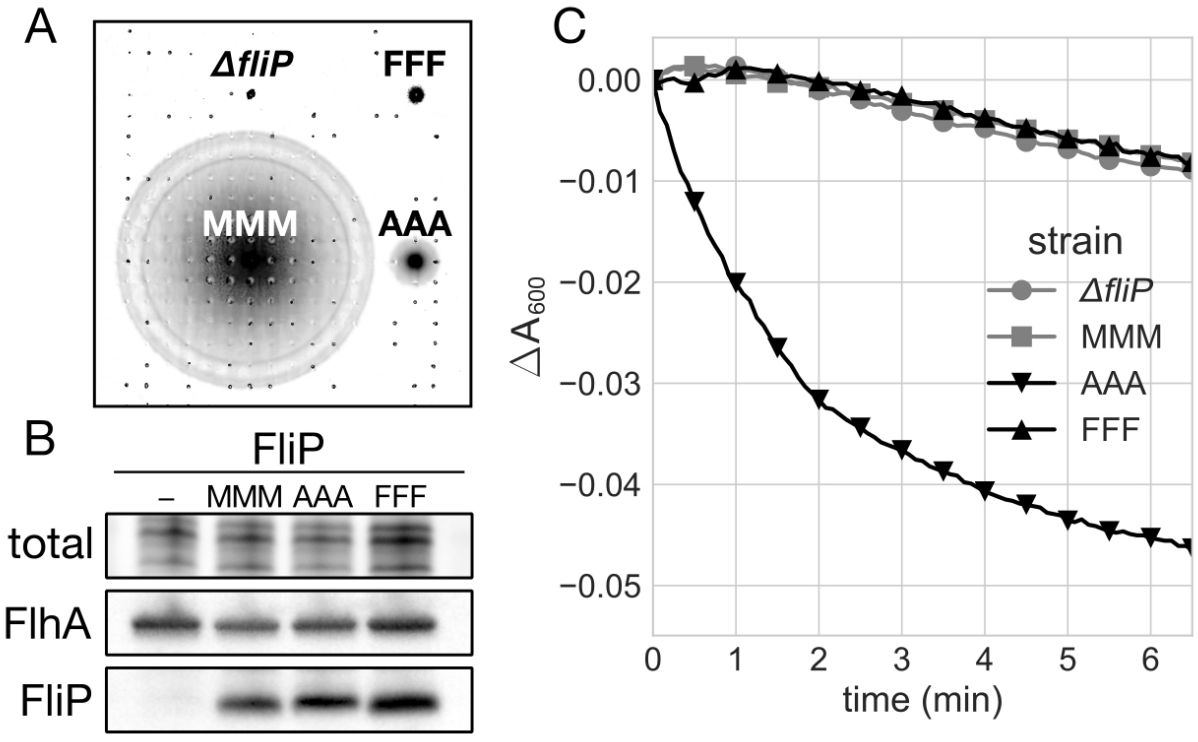


Figure S11. Influx of guanidinium into cells of the Δ*flhDC* strain, with either wild-type FliP or ΔMet-FliP expressed from a plasmid. Where indicated, protein expression was induced with 10 μM salicylate. In this background, FliP (either w.t. or mutant) is the only flagellar protein present, but guanidinium flow is still observed.