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Supplemental Information

A Ubiquitous Platform

for Bacterial Nanotube Biogenesis

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Supplemental information

Supplementary Figures

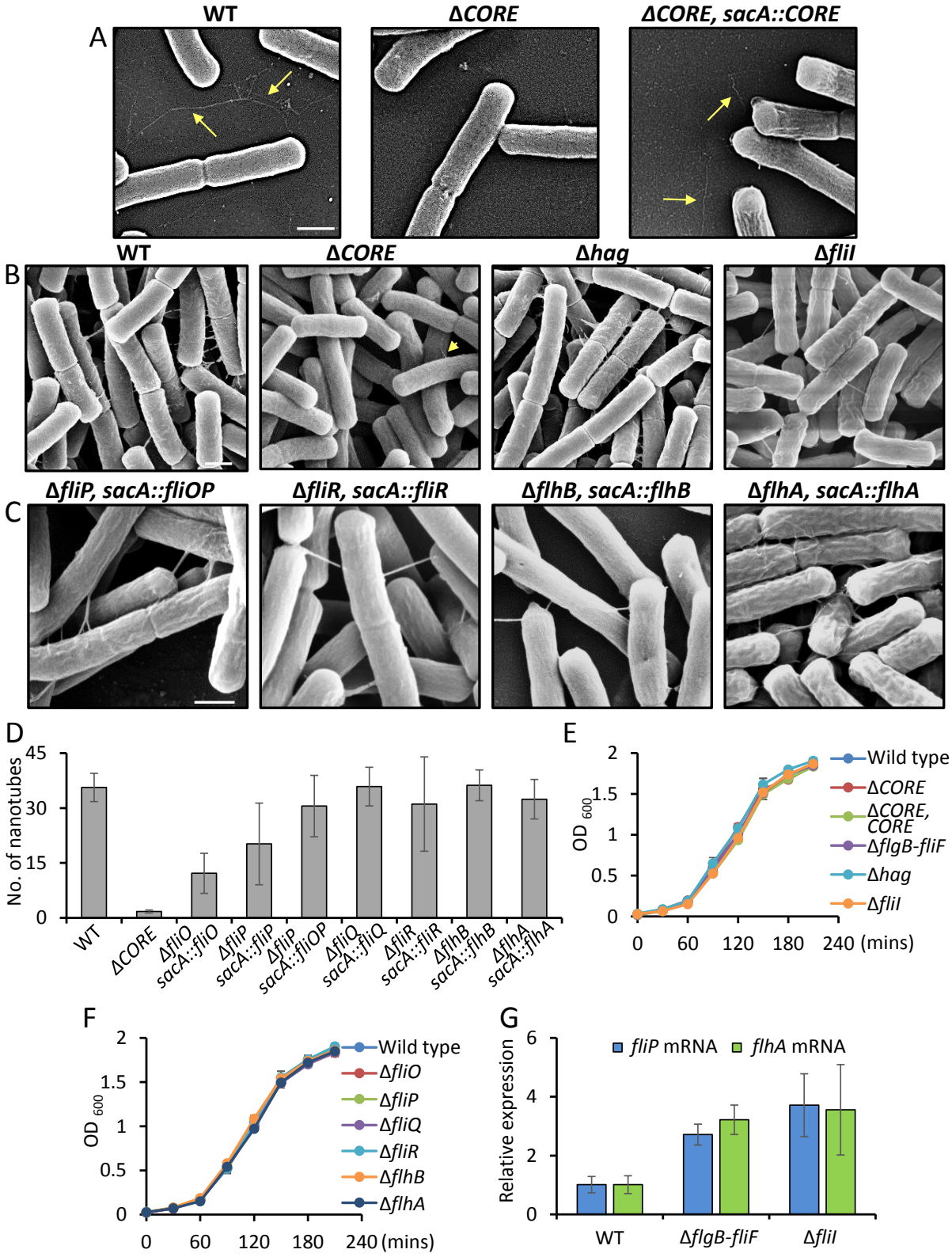

Figure S1: *Bs CORE* mutants are impaired in nanotube formation. Related to Figures 1 and 2
(**A**) PY79 (wild type), SH9 (Δ*CORE*) and SH33 (Δ*CORE*, *sacA::CORE*) strains were visualized by XHR-SEM to monitor the formation of extending nanotubes at low cell density. Strains were grown to mid logarithmic

phase, spotted onto EM grids at low cell density (x0.1 dilution), incubated on LB agar plates for 2 hrs at 37°C, and visualized by XHR-SEM. Arrows indicate extending nanotubes. Scale bar represents 500 nm.
(**B**) The indicated *Bs* mutant strains were visualized by XHR-SEM to monitor the formation of intercellular nanotubes. Strains were grown to mid logarithmic phase, spotted onto EM grids, incubated on LB agar plates for 4 hrs at 37°C, and visualized by XHR-SEM. PY79 (wild type) and SH9 (Δ*CORE*) are larger fields of the images displayed in Figure 1C. Scale bar represents 500 nm.
(**C**) *Bs CORE* mutants were complemented with the corresponding *CORE* genes, as indicated, and were visualized by XHR-SEM to monitor the formation of intercellular nanotubes. Cells were processed for XHR-SEM as in (B). Shown are representative examples of the indicated complemented strains. Scale bar represents 500 nm.
(**D**) Quantification of the average number of nanotubes displayed per 50 cells by the indicated strains following XHR-SEM analysis described in (C). Shown are average values and SD of at least 3 independent experiments ($n \geq 200$ for each strain).
(**E-F**) Growth kinetics of *Bs CORE* mutants. The indicated mutant strains were incubated in LB medium at 37°C and cell growth was followed by measuring OD_{600} at the indicated time points.
(**G**) Expression of *CORE* genes in *Bs* non-*CORE* flagellar basal body mutants. RNA was isolated from *Bs* wild type (PY79), Δ*flgB-fliF* (SH8) and Δ*fliI* (SH177) cells grown to the mid logarithmic phase and the expression of *fliP and flhA* was determined by qRT-PCR. Transcript levels are relative to wild type (PY79). Each bar represents an average value and SD of three independent experiments.

