Supporting Information

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SI Extended Experimental Procedures

Strains and Growth Conditions. *B. subtilis* strains were grown in LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g NaCl/L) or on LB plates fortified with 1.5% Bacto Agar (Difco) at 37 °C. When appropriate, antibiotics were included at the following concentrations: 10 μg/mL tetracycline, 100 μg/mL spectinomycin, 5 μg/mL chloramphenicol, 5 μg/mL kanamycin, and 1 μg/mL erythromycin plus 25 μg/mL lincomycin (*mls*). IPTG (Sigma) was added to the medium at the indicated concentration when appropriate.

Strain Construction. All constructs were either first introduced into strain PY79 or DS2569 by natural competence and then were transferred to the 3610 background using SPP1-mediated generalized phage transduction or were transformed directly into the competent 3610-derivative DK1042 (43, 44). All plasmids used in this study are listed in Table S1. All primers used in this study are listed in Table S2. All strains used in this study are listed in Table S3.

The *pdeH::tet* insertion–deletion mutation was generated using isothermal assembly of amplicons (using primer pairs 3428/3429 and 3430/3431) and DNA containing a tetracycline resistance gene amplified from pDG1515 (45) with primer pair 3250/3251. Assembled DNA was transformed into strain DS2569 and transferred into recipients using SPP1-mediated generalized transduction.

To generate the *thrC::$P_{constitutive}$-MotI* plasmid pXG126, pXG095 (*amyE::$P_{constitutive}$-MotI spec*) (23) was digested with EcoRI and BamHI, and the *P_c-MotI* fragment was purified and cloned into EcoRI and BamHI sites of pDG1731 containing a multiple cloning site (MCS) and a spectinomycin resistance marker between two arms of the *thrC* gene (46).

To generate the *lacA::$P_{hyspank}$-cd1420* plasmid pXG128, pXG104 (thrC::*$P_{hyspank}$ -cd1420 mls*) (23) was used as the PCR template, DNA was amplified with primer pair GXH620/GXH621, and the amplicon was cloned via isothermal assembly into sites EcoRI and BamHI in pDR183 containing an MCS and an erythromycin resistance marker between two arms of the *lacA* gene (47).

To generate the *amyE::$P_{hyspank}$-motI-sfGFP* plasmid pSS891, three fragments were generated: (*i*) the upstream portion of the *motI* (1-348) gene was amplified from the plasmid pXG095 with primer pair GXH624/SS471; (*ii*) the *sfGFP* gene was amplified from pSR-sfgfp with primer pair SS472/SS473; and (*iii*) the downstream portion of the gene encoding *motI* (349-651) was amplified from the plasmid pXG095 with primer pair SS474/GXH631. Additionally, three fragments were designed with overlapping sequences at their termini to facilitate the generation of a full-length amplicon, MotI(1-116)-sfGFP-MotI(116-217), using primers GXH624 and GXH631. This amplified product was purified and cloned via isothermal assembly into sites HindIII and SphI of pDR111 containing an MCS and a spectinomycin resistance marker between two arms of the *amyE* gene (the generous gift of David Rudner, Harvard Medical School, Boston).

To generate the *amyE::$P_{hyspank}$-$motI^{\Delta 4}$* plasmid pSS889, the gene encoding *motI* was amplified from the plasmid pXG095 with primer pair GXH624/SS466. The amplified product was purified and cloned via isothermal assembly into sites HindIII and SphI of pDR111 containing an MCS and a spectinomycin resistance marker between two arms of the *amyE* gene.

To generate t*hrC::P_{motA}-motI* plasmid pSS907, the gene encoding *motI* was amplified from the plasmid pXG126 with primer pair SS605/SS608, and the promotor region of *motA* was amplified from plasmid pEC6 with primer pair SS602/SS609. The amplified product was purified and cloned via isothermal assembly into sites BamHI and SphI of pDG1731 containing an MCS and a spectinomycin resistance marker between two arms of the *thrC* gene.

