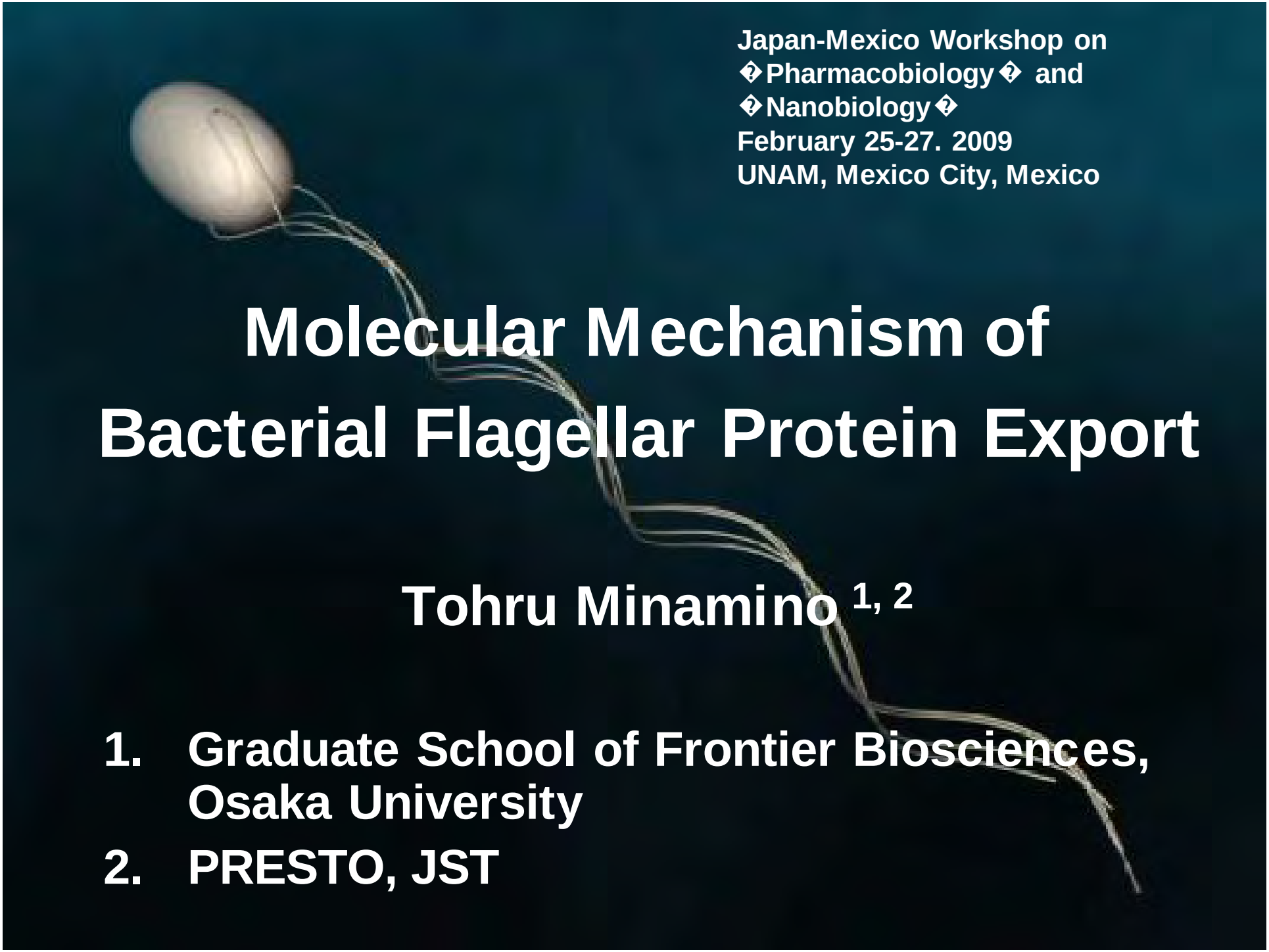

Japan-Mexico Workshop on
"Pharmacobiology" and
"Nanobiology"
February 25-27. 2009
UNAM, Mexico City, Mexico

Molecular Mechanism of Bacterial Flagellar Protein Export

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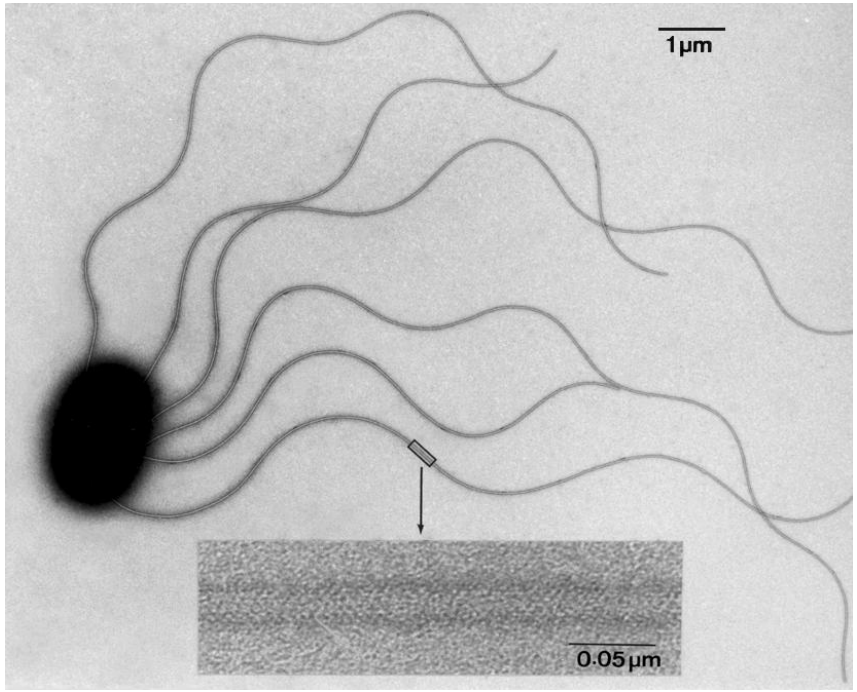
Bacterial flagella are filamentous organelles extended from the cell surface and is responsible for bacterial motility

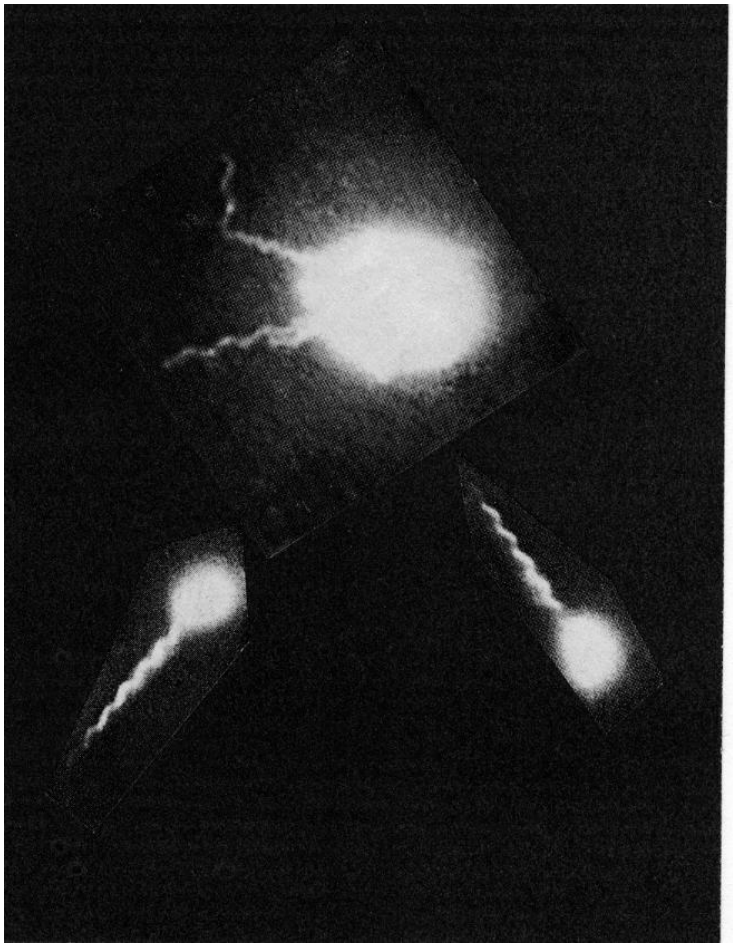
Electron micrograph of *Salmonella enterica serovar* Typhimurium

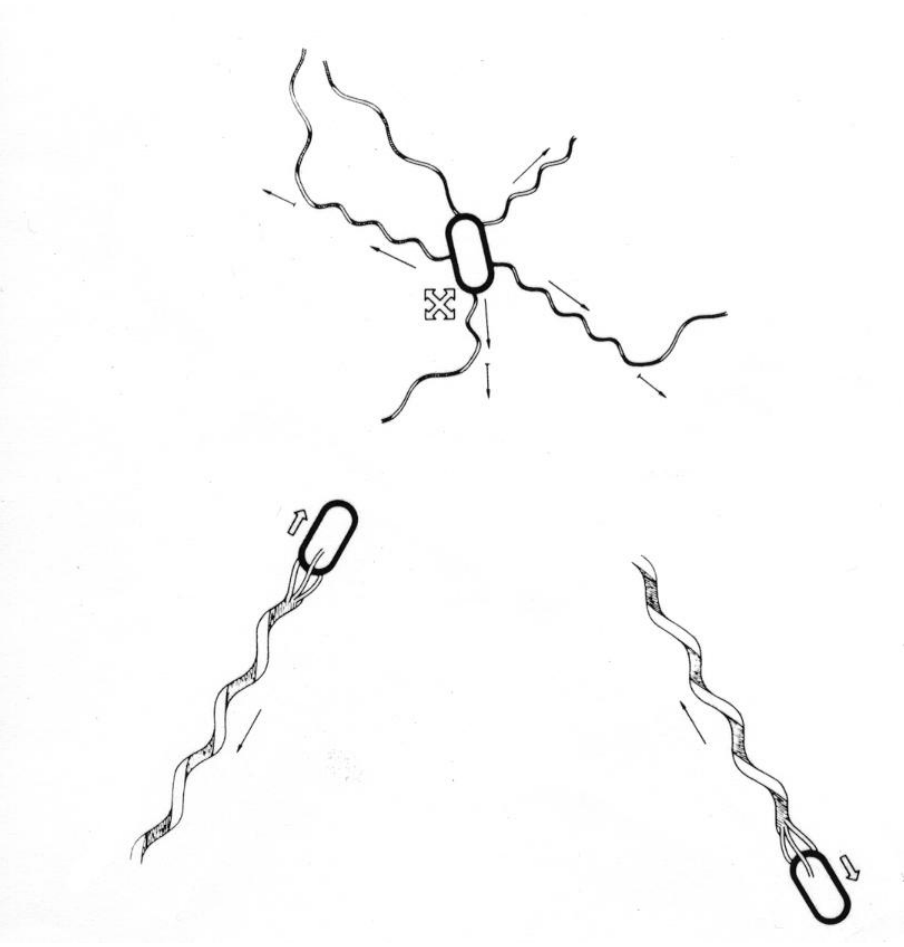


A reversible rotary motor, which is located at the base of the filament, is powered by the electrochemical potential gradient of proton across the cytoplasmic membrane

Swimming behavior of *Salmonella enterica serovar* Typhimurium in aqueous environments