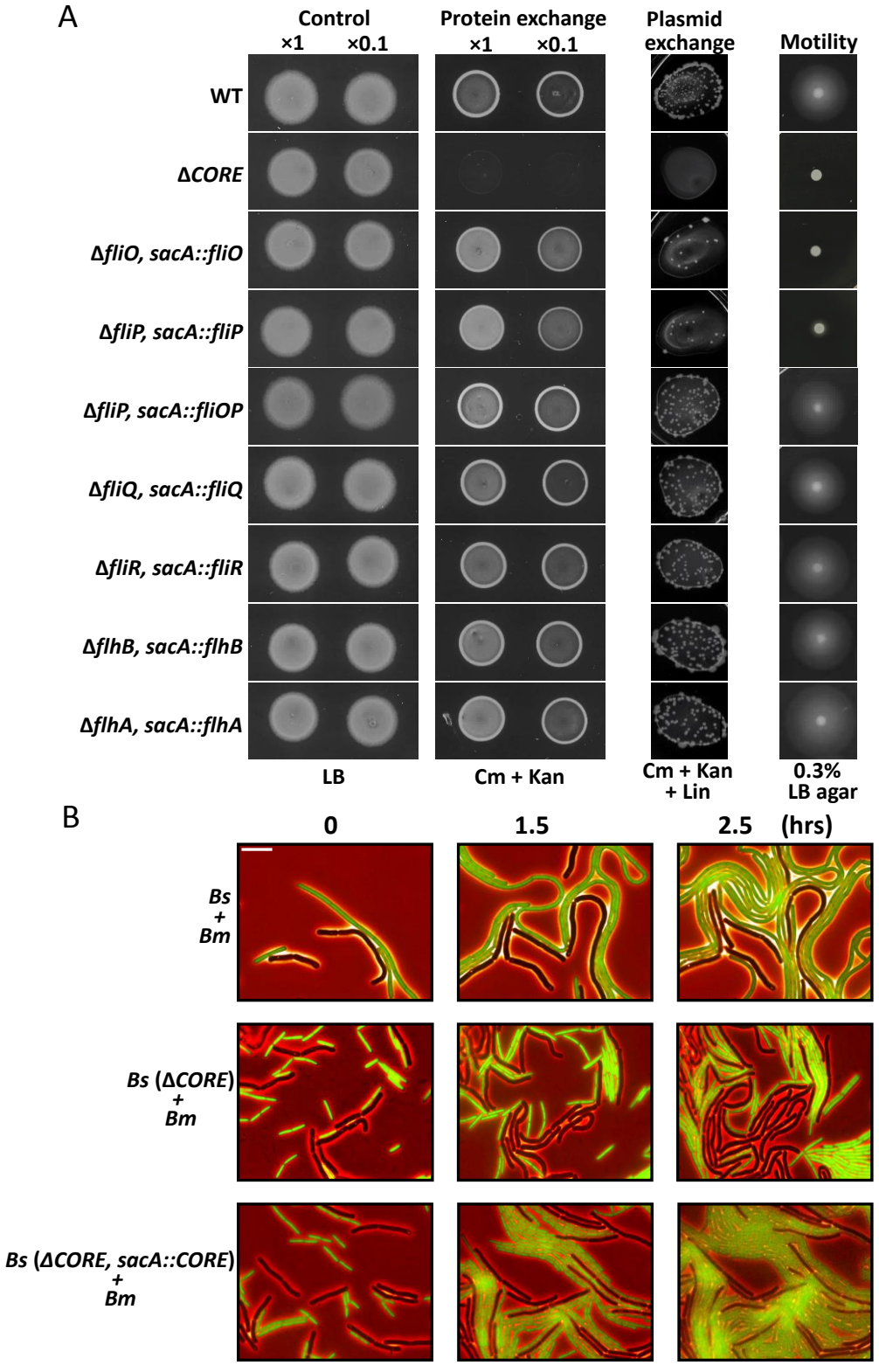

Figure S2: *Bs CORE* mutants are impaired in nanotube mediated molecular exchange. Related to Figure 2