To generate the *amyE::$P_{hyspank}$-motI-mNeonGreen* plasmid pSS913, three fragments were generated: (*i*) the upstream portion of the *motI* (1-348) gene was amplified from the plasmid pXG095 with primer pair GXH624/SS642; (*ii*) the *mNeonGreen* gene (a generous gift from Ethan Garner, Harvard University, Boston) was amplified with primer pair SS641/SS644; (*iii*) the downstream portion of gene encoding *motI* (349-651) was amplified from the plasmid pXG095 with primer pair SS643/GXH631. Additionally, three fragments were designed with overlapping sequences at their termini to facilitate the generation of a full-length amplicon, MotI(1-116)-mNeonGreen-MotI(116-217), using primers GXH624 and GXH631. This amplified product was purified and cloned via isothermal assembly into sites HindIII and SphI of pDR111 containing an MCS and a spectinomycin resistance marker between two arms of the *amyE* gene (generous gift of David Rudner, Harvard Medical School, Boston).

SPP1 Phage Transduction. To 0.2 mL of dense culture grown in TY broth (LB broth supplemented after autoclaving with 10 mM $MgSO_4$ and 100 μM $MnSO_4$), serial dilutions of SPP1 phage stock were added and statically incubated for 15 min at 37 °C. To each mixture, 3 mL TYSA (molten TY supplemented with 0.5% agar) was added, poured atop fresh TY plates, and incubated at 37 °C overnight. Top agar from the plate containing nearly confluent plaques was harvested by scraping into a 50-mL conical tube, vortexed, and centrifuged at 5,000 × *g* for 10 min. The supernatant was treated with DNase (final concentration 25 μg/mL) before being passed through a 0.45-μm syringe filter and stored at 4 °C.

Recipient cells were grown to stationary phase in 2 mL TY broth at 37 °C. Then cells (0.9 mL) were mixed with 5 μL of SPP1 donor phage stock and allowed to stand at 37 °C for 30 min. The transduction mixture was then centrifuged at 5,000 × *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in the remaining volume. The cell suspension (100 μL) was then plated on TY fortified with 1.5% agar, the appropriate antibiotic, and 10 mM sodium citrate.

Swarm Expansion Assay. Cells were grown to midlog phase at 37 °C in LB broth and were resuspended to 10 OD_{600} in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 8.0) containing 0.5% India ink (Higgins). Freshly prepared LB containing 0.7% Bacto Agar (25 mL per plate) was dried for 20 min in a laminar flow hood, centrally inoculated with 10 μL of the cell suspension, dried for another 10 min, and incubated at 37 °C. The India ink demarked the origin of the colony, and the swarm radius was measured relative to the origin. For consistency, an axis was drawn on the back of the plate, and swarm radii measurements were taken along this transect. For experiments including IPTG, cells were propagated in broth in the presence of IPTG, and IPTG was included in the swarm agar plates.

MotI Purification. The expression construct for $MotI^{\Delta 4}$ was generated by amplification using primers GXH482/GXH513 with genomic DNA as the template. The amplified product was purified and cloned into the NcoI and EcoRI sites of pHIS-parallel (48) containing an N-terminal 6×His tag along with a tobacco