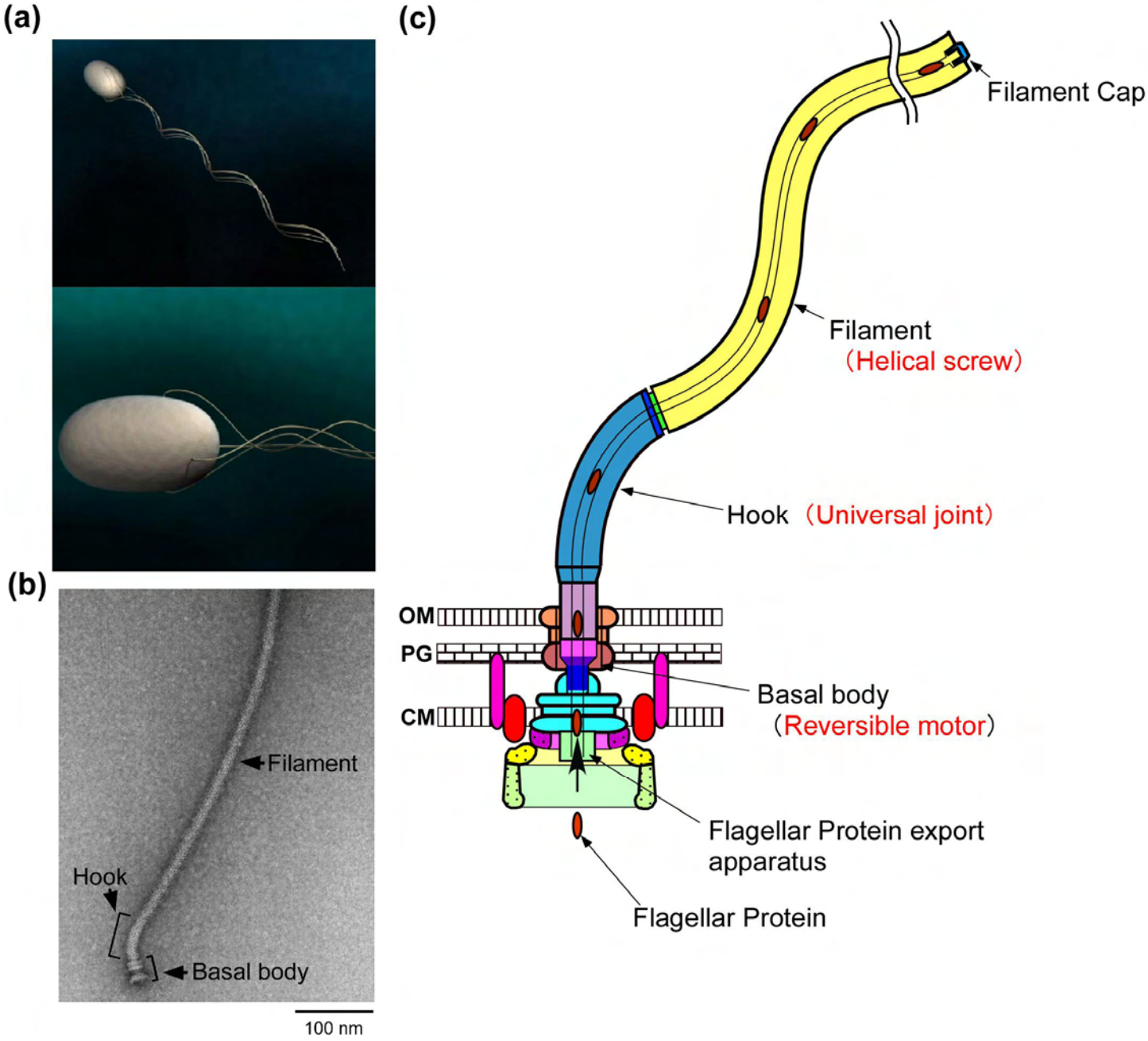
Flagellar bundle is disrupted by quick reversal change of the direction of flagellar motor rotation, changing the swimming direction of bacteria



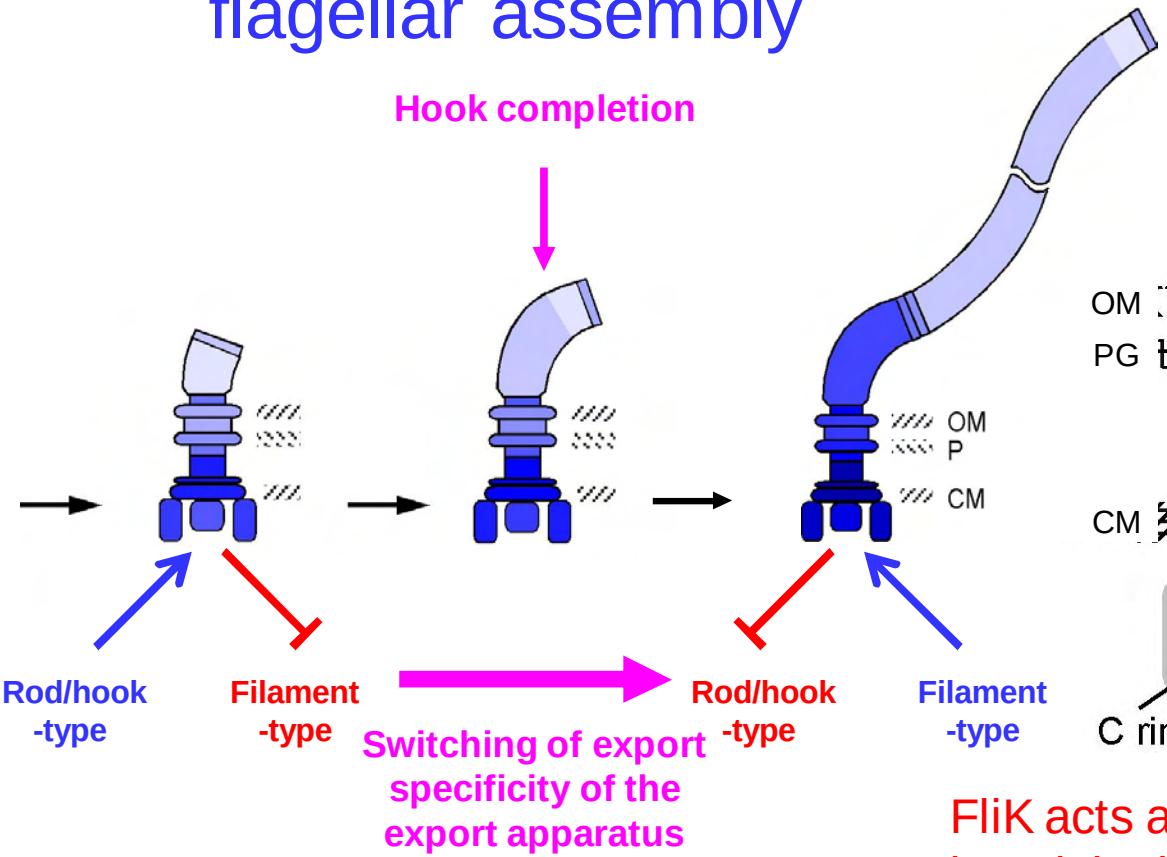


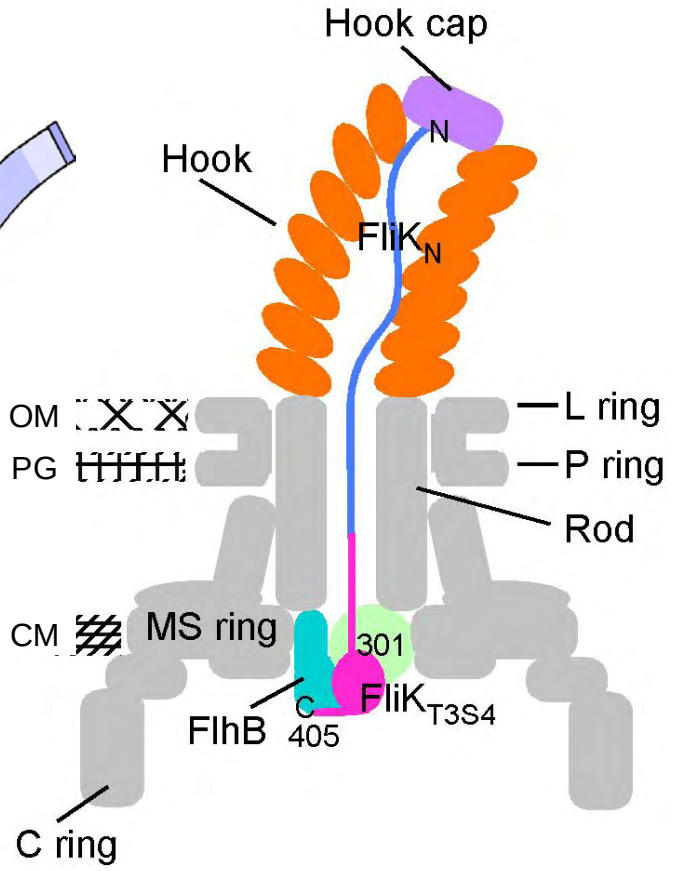
(Macnab & Ornston, *J. Mol. Biol.* 1977)

Bacterial flagellum



The order of flagellar protein export exactly parallels that of flagellar assembly

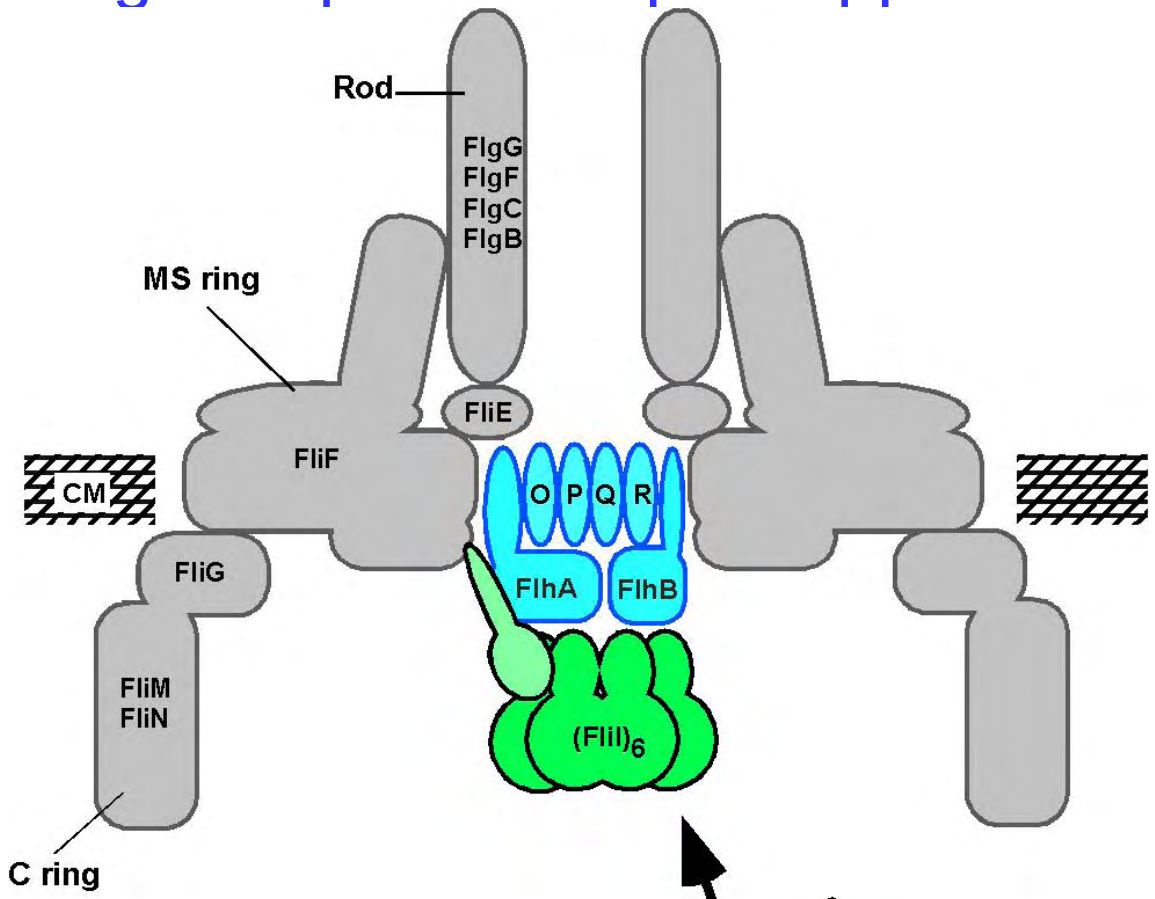


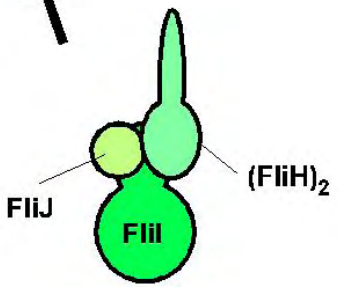


(Kutsukake, Minamino *et al*., *J. Bacteriol*. 1994; Minamino *et al*., *Mol. Microbiol.* 1999; Minamino *et al*., *J. Mol. Biol.* 2004 ; Minamino *et al*., *J. Mol. Biol.* 2006a; Moriya, Minamino *et al*., *J. Mol. Biol.* 2006)

FliK acts as a ruler to measure hook length in the cell exterior and switches export specificity of FlhB, an integral membrane component of the export apparatus, allowing such huge and complex architecture to be built efficiently in a well regulated manner.