(**A**) Assessing molecular exchange in *Bs CORE* complemented strains. For protein exchange assay, pairs of a donor (SB463: *amyE*::$P_{hyper\text{-}spank}$-*cat-spec*) (Cm^R, $Spec^R$) and a recipient (SB513: *amyE*::$P_{hyper\text{-}spank}$-*gfp-kan*) (Kan^R) parental strains (wild type) were used. The investigated complemented strains harbor the corresponding genotypes of both donor and recipient strains. Donor and recipient strains were mixed in 1:1 ratio (at two concentrations x1, x0.1) and incubated in LB supplemented with 1 mM IPTG for 4 hrs at 37°C with gentle

shaking. Equal numbers of cells were then spotted onto LB agar (Control) and LB agar containing chloramphenicol (Cm) and kanamycin (Kan) (Protein exchange), and photographed after 18 hrs. For plasmid exchange assay, pairs of a donor (GD110: *amyE*::$P_{hyper\text{-}spank}$-*cat-spec*, pHB201/*cat, erm*) (Cm^R, $Spec^R$, Mls^R) and a recipient (SB513: *amyE*::$P_{hyper\text{-}spank}$-*gfp-kan*) (Kan^R) parental strains (wild type) were used. The investigated complemented strains harbor the corresponding genotypes of both donor and recipient strains. Cells were mixed in 1:1 ratio (concentration x1), processed as described for protein exchange, and spotted onto LB agar containing Cm, Kan and lincomycin (Lin) (Plasmid exchange). Cells were incubated at 37ºC and colonies were photographed after 36 hrs of incubation. For motility assay, wild type (PY79) and the indicated strains were grown to the mid logarithmic phase and spotted onto LB plates containing 0.3% agar and photographed after 7 hrs of incubation at 37ºC (Motility).

(**B**) *Bm* inhibition by *Bs* is CORE dependent. Representative time lapse microscopy images displaying mixtures of *Bs* (AR16: *amyE*::P_{rrnE}-*gfp*) and *Bm* (OS2) (upper panels), *Bs* (SH30: Δ*CORE*, *amyE*::P_{rrnE}-*gfp*) and *Bm* (OS2) (middle panels), and *Bs* (SH41: Δ*CORE, sacA::CORE, amyE*::$P_{hyper\text{-}spank}$-*gfp*) and *Bm* (OS2) (lower panels). Shown are overlay of fluorescence from GFP (green) and phase contrast (red) images, captured at the indicated time points. *Bs* cells are shown in green while *Bm* cells are shown in black. Scale bar represents 5 μm.

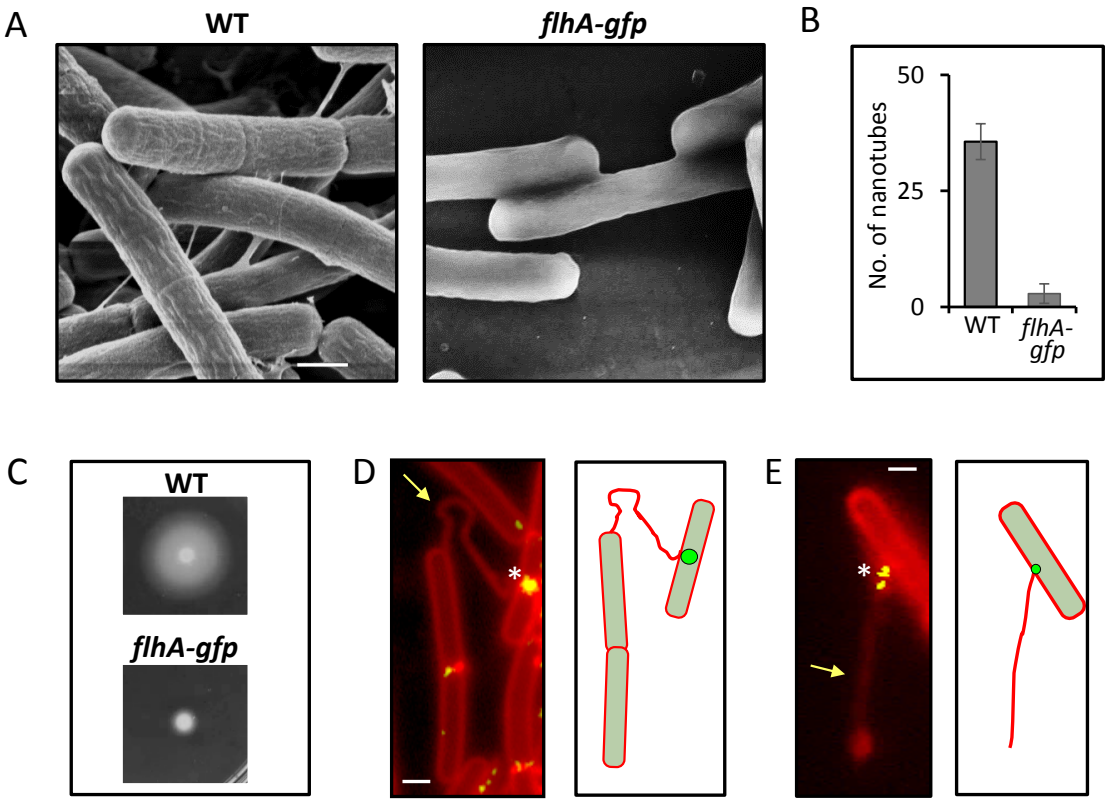

Figure S3: FlhA localizes to the base of nanotubes. Related to Figure 3
(**A**) WT (PY79) and SH47 (*flhA-gfp*) strains were visualized by XHR-SEM to monitor the formation of intercellular nanotubes. Strains were grown to mid logarithmic phase, spotted onto EM grids, incubated on LB agar plates for 4 hrs at 37°C, and visualized by XHR-SEM. Scale bar represents 0.5 µm.
(**B**) Quantification of the average number of nanotubes displayed per 50 cells by the indicated strains following XHR-SEM analysis described in (A). Shown are average values and SD of at least 3 independent experiments (n≥70 for each strain).
(**C**) Motility assay for PY79 (wild type) and SH47 (*flhA-gfp*) strains. Cells were grown to mid logarithmic phase, spotted onto LB plates containing 0.3% agar, and photographed after 7 hrs of incubation at 37ºC.
(**D-E**) SH55 (*flhA-gfp*, *amyE*::$P_{hyper\text{-}spank}$-*ymdB*, Δ*ymdB*, Δ*hag*) cells were grown in liquid LB to mid logarithmic phase, stained with a membrane dye (FM 4-64) and visualized by fluorescence microscopy. Shown are overlays of signals from FlhA-GFP (green) and FM 4-64 (red). Arrows denote nanotubes, and asterisks highlight FlhA foci at sites proximal to nanotube emanation. Schematics depict cells and nanotubes layouts (red), as well as FlhA-GFP signal (green) at sites of nanotube origin. Scale bars represent 0.5 µm.

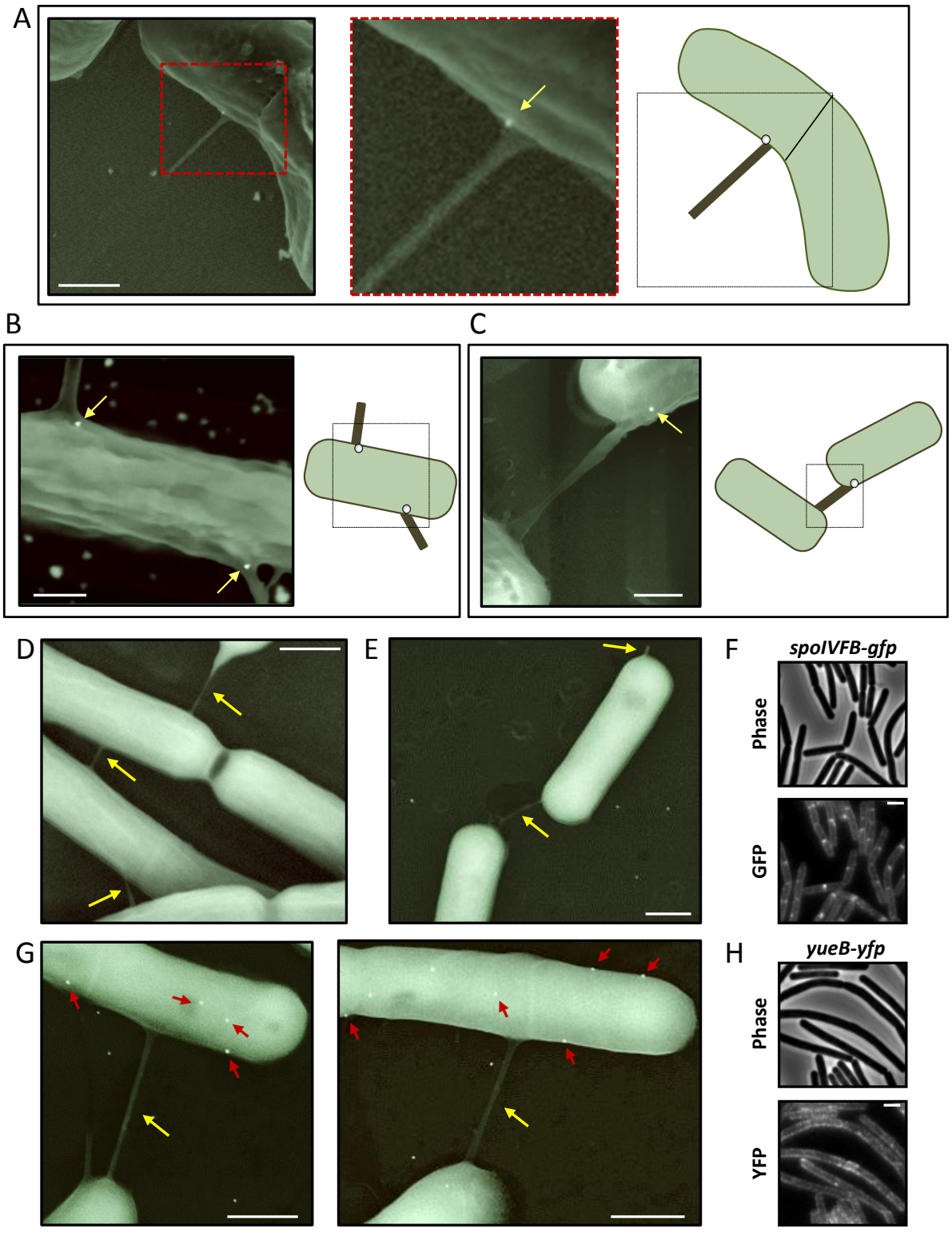

Figure S4: FliP localizes to sites of nanotube emanation. Related to Figure 3
(**A**) Cells expressing HA tagged FliP (SH110: *amyE*::P$_{hyper\text{-}spank}$-*fliP*$_{2xHA}$, *sacA*::P$_{hyper\text{-}spank}$-*ymdB, Δmbl*) were spotted onto EM grids and subjected to immuno-gold XHR-SEM using primary antibodies against HA and secondary gold-conjugated antibodies. Samples were not coated before observation. Shown are overlays of XHR-SEM images that were acquired using TLD-SE (Through lens detector- secondary electron) for nanotube visualization and vCD (low-kV high-contrast detector) for gold particle detection. An example of FliP$_{2xHA}$