Flagellar protein export apparatus





Export components

<Integral membrane proteins>

FlhA, FlhB, FliO, FliP, FliQ, FliR (Export gate)

<Cytoplasmic proteins>

FliH (ATPase regulator), FliI (ATPase), FliJ (Putative chaperone)

(Minamino & Macnab, *J. Bacteriol*. 1999; Minamino & Macnab, *Mol. Microbiol*. 2000)

The flagellar export pathway is one example of a type III pathway

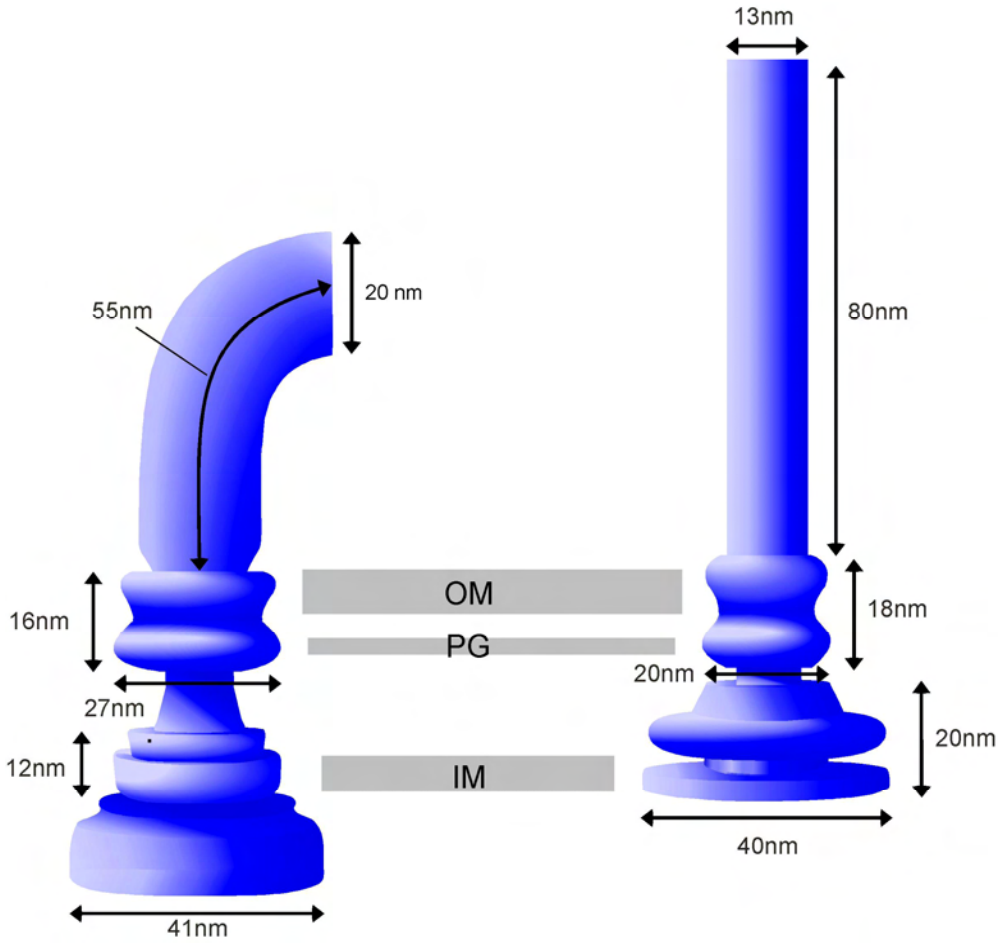
Flagellar protein export		Secretion of virulence factors (effectors)				
SALTY	Function	YEREN (Ysc)	SALTY (SPI-1)	EPEC	SHIFL	PSESH
FlhA	Export gate	LcrD	InvA	EscV	MxiA	HrcV
FlhB	Export gate	YscU	SpaS	EscU	Spa40	HrcU
FliO	Export gate	?	?	?	?	?
FliP	Export gate	YscR	SpaP	EscR	Spa24	HrcR
FliQ	Export gate	YscS	SpaQ	EscS	Spa9	HrcS
FliR	Export gate	YscT	SpaR	EscT	Spa29	HrcT
FliH	Regulator	YscL	?	?	MxiN?	HrpF?
FliI	ATPase	YscN	InvC	EscN	Spa47	HrcN
FliJ	General chaperone	YscB?	InvI	Orf15?	Spa13?	HrcP?

Component proteins of the flagellar export apparatus share substantial sequence similarities with those of type III secretion system (injectisome) of pathogenic bacteria such as *Yersinia, Salmonella*, EPEC, *Shigella,* and *Pseudomonus,* which is responsible for direct secretion of virulence factors into host cells.

Hook-basal body complex and Injectisome (secretes virulence factors) look similar to each other.

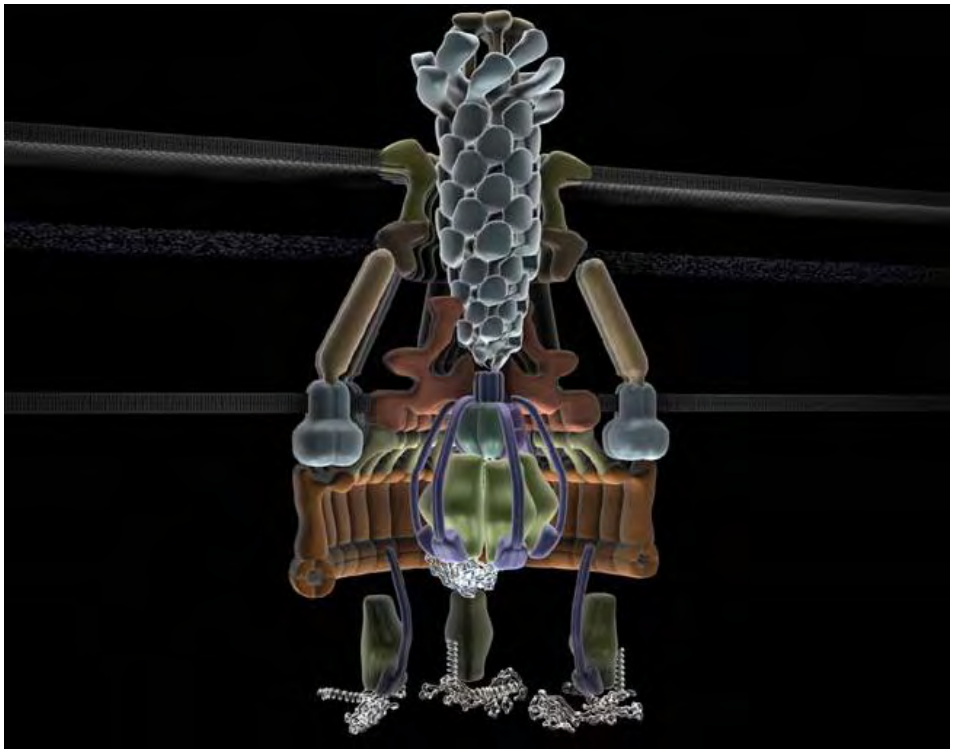
Hook-basal body

Injectisome



The sequence and structural similarities between the flagellum and the injectisome suggest an evolutional origin shared by these molecular machines.