localization (white dot), at the site of emergence of an extending nanotube is displayed. Right panel is a magnification of the red inset in the left panel. Scale bar represents 250 nm.
(**B**) An example of $FliP_{2xHA\text{-}LOOP}$ localization (white dots), at the sites of nanotube emergence. SH161 (*amyE*::$P_{hyper\text{-}spank}$-*fliP*$_{2xHA\text{-}LOOP}$-*spec*, *sacA*::$P_{hyper\text{-}spank}$-*ymdB-kan*, *Δmbl::erm*) cells were processed for immuno XHR-SEM as in (A). Shown is an overlay image of TLD-SE and vCD modes. Scale bar represents 250 nm.
(**C**) An example of localization of $FliP_{2xHA}$ (white dot) expressed as a sole copy, at the site of nanotube emergence. SH247 (Δ*fliP::tet*, $P_{fla/che}$-*fliQ-cheD*, *sacA::*$P_{fla/che}$-*fliO-fliP*$_{2xHA}$-*spec, Δmbl::erm*) cells were processed for immuno XHR-SEM as in (A). Shown is an overlay image of TLD-SE and vCD modes. Scale bar represents 250 nm.
Schematics in (A-C) depict the interpretive cell layout and highlights the nanotube region with gold signal (black dashed box) captured by XHR-SEM. Arrows indicate gold signal from HA-tagged FliP.
(**D**) GB168 (*ΔymdB, Δhag, amyE*::$P_{hyper\text{-}spank}$-*ymdB*) cells, lacking HA tag, were processed for immuno XHR-SEM as in (A). Shown is an example of an overlay of XHR-SEM images that were acquired using TLD-SE and vCD modes. No gold signal was obtained from cell surface and/or nanotubes. Arrows indicate nanotubes. Scale bar represents 500 nm.
(**E**) SH258 (*amyE*::P_{xylA}-*spoIVFB-gfp-cat*, *sacA*::$P_{hyper\text{-}spank}$-*ymdB-kan, Δmbl::erm*) cells, were spotted onto EM grids and subjected to immuno-gold XHR-SEM using primary antibodies against GFP and secondary gold-conjugated antibodies. Samples were not coated before observation. Shown is an example of an overlay of XHR-SEM images that were acquired using TLD-SE and vCD modes. No gold signal was obtained from cell surface and/or nanotubes. Arrows indicate nanotubes. Scale bar represents 500 nm.
(**F**) BDR524 (*amyE*::P_{xylA}-*spoIVFB-gfp-cat*) cells were grown in liquid LB to mid logarithmic phase and visualized by fluorescence microscopy. Shown are images from phase contrast and GFP fluorescence. SpoIVFB-GFP protein localizes to the cell membrane facing the cytoplasm. Scale bar represents 1 μm.
(**G**) SH257 (*amyE*::$P_{hyper\text{-}spank}$-*yueB-yfp-spec*, *sacA*::$P_{hyper\text{-}spank}$-*ymdB-kan, Δmbl::erm*) cells, were processed for immuno XHR-SEM as in (E). Shown are examples of overlays of XHR-SEM images that were acquired using TLD-SE and vCD. Yellow arrows indicate nanotubes. Red arrows indicate signal from YueB-YFP. Scale bar represents 500 nm.
(**H**) ET13 (*amyE*::$P_{hyper\text{-}spank}$-*yueB-yfp-spec*) cells were grown in liquid LB to mid logarithmic phase and visualized by fluorescence microscopy. Shown are images from phase contrast and YFP fluorescence. YueB-YFP receptor protein localizes to the cell circumference. Scale bar represents 1 μm.

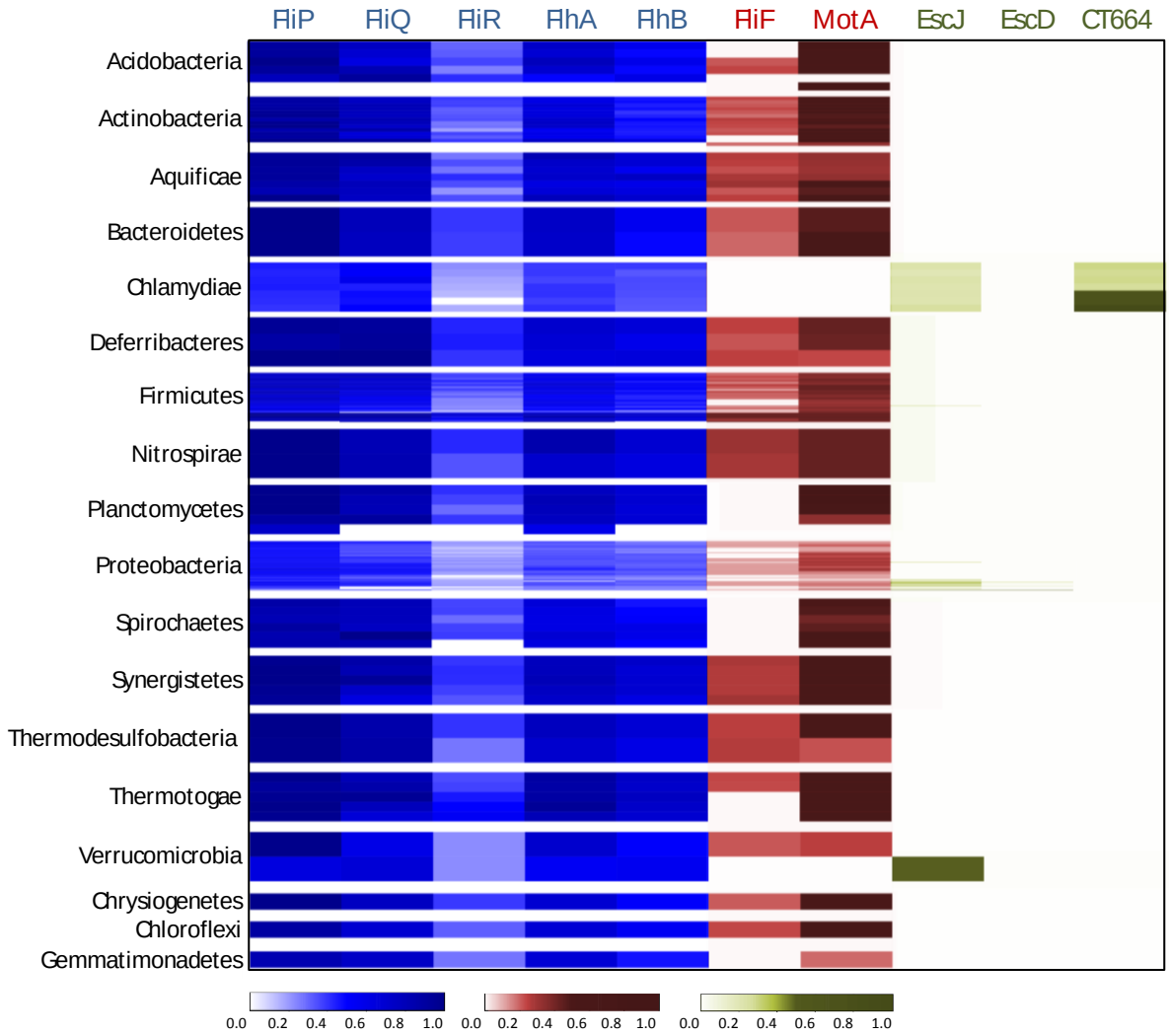

Figure S5: The spread of CORE complex in diverse bacterial phyla. Related to Figure 4
Heatmap showing the conservation of proteins comprising flagella or injectisome in different bacterial phyla. Each row depicts a single species from the STRING database representative genomes, which is included in the respective phylum listed on the left. Each column represents a single protein (blue: CORE proteins; red: exclusive proteins of the flagella apparatus; green: proteins that are unique to the injectisome apparatus). The proteins used as a reference derived from *Bs* flagella (blue and red proteins), from Enterohemorrhagic *E. coli* injectisome (EscJ,D) and from *C. trachomatis* injectisome (CT664). The sequence similarity scores are based on homology data extracted from the STRING database. Color ranges at the bottom represent the STRING sequence similarity scores. See also Table S2 for detailed analysis.

Table S1: Conservation of CORE proteins in distinct bacterial species. Related to Figure 4
Conservation analysis of CORE proteins in *Bm* (DSM319), *Lm* (10403S) and *Ec* (K12 MG1655) by NCBI Protein BLAST, using the corresponding *Bs* PY79 CORE protein sequences as query.

		Bm	***Lm***	***Ec***
FliO	Query Coverage (%)	82	-	10
	Identities (%)	36	-	36
	Positives (%)	62	-	48
	E- value	$7e^{-32}$	-	1.2
FliP	Query Coverage (%)	100	95	95
	Identities (%)	79	34	54
	Positives (%)	92	62	72
	E- value	$2e^{-130}$	$3e^{-45}$	$2e^{-76}$
FliQ	Query Coverage (%)	100	50	100
	Identities (%)	72	44	51
	Positives (%)	88	62	69
	E- value	$1e^{-38}$	$5e^{-08}$	$4e^{-14}$
FliR	Query Coverage (%)	99	91	88
	Identities (%)	55	24	28
	Positives (%)	75	49	50
	E- value	$5e^{-86}$	$1e^{-16}$	$1e^{-16}$
FlhB	Query Coverage (%)	99	93	95
	Identities (%)	59	34	39
	Positives (%)	76	59	62
	E- value	$7e^{-149}$	$3e^{-62}$	$3e^{-73}$
FlhA	Query Coverage (%)	100	99	97
	Identities (%)	97	36	45
	Positives (%)	86	59	64
	E- value	0	$3e^{-128}$	$3e^{-175}$

Table S2: Table showing the conservation of proteins comprising different flagella or type III secretion system (T3SS) subunits in different bacterial species. Related to Figure 4

(Provided as a separate Excel file)

Each row represents a single species from the STRING core genomes and each column represents one protein. The protein names are followed by the species of origin (Bacillus: *B. subtilis* proteins; EHEC: Enterohemorrhagic *E. coli* proteins; Chlamydia: *C. trachomatis* protein). The sequence similarity scores are based on the STRING database homology data. Species lacking any homolog were excluded from the analysis.