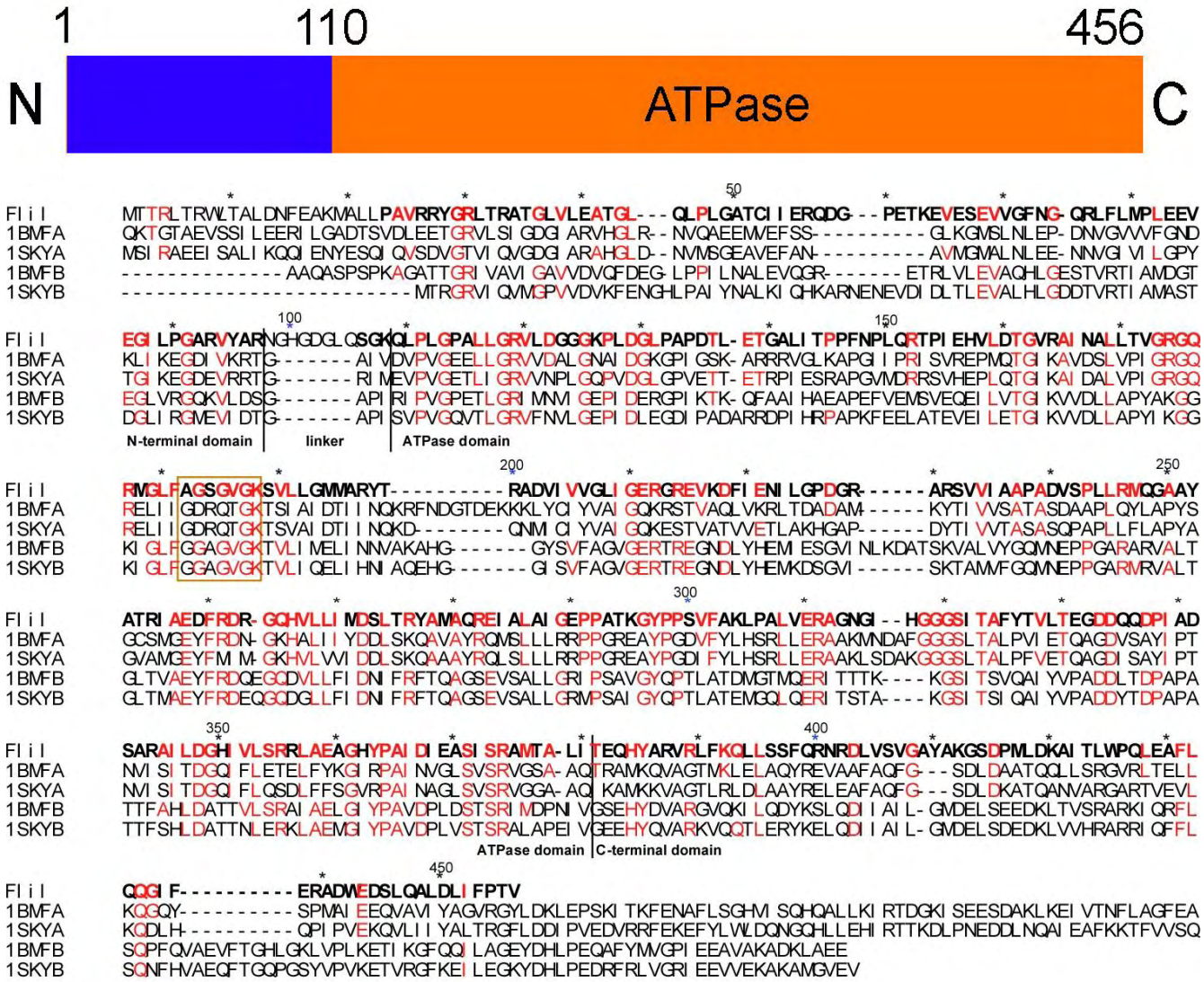
Todayôs topics



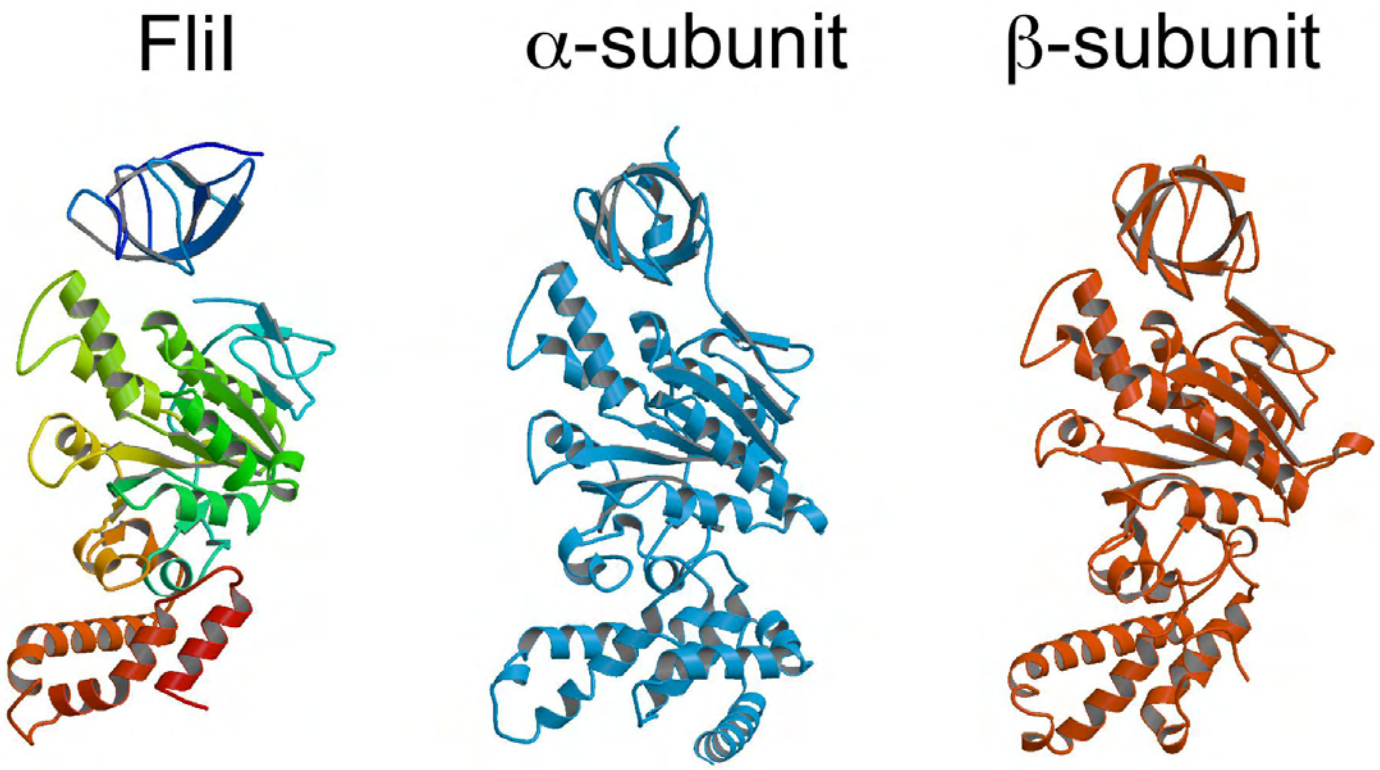
1. Dynamic, specific and cooperative interaction between export component proteins involved in the early stages of flagellar protein export.

2. Energy source for flagellar protein export

Sequence similarity between FliI ATPase and the α/β subunits of the proton-translocating F1 ATPase



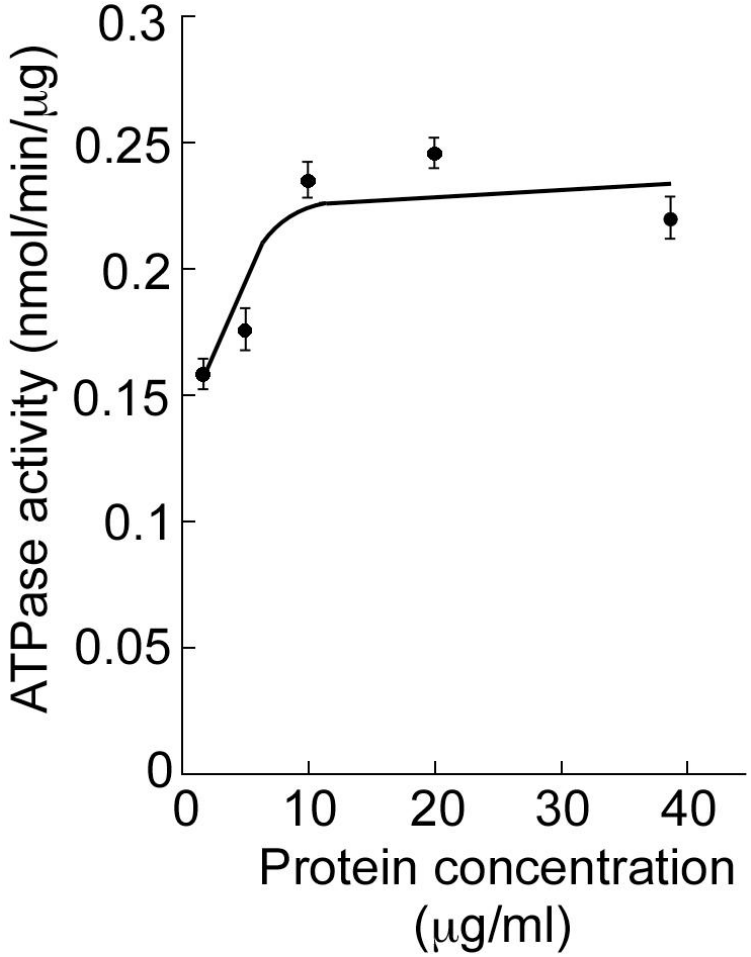
FliI can be superimposed to the F1 ATPase α/β subunits



The whole structure of FliI shows a striking similarity to the α and β subunits of F_1 ATPase and nucleotide binds to the P-loop in FliI in a similar way as in the F1-α/β subunits, implying a similarity in the functional mechanism between FliI and F_1-ATPase.

(Imada, Minamino *et al*., *PNAS*. 2007)

Enzymatic characteristics of FliI ATPase

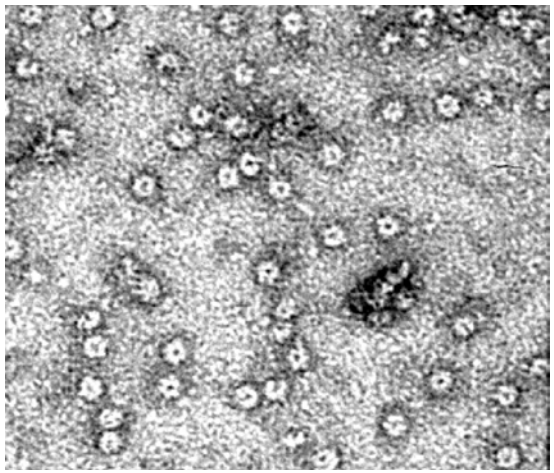


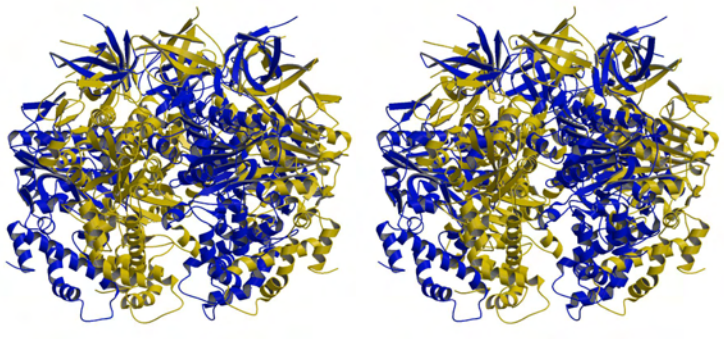
Km: 0.71

Hill's cooperativity coefficient: 1.78

(Minamino *et al*., *J. Mol. Biol*. 2006)

FliI hexamer



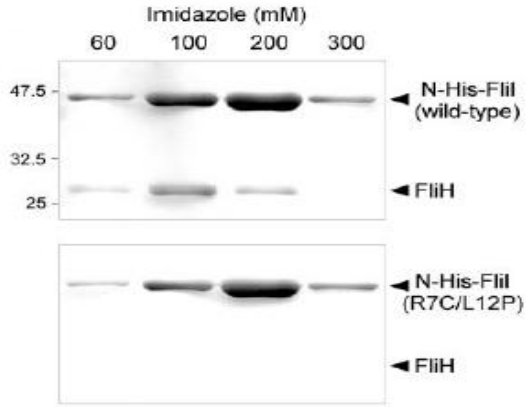


(Imada, Minamino *et al*., *PNAS*. 2007)

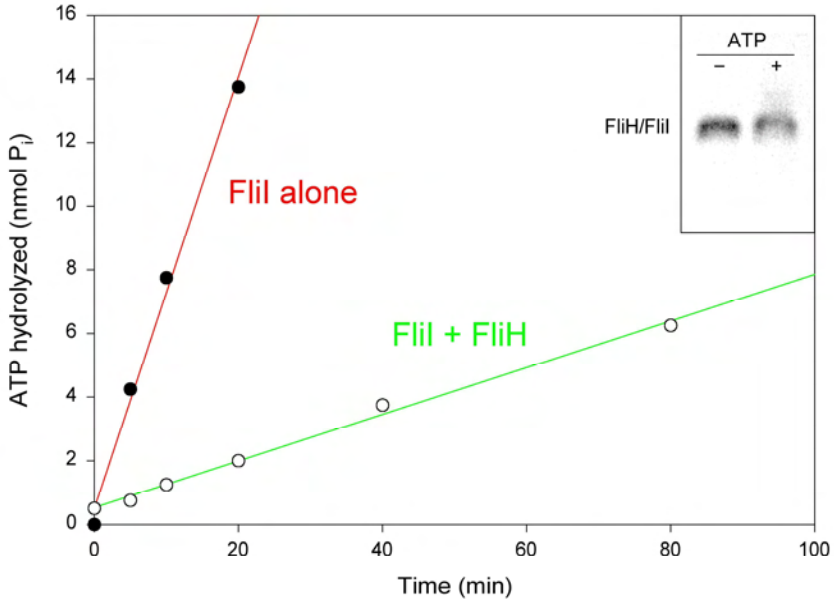
Unlike F1-ATPase, FliI can self-assemble into homohexamer and hence fully exerts its ATPase activity.

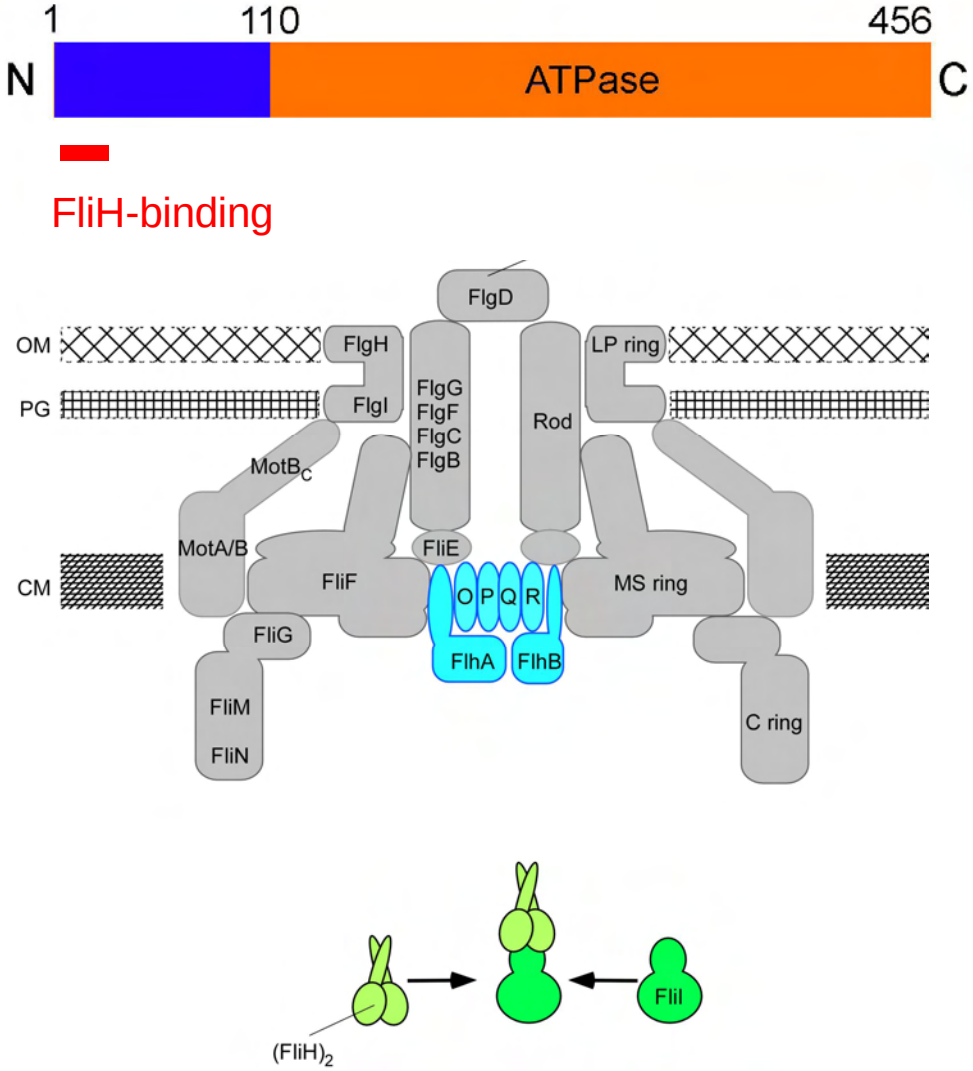
FliH binds to the extreme N-terminal region of FliI and suppresses the ATPase activity of FliI

Pull down assays with Ni-NTA affinity chromatography



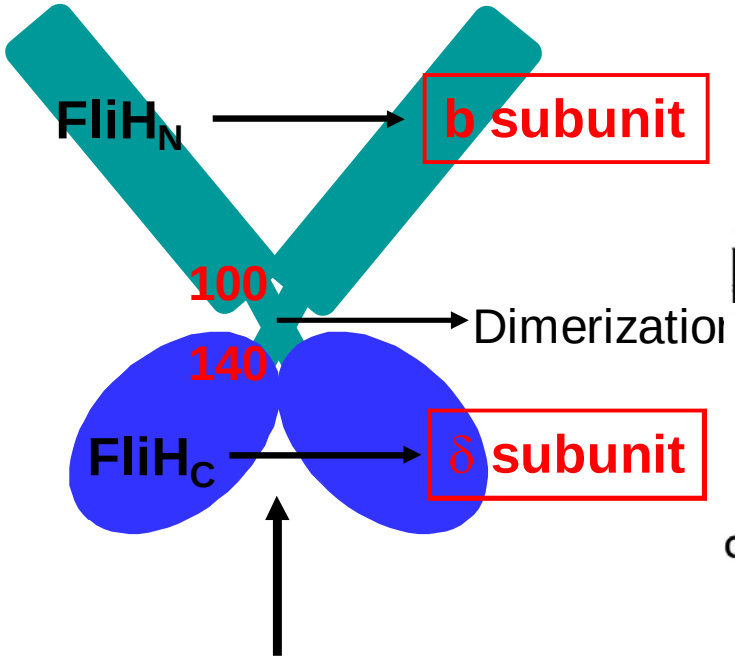
ATPase activity of the FliH/FliI complex

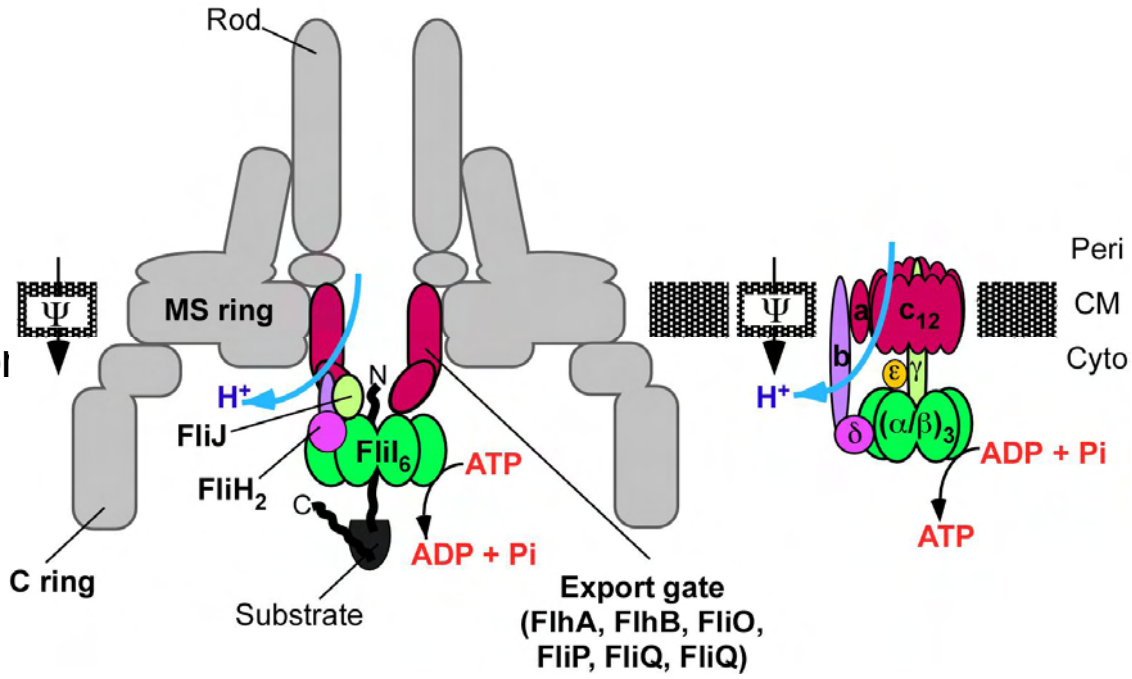




(Minamino & Macnab. *Mol. Microbiol.* 2000b; Okabe, Minamino et al., *FEBS lett*. 2009)

Sequence similarity between FliH and F_0F_1 ATP synthase components





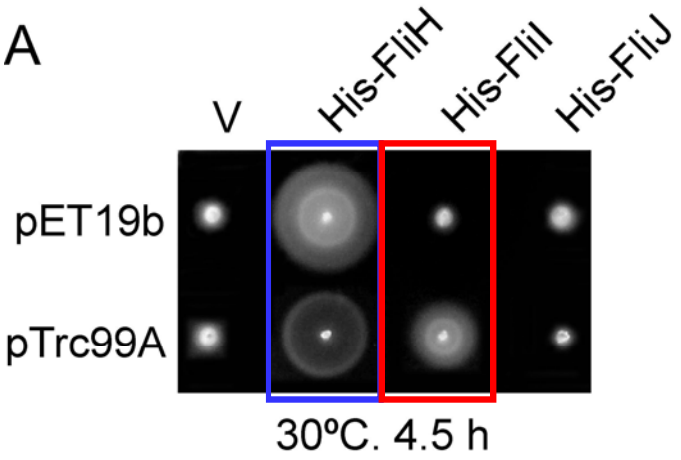
Interaction with FliI ATPase

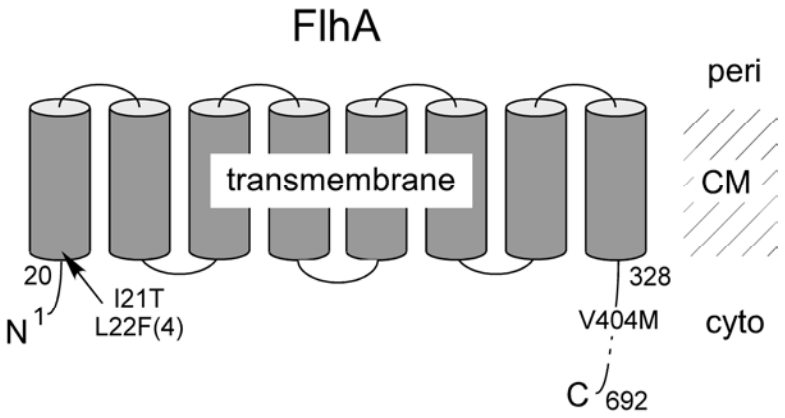
(González-Pedrajo *et al*., *Mol. Microbiol.* 2002; Minamino *et al*., *J. Mol. Biol.* 2002)

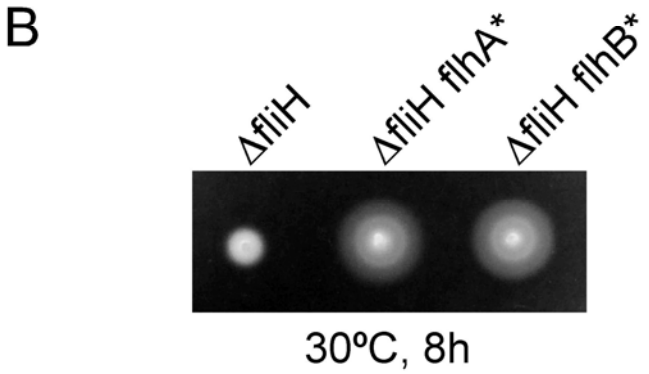
FliH represents a fusion of domains homologous to second stalk proteins of the F_0F_1 syntase (the b and δ subunits) essential for connecting F_1 with F_0

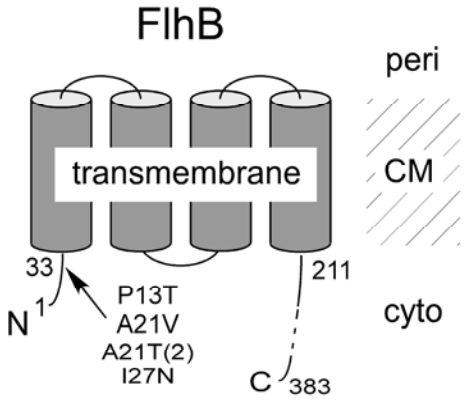
(Pallen *et al*., *Protein Sci*. 2006)

Bypass effects on the FliH defect









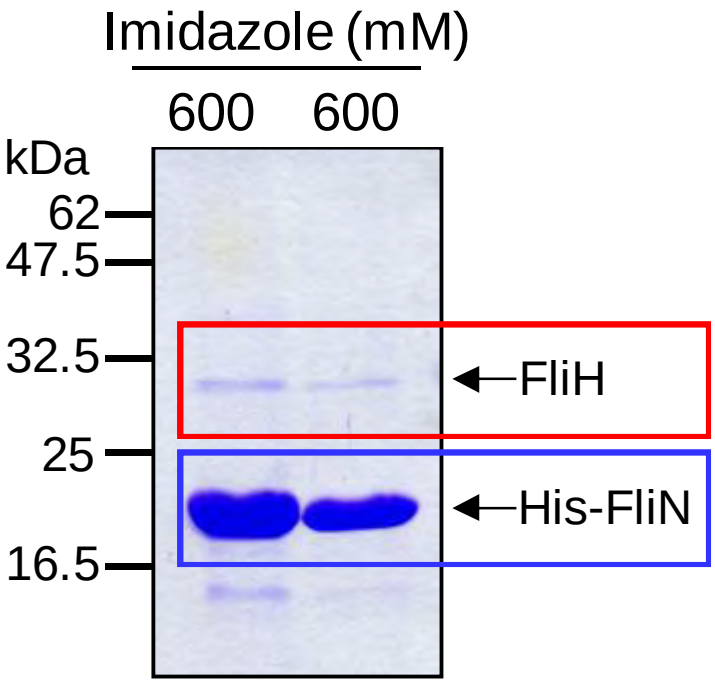
A FliH defect can be bypassed by overproduction of FliI or by a second-site mutation in FlhA or FlhB, integral-membrane components of the export apparatus, suggesting that FliH plays an important role in the effective docking of FliI ATPase to the FlhA-FlhB platform.