Table S3. List of primers used in this study. Related to STAR Methods

Primer name	**Primer sequence (5'-3')**
P_{flgB}1	GTTGACCAGTGCTCCCTGCAATAAGCTTTATTTCCTGGGTTG
P_{flgB}2	GCTCAAATCCACTTACCTCCA
flgB-fliF KO P1	CCAGCAAATTGAAAATGATCG
flgB-fliF KO P2	CTGAGCGAGGGAGCAGAACAAATCCACTTACCTCCATTTCA
flgB-fliF KO P3	TGGAGGTAAGTGGATTTGAGCATGGCGAGACGTGATCAAGATA
flgB-fliF KO P4	ATATCATCCCCTCCGCCTCT
CORE KO P1	TGATGATGAAATGCTGGTGAA
CORE KO P2	CTGAGCGAGGGAGCAGAATTTCAACAGTCGTACACCCTTT
CORE KO P3	TGGAGGTAAGTGGATTTGAGCATGAAAATAAAAAAATTTACTGCTGCT
CORE KO P4	CACATTCTGGCCGTTAGTCA
sacA P1	CAGCATTTTCCGCTTTTCTC
sacA P2	AGCCTGCCCTTTCAAATTCT
sacA P3	GTTGACCAGTGCTCCCTGTGTTTTCCCTTCAAGGATCG
sacA P4	TTTTCCAAACATTCCGGTGT
Comp P_{flgB}1	AGAATTTGAAAGGGCAGGCTCAATAAGCTTTATTTCCTGGGTTG
Comp P_{flgB}2	CAATAAAATATTGACTCTTTTTCATGCTCAAATCCACTTACCTCCA
Comp P_{flgB}2a	GCACAGTAGCATGGTTATTCATGCTCAAATCCACTTACCTCCA
Comp P_{flgB}R	GCTCAAATCCACTTACCTCCA
Bs CORE comp1	ATGAAAAAGAGTCAATATTTTATTG
Bs CORE comp2	CTGAGCGAGGGAGCAGAATTAAATATCCACCACTCCAATG
Comp P_{HS}1	AGAATTTGAAAGGGCAGGCTCTCGAGGGTAAATGTGAGCA
Comp P_{HS}2	CAATAAAATATTGACTCTTTTTCATCATAGTAGTTCACCACCTTTAAGC
ymdB R	CTGAGCGAGGGAGCAGAACTATTCAAAGAACATGTGATCATCG
fliO KO P1	TGATGATGAAATGCTGGTGAA
fliO KO P2	CTGAGCGAGGGAGCAGAATTTCAACAGTCGTACACCCTTT
fliO KO P3	TGGAGGTAAGTGGATTTGAGCATGAATGAGTTTATAAATATTTTCAGTTC
fliO KO P4	AAAAAGCAGGAAATAAGTCAATAA
fliP KO P1	GCAGCATTTATGCGAATGAT
fliP KO P2	CTGAGCGAGGGAGCAGAAGATGACGTGGGCCTTTCTT
fliP KO P3	TGGAGGTAAGTGGATTTGAGCGTGAGTTCAGAATTTGTAATTTCTATGG
fliP KO P4	AAAACATTCCGGACAACGAC
fliQ KO P1	AATATTGGCGGCACATCG
fliQ KO P2	CTGAGCGAGGGAGCAGAAAACTCACGTTTAGCACCTACCC
fliQ KO P3	TGGAGGTAAGTGGATTTGAGCATGAATTCAATTATTGACTTATTTCCTGC
fliQ KO P4	TCATTGTGAGCGATTCGGTA
fliR KO P1	AACAAAACCGTTCTGAAGGAAA
fliR KO P2	CTGAGCGAGGGAGCAGAATTCATCTCTACATTACCCTGCAAA
fliR KO P3	TGGAGGTAAGTGGATTTGAGCATGAAGCTTAGAGTTGACCTGCAG
fliR KO P4	GCCTGGTCAATTTCCACTTG
flhB KO P1	CGGGCCATTATTGGCTATT
flhB KO P2	CTGAGCGAGGGAGCAGAACTTCATTAAGAAACACCGACCA
flhB KO P3	TGGAGGTAAGTGGATTTGAGCATGTCAACAAGAGATTTATCCGTT
flhB KO P4	GAACAACACTTTCAGGGCTTTT
flhA KO P1	CCGCCGTTTCTTTACTGGT
flhA KO P2	CTGAGCGAGGGAGCAGAACTCTTGTTGACATGCTGTTTTT
flhA KO P3	TGGAGGTAAGTGGATTTGAGCATGAAAATAAAAAAATTTACTGCTGCT
flhA KO P4	CACATTCTGGCCGTTAGTCA
Ec CORE comp1	ATGAATAACCATGCTACTGTGC
Ec CORE comp2	CTGAGCGAGGGAGCAGAACTTGCACCAGCAGCGGAAAC
fliI KO P1	GAGAGTCATTCGCGATGTTG
fliI KO P2	CTGAGCGAGGGAGCAGAATCAGCTGCACCTGCTTCC
fliI KO P3	TGGAGGTAAGTGGATTTGAGCGTGGCTTATCAATTTAGATTCCAAAAG
fliI KO P4	ATAGGTTGCTGCTTGCTCAG
flhA CT-F-*Eco*RI	TAGTAGGAATTCCAGTAGTTGATCCTGCATCAGTC
flhA CT-R-*Xho*I	CTACTACTCGAGAATATCCACCACTCCAATGCTT