(Minamino *et al.*, *J. Bacteriol*. 2003)

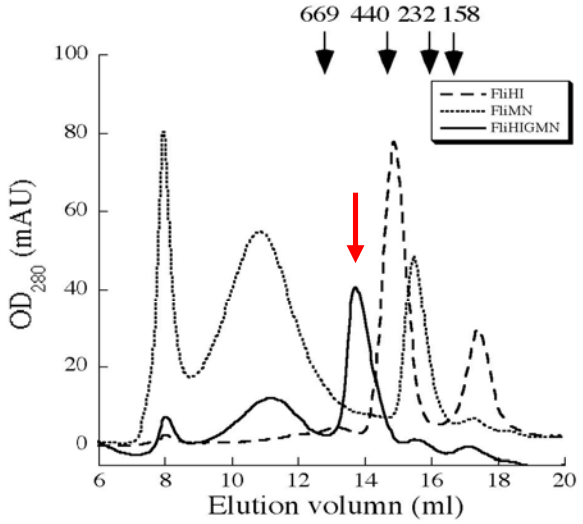
Interaction of FliH and FliN (one of the switch proteins, which participate not only in the motor function but also in the flagellar/assembly export process)

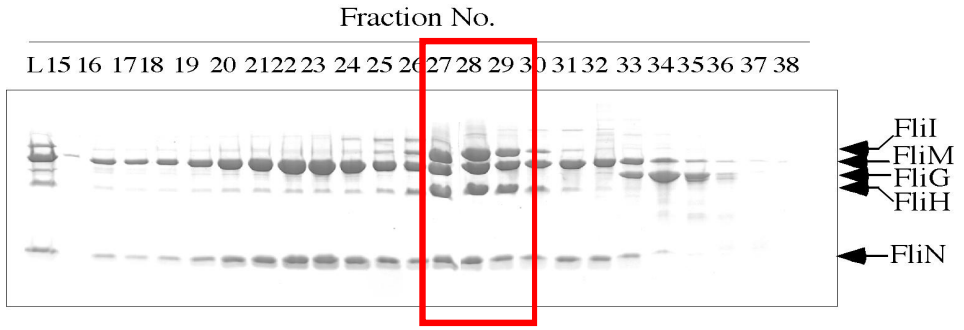
Pull down assays

FliH + His-FliN



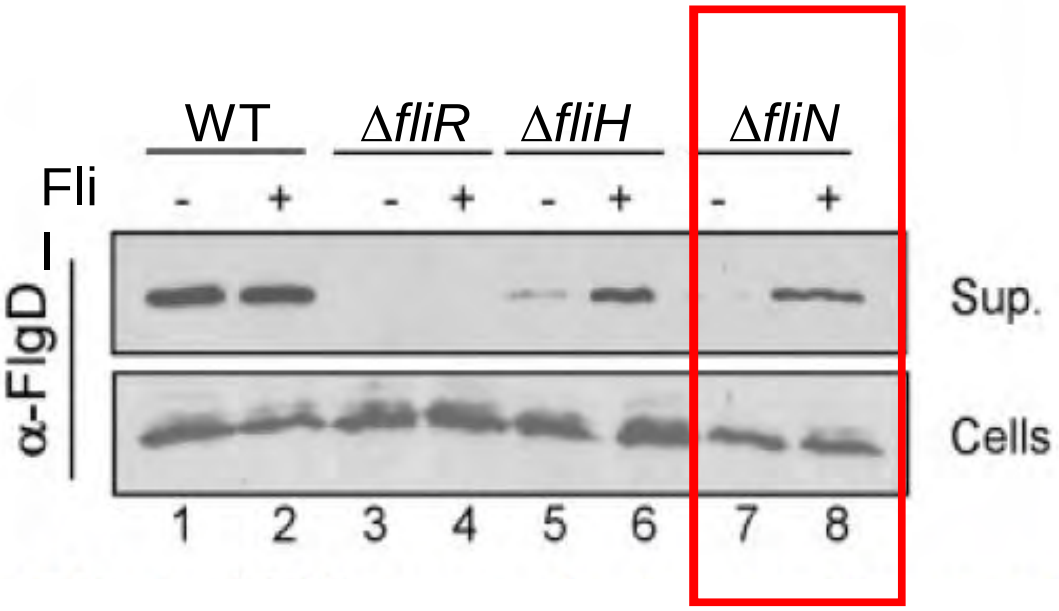
Gel filtration chromatography





(González-Pedrajo, Minamino *et al*., *Mol. Microbiol*. 2006)

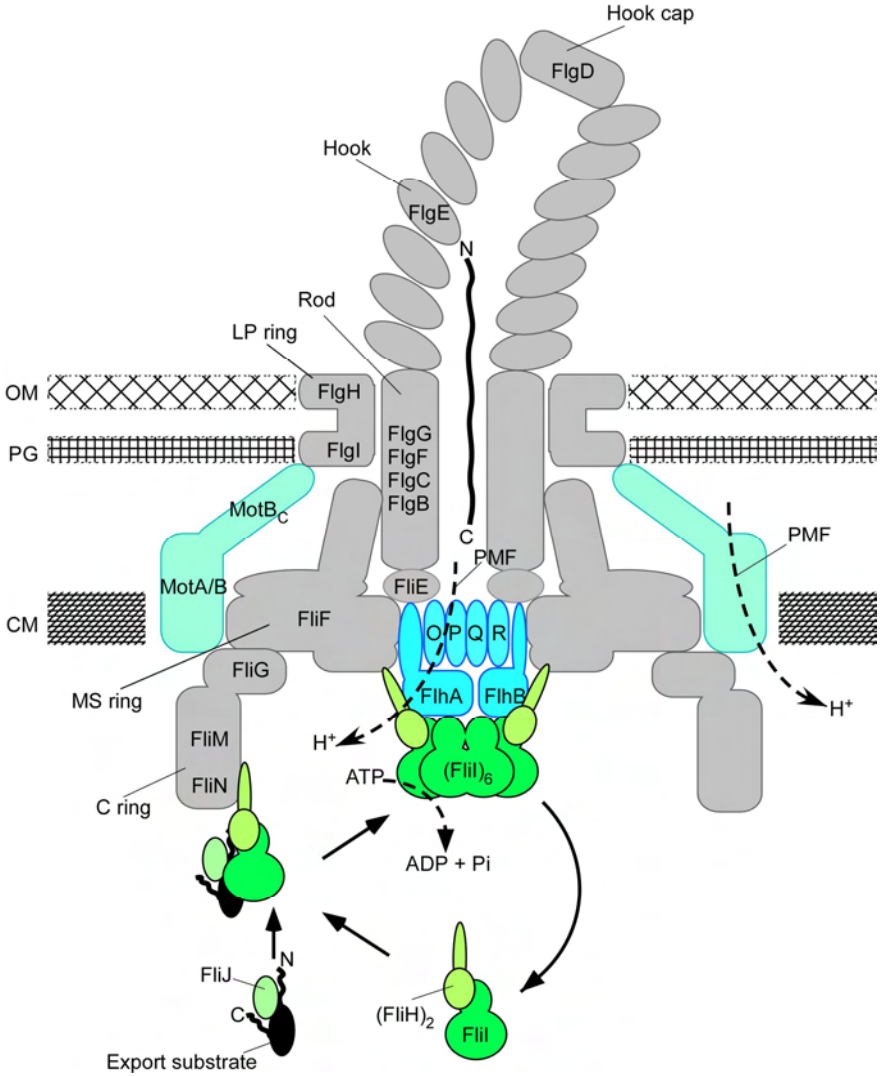
The reduced secretion activity of a *fliN* null mutant is partially recovered by overproduction of FliI ATPase.



The C ring seems to provide docking sites for the FliH-FliI complexes near the export gate so that they can efficiently dock to the FlhA-FlhB platform of the export gate.

(McMurry *et al*., Biochemistry. 2006)

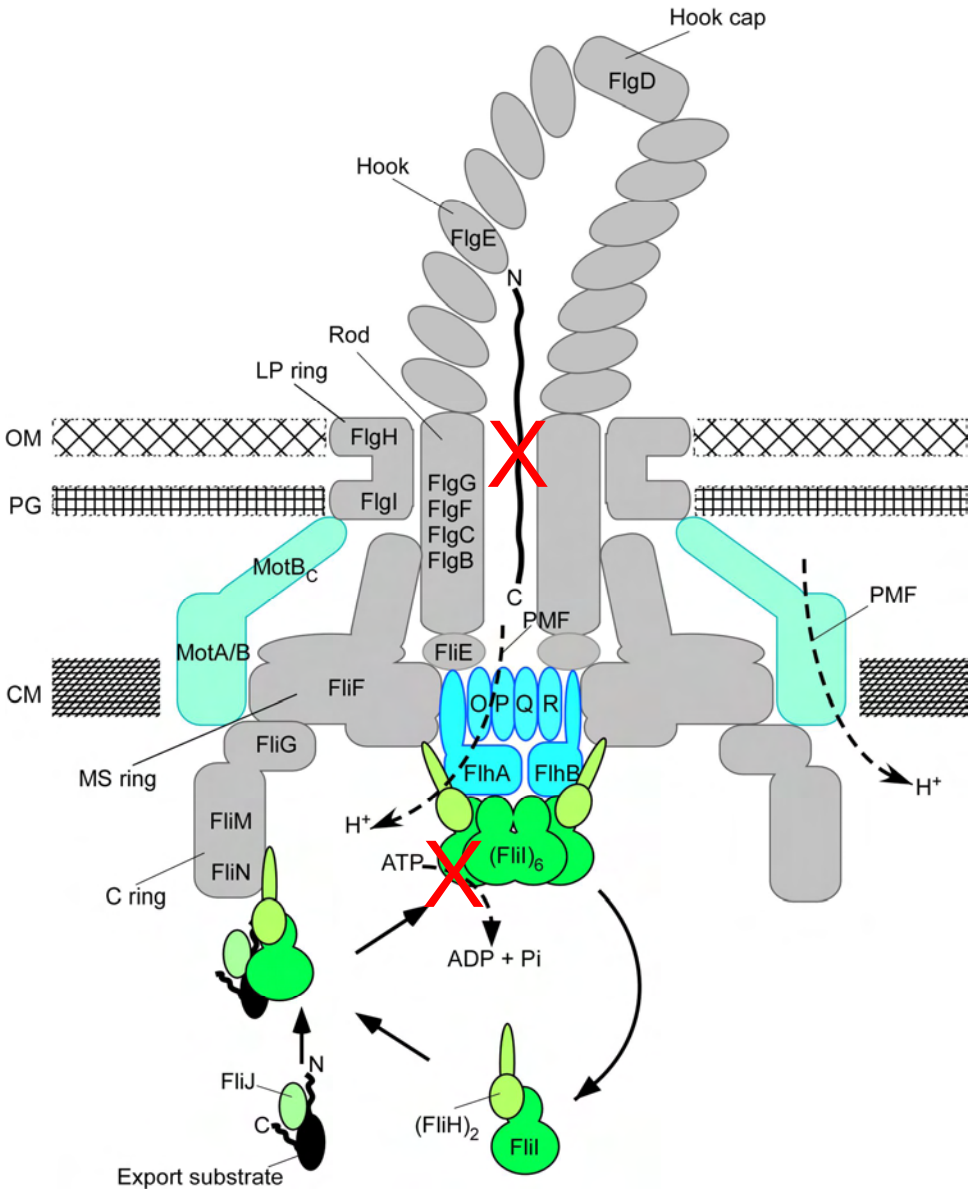
FliH/FliI complex acts as a pilot to deliver export substrate into the export gate



1. The $FliH_2FliI$ complex binds to the chaperone-export substrate complex in the cytoplasm.
2. The $FliH_2FliI$-chaperone-substrate complex docks to the C ring through the FliH-FliN interaction.
3. The $FliH_2FliI$-chaperone-substrate complex can efficiently dock to the platform made of integral membrane export components, where FliI forms the hexamer ring and its specific binding to the FlhA-FlhB platform promotes initial entry of the N-terminal segment of the substrate.

(González-Pedrajo, Minamino *et al*., *Mol. Microbiol*. 2006; Minamino & Macnab, *Mol. Microbiol*. 2000a, b; Minamino *et al*., *J. Bacteriol*. 2003; Minamino *et al*., *J. Mol. Biol*. 2006.)

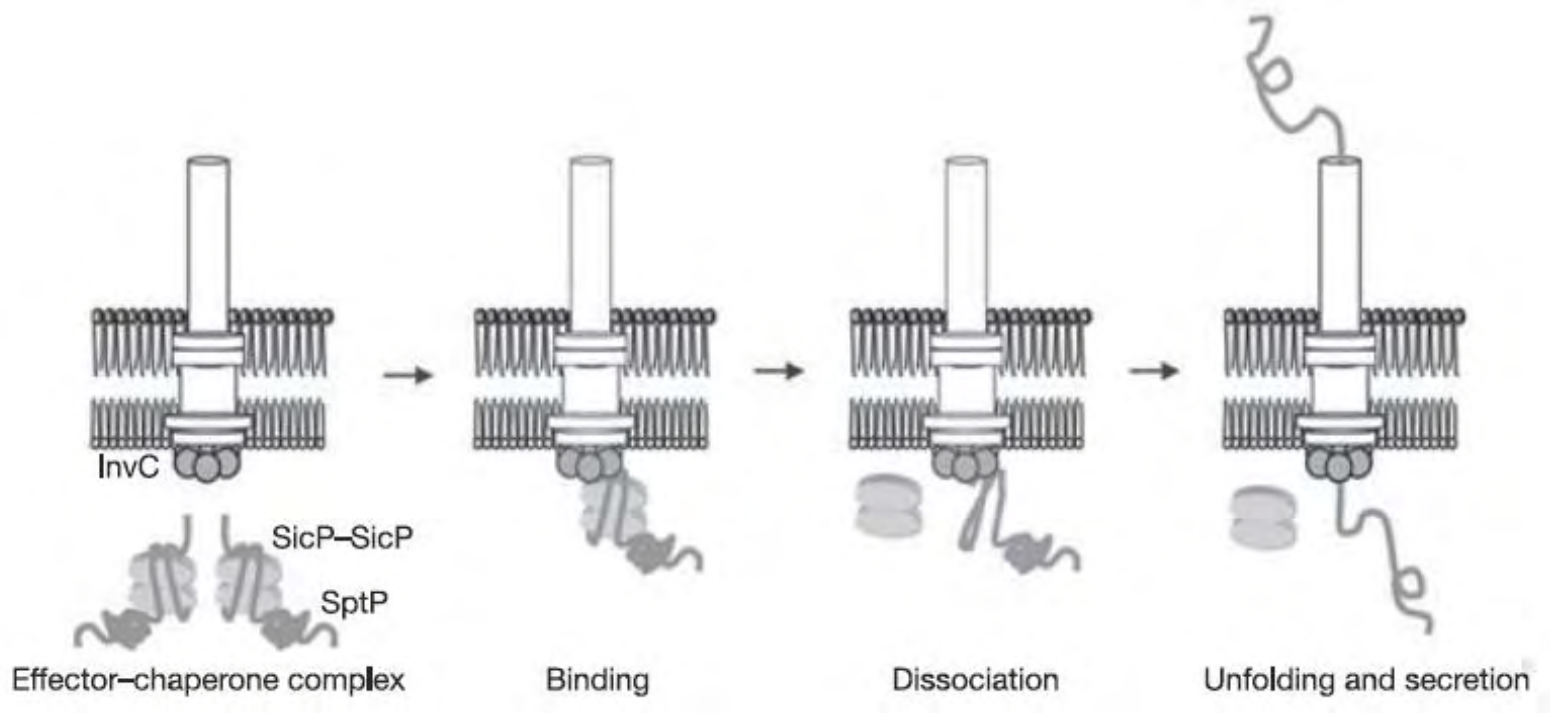
What is energy source for flagellar protein export ?



Translocation of many soluble proteins across cell membranes requires bioenergies such as ATP and proton motive force.

Since *fliI* mutants cannot export any flagellar proteins, FliI has been thought to provide the energy for the translocation of export substrates into the narrow channel of the growing flagellar structure.

Role of *Salmonella* InvC (FliI homolog) in type III secretion of virulence factor



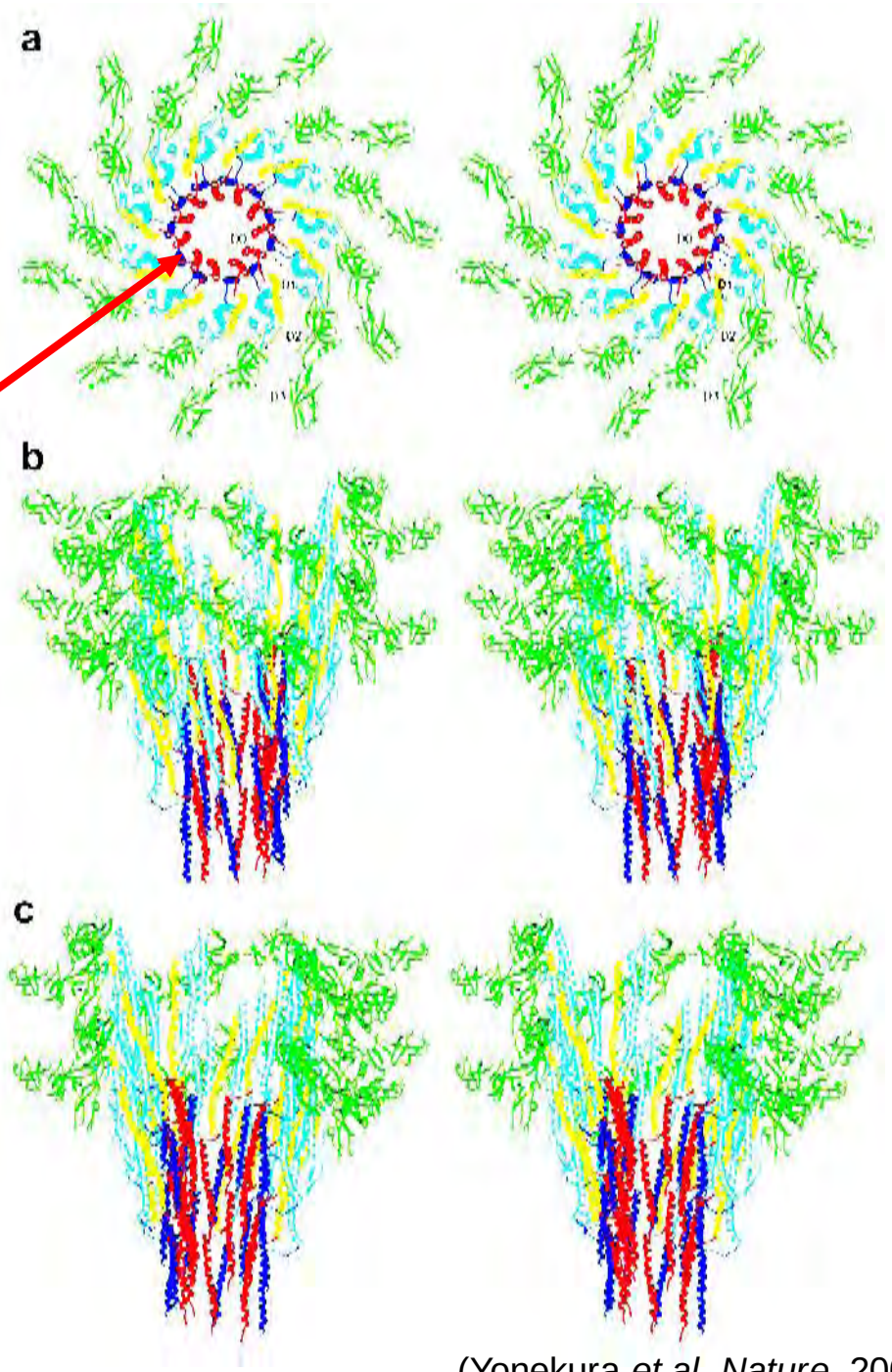
InvC binds to chaperone-effector complexes and acts as an unfoldase to induce chaperone release from and unfolding of the effector to be secreted in an ATPase-dependent manner

(Akeda, Y. & Galán, J. E. *Nature*. 2005)

Atomic model of the flagellar filament structure solved by cryo-EM and helical image analysis

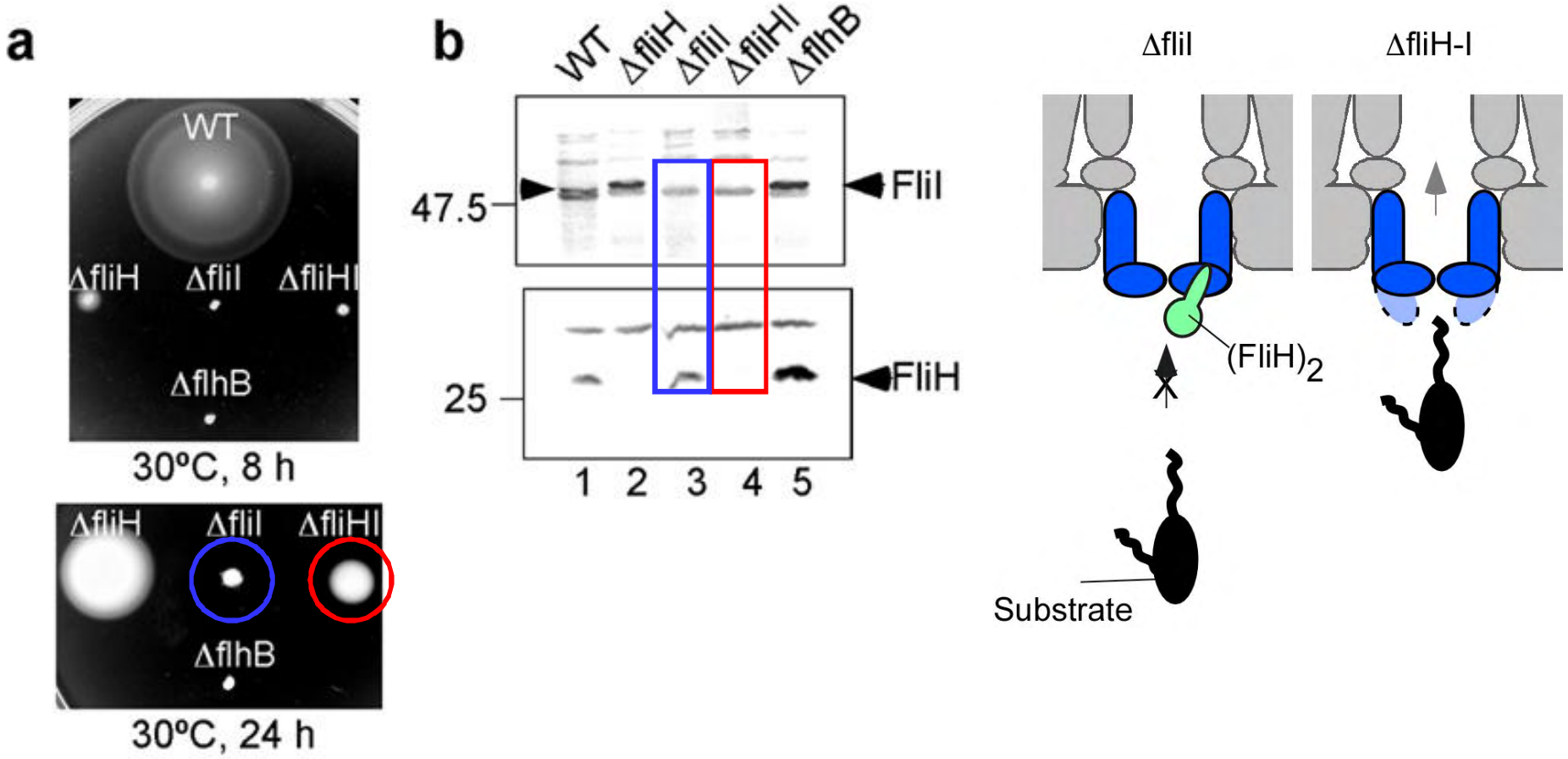
The diameter of the central channel, which is the physical path for flagellar protein export, is only 2 nm.