fliP ORF F (*Hind*III)	AAACCCAAGCTTAAAGGTGGTGAACTACTATGAATGAGTTTATAAATATTTTCAGTTC
fliP ORF R (2X HA)	CATAGGGATAGCCAGCGTAATCTGGAACATCATATGGGTAAAAGCTCTGAAGCAAAGATTTC
2X HA R (*Sph*I)	ACCTAGGCATGCTTATGCGTAGTCCGGGACGTCATAGGGATAGCCAGCGTAA
2X HA R	CTGAGCGAGGGAGCAGAATTATGCGTAGTCCGGGACGTCATAGGGATAGCCAGCGTAA
fliP ORF F (pDR111)	AATTGTGAGCGGATAACAATTAAGCTTAAAGGTGGTGAACTACTATGAATGAGTTTATAAATATTTTCAGTTC
fliP NT R (GSS/2X HA)	CATAGGGATAGCCAGCGTAATCTGGAACATCATATGGGTAGGAACTACCATCCATTTTCGCGTAATTCA
fliP CT F (GSS/2X HA)	TTACGCTGGCTATCCCTATGACGTCCCGGACTACGCAGGATCGAGTAAACCTGAATCATTAAAGGATATT
fliP CT R (pDR111)	CCACCGAATTAGCTTGCATGCCTAAAAGCTCTGAAGCAAAGA
Ec fliO-flhA F (pDR111)	AATTGTGAGCGGATAACAATTAAGCTTAAAGGTGGTGAACTACTATGAATAACCATGCTACTGTGC
Ec fliO-flhA R (pDR111)	CCACCGAATTAGCTTGCATGCCTTGCACCAGCAGCGGAAAC
Lm CORE P1	CCCAAACTCGAGTCAGAGATATCAAGGACTTTC
Lm CORE P2	ATCTTTTCTACTAAATGTTTTCCCATTGCCTTAGTCTCCTCTTTCAAAAG
Lm CORE P3	CTTTTGAAAGAGGAGACTAAGG CAATGGGAAAACATTTAGTAGAAAAGAT
Lm CORE P4	AAACCCGTCGACCCAGAAGAAAATGTAAAGCACGTTA
fliO comp1	TGGAGGTAAGTGGATTTGAGCATGAAAAAGAGTCAATATTTTATTG
fliO comp2	CTGAGCGAGGGAGCAGAATCATTCATGATGACGTGG
fliP comp1	TGGAGGTAAGTGGATTTGAGCATGAATGAGTTTATAAATATTTTCAGTTC
fliP comp2	CTGAGCGAGGGAGCAGAACTAAAAGCTCTGAAGCAAAGATTTC
fliQ comp1	TGGAGGTAAGTGGATTTGAGCATGAGTTCAGAATTTGTAATTTCT
fliQ comp2	CTGAGCGAGGGAGCAGAATTACCCTGCAAAACGATTTA
fliR comp1	TGGAGGTAAGTGGATTTGAGCATGAATTCAATTATTGACTTATTTCC
fliR comp2	CTGAGCGAGGGAGCAGAATTAAGAAACACCGACCAATG
flhB comp1	TGGAGGTAAGTGGATTTGAGCATGAAGCTTAGAGTTGACCT
flhB comp2	CTGAGCGAGGGAGCAGAATCAATATACTTTTTGTTTTGTTTTATATACG
flhA comp1	TGGAGGTAAGTGGATTTGAGCATGTCAACAAGAGATTTATCCGTT
flhA comp2	CTGAGCGAGGGAGCAGAATTAAATATCCACCACTCCAATGC
Lm CORE comp1	TGGAGGTAAGTGGATTTGAGCATGCGTAAAATAGCCTCTAGACGA
Lm CORE comp2	CTGAGCGAGGGAGCAGAATCAAGTTGGTTCAATCAGTGC
Bm CORE comp1	TGGAGGTAAGTGGATTTGAGCATGCTGCGAAAGTTCGTAATTG
Bm CORE comp2	CTGAGCGAGGGAGCAGAATCAGATATCCACCACTCCAACA
Lm flaA int F	TACCGGGCCCCCCCTCGAGGTCGACCTTGCAACGTATGCGTCAAT
Lm flaA int R	TATCAAGCTTATCGATACCGCGATGGATTGATTGTTCACG
Bm hag int F (*Sal*I)	AAACCCGTCGACTCAGGTCAGCGCATTAACAA
Bm hag int R (*Bam*HI)	AAACCCGGATCCTAGCGGCATCTGCACTTAAA
311	GGTGGAAACCCAATACGTAATCAACGACTTGCAATATAGGATAACGAATCGTGTAGGCTGGAGCTGCTTC
312	ATCAGGCAATTTGGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACAACGACATATGAATATCCTCCTTA
3907	ACCGATATCATTACCCCGTCCGAGCGGATGCGCCGCCTGAGCCGTTAGTGTCCTAATTTTTGTTGACACTCTATC
3908	GATATTATTTTTCGGATAATCCTTAGGGTAGCATGATAAACGTTACGGAAATCAAAGGGAAAACTGTCCATA
3909	GAAAGCGATTAATCCGCTGG
3912	GTCACATTATCCGTCAGTCG
3950	CATGATAAACGTTACGGAACACTAACGGCTCAGGCGGCGCATTCG
3951	CGAATGCGCCGCCTGAGCCGTTAGTGTTCCGTAACGTTTATCATG
3914	TTTGCGCTCTGGCATCATTTACGCTCAATACTCTTTCCAGGATTGGCGACTCCTAATTTTTGTTGACACTCTATC
3915	GCGGCTTGCACCAGCAGCGGAAACAATAATATTGCTAATAAGGCTCTCATATCAAAGGGAAAACTGTCCATA

3952	GTGGCTATGTGGTGAAGCC
3953	GCTAATAAGGTTCTCATGTCGCCAATCCTGGAAAGAG
3954	CTCTTTCCAGGATTGGCGACATGAGAACCTTATTAGC
3955	CGTGATCAAATCATGCCTGC

All primers were designed during this study, and synthesized by Integrated DNA Technologies (IDT).