Does FliI act as an unfoldase to drive flagellar protrein export in an ATP-dependent manner?



(Yonekura *et al*.,*Nature*, 2003)

Motility assays of a *fliH-fliI* double null mutant



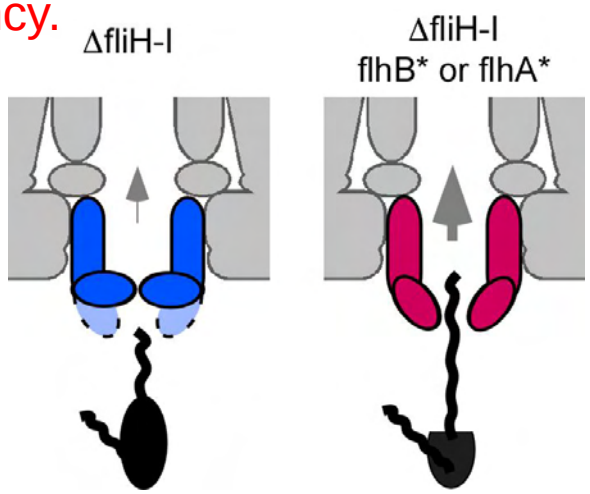
Unlike a *fliI* null mutant, *Salmonella* cells missing both FliH and FliI formed swarms on soft agar plates after prolonged incubation, suggesting that FliI is not absolutely required for flagellar protein export.

(Minamino & Namba, *Nature*. 2008)

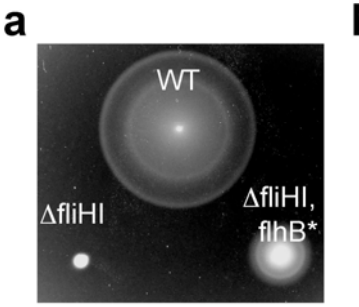
Isolation of pseudorevertants from the Δ*fliH-fliI* mutant.

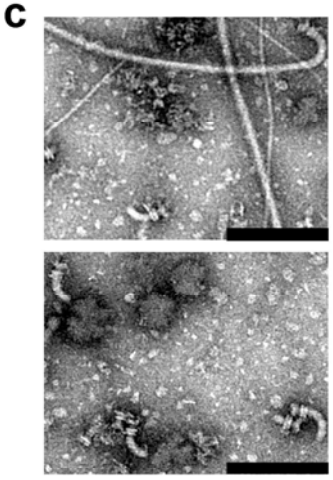
The second-site mutations in FlhB and FlhA substantially improved both flagellar protein export and motility of the *fliH-fliI* double null mutant.

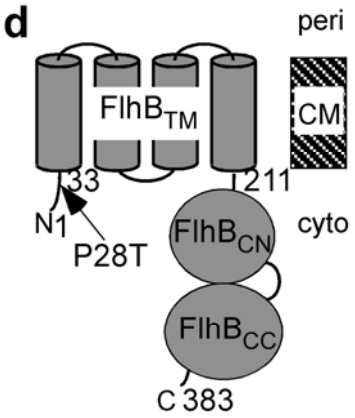
These gain-of-function mutations increase the probability of flagellar protein entry into the export gate, thereby increasing export efficiency.

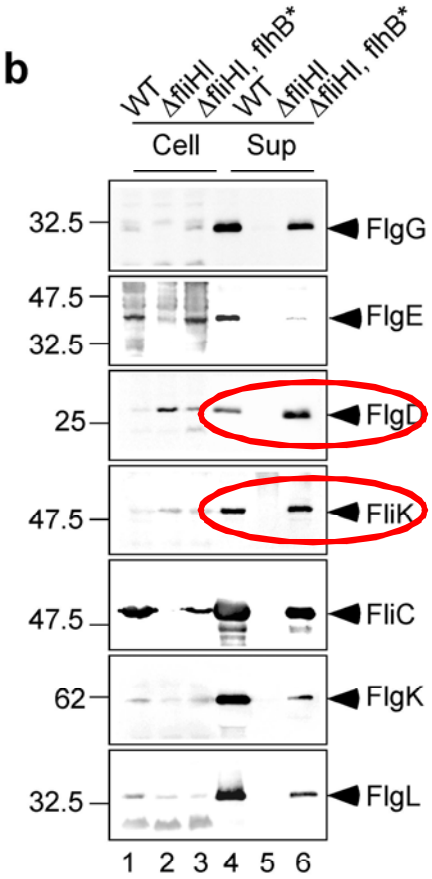


(Minamino & Namba, *Nature*. 2008)



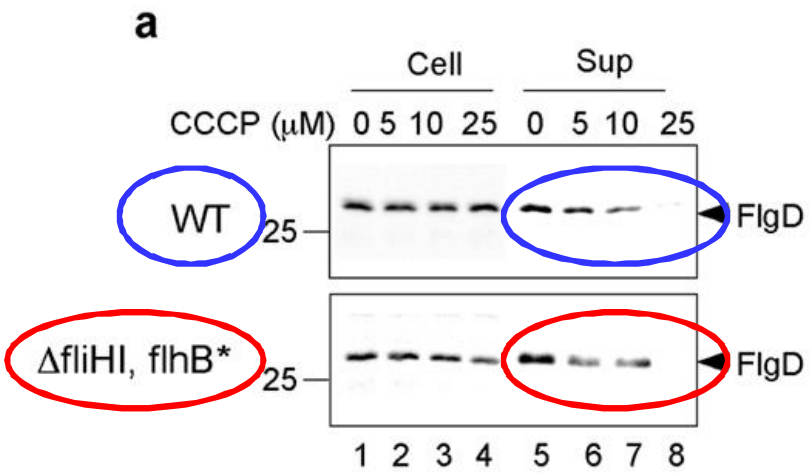




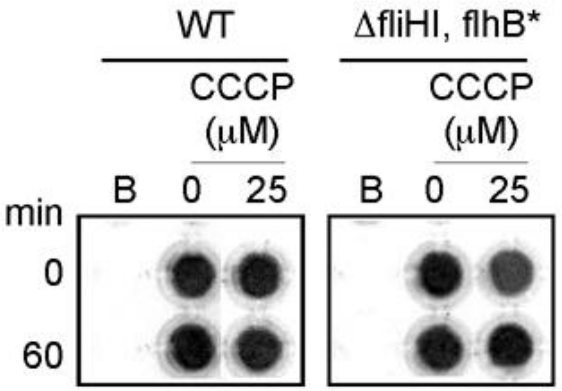


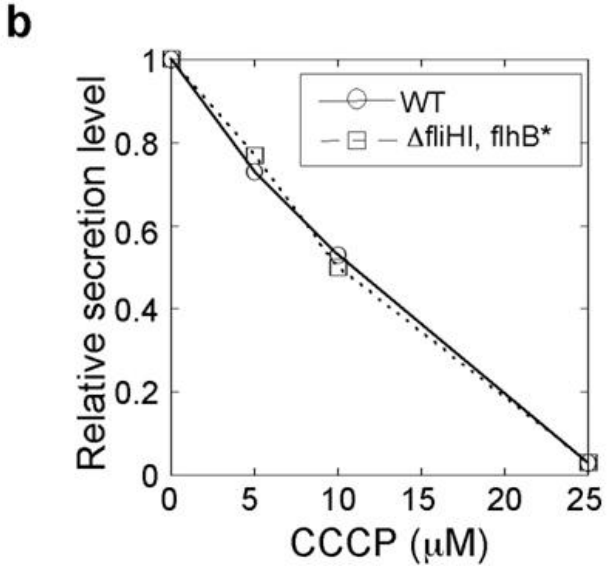
The amounts of FlgD and FliK secreted by the pseudorevertants were even larger than those of wild-type, suggesting that the translocation of export substrate is not powered by the chemical energy derived from ATP hydrolysis by FliI.

Effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on secretion of flagellar proteins



Intracellular ATP level

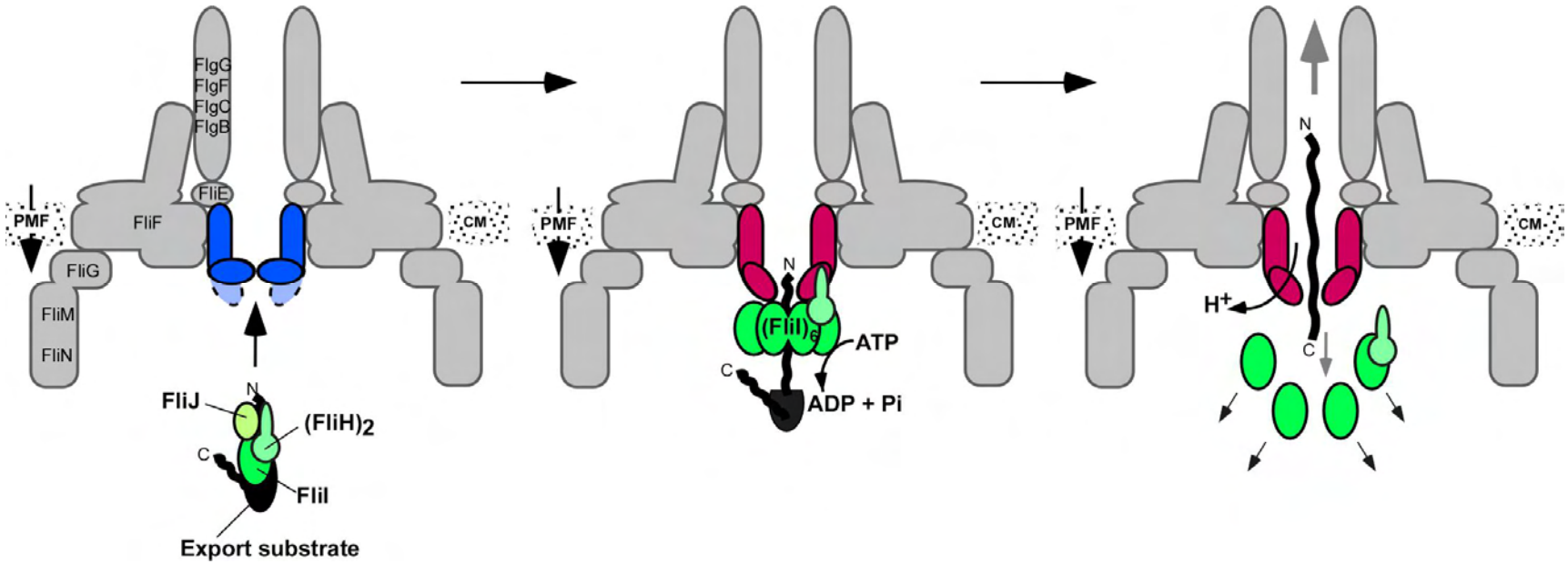




When PMF was gradually collapsed by adding CCCP, the secretion levels of export substrates decreased significantly at the CCCP concentration above 10 μM and diminished at 25 μM although the intracellular levels of export substrates and ATP were maintained. These results indicate that PMF is absolutely essential for the export process regardless of the presence or absence of FliH and FliI.

(Minamino & Namba, *Nature*. 2008)

Distinct roles of FliI ATPase and proton motive force in bacterial flagellar protein export



1. The FliH/I complex facilitates only the initial entry of export substrates into the gate.
2. The rest of the successive unfolding/translocation process of the substrates is driven by proton motive force.
3. The energy of ATP hydrolysis by FliI seems to be used to disassemble and release the FliH/I complex from the export gate and the protein about to be exported.

(Minamino & Namba, *Nature*. 2008)

Similarity between flagellar protein export apparatus and F_0F_1ATPsynthase

1. The entire structure of FliI is almost identical to the α and β subunits.
2. Sequence similarity between FliH and the b/δ subunits.
3. Energy source for both the translocation of flagellar protein and ATP synthesis is proton motive force across the cytoplasmic membrane.

These two remotely related systems may be similar to each other for their entire structural architectures.

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