etch virus (TEV) protease recognition site and an ampicillin resistance marker to create pXG093. For expression of MotI$^{\Delta4}$, the construct pXG093 was transformed into Rosetta (DE3) pLysS cells and grown to an OD_{600} of ∼0.6 in LB medium at 37 °C. Cells were then induced with 0.5 mM IPTG and grown at 20 °C overnight. For expression of the SeMet-MotI$^{\Delta4}$ construct, pXG093 was transformed into Rosetta (DE3)pLysS cells and grown at 37 °C to an OD_{600} of ∼0.6 in minimal medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 19 mM NH_4Cl, 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2 X Metals) (49). Amino acids (100 mg/L L-lysine, 100 mg/L L-phenylalanine, 100 mg/L L-threonine, 60 mg/L L-SeMet, 50 mg/L L-isoleucine, 50 mg/L L-leucine, and 50 mg/L L-valine) were then added to the medium, and the cells were then induced with 0.5 mM IPTG and grown at 20 °C overnight. Purification of MotI$^{\Delta4}$ and SeMet-MotI$^{\Delta4}$ protein was carried out in identical buffers, except that the buffer for SeMet-MotI$^{\Delta4}$ contained 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP). Cells were then harvested by centrifugation and resuspended in lysis buffer [20 mM Tris (pH 8.0), 350 mM NaCl, 0.3 mM TCEP, 10 mM $CaCl_2$, and 10 mM $MgCl_2$]. Lysis was carried out using a pressurized cell homogenizer, and lysates were loaded onto Ni-NTA resin (Qiagen) in a gravity column (Bio-Rad). The Ni-NTA column was washed with 10 column volumes of wash buffer 1 [25 mM Tris·HCl (pH 8), 300 mM NaCl, and 0.3 mM TCEP] followed by 10 column volumes of wash buffer 2 [25 mM Tris·HCl (pH 8), 300 mM NaCl, 25 mM imidazole, and 0.3 mM TCEP], and MotI$^{\Delta4}$/SeMet- MotI$^{\Delta4}$ protein was eluted with elution buffer [25 mM Tris·HCl (pH 8), 300 mM NaCl, 25 mM imidazole, and 0.3 mM TCEP]. The protein was then buffer-exchanged into the TEV digestion buffer [20 mM Tris (pH 8.0), 300 mM NaCl, 0.3 mM TCEP], and the digestion reaction was carried out using TEV protease overnight. The cleavage reaction was then loaded onto Ni-NTA resin (Qiagen) in a gravity column (Bio-Rad), and TEV protease–cleaved MotI$^{\Delta4}$/SeMet-MotI$^{\Delta4}$ protein came in flow through. MotI$^{\Delta4}$/SeMet-MotI$^{\Delta4}$ protein was then incubated overnight with a twofold molar ratio of c-di-GMP (Biolog catalog no. C 057). Final purification was conducted via size-exclusion chromatography on a Superdex 75 16/60 column (GE Healthcare) using gel filtration buffer [10 mM Tris (pH 7.5), 300 mM Nacl, and 0.3 mM TCEP].

MotI Structure Determination. Crystallization trials were set up via sitting-drop vapor diffusion with 1 μL protein (MotI$^{\Delta4}$ at 8.3 mg/mL or SeMet-MotI$^{\Delta4}$ at 13.4 mg/mL) and 1 μL crystallant [23–24% PEG3350, 0.1 M Bis-Tris propane (pH 6.5), and 0.2 M Na-K tartarate]. Plates were incubated at 20 °C, and rod-shaped crystals were obtained within a few days for both MotI$^{\Delta4}$ and SeMet-MotI$^{\Delta4}$ proteins. Crystals were frozen in liquid nitrogen after transfer to a cryoprotectant containing mother liquor and either 10% ethylene glycol or 10% glycerol. Data collection was performed at Lawrence Berkeley National Laboratory Advanced Light Source beamline 4.2.2 using the Taurus CMOS detector. All data were reduced in space group $P6_522$ (HKL2000) (50). Initial maps were obtained via SAD phasing of a SeMet-MotI$^{\Delta4}$ dataset using autoSHARP (51). Phases from the autoSHARP SeMet dataset were used with amplitudes from the native dataset in PHENIX to generate a suitable map for model building using Coot. Subsequent rounds of refinement were carried out using phenix.refine in PHENIX (52–54).

Microscopy. Cells were grown overnight at 22 °C in LB, the culture was diluted 1:100 into fresh medium and was grown at 37 °C to OD_{600} 0.6–1.0, and 1 mL was washed once in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 8.0), pelleted, and resuspended in 50 μL PBS buffer. Samples were observed by spotting 3 μL of the suspension on an agarose pad and then covered with a coverslip. Agarose pads were created by making a 1% solution of agarose (Fisher Brand Electrophoresis Grade) in PBS buffer, applying 400 μL of the molten solution to a glass slide, covering the slide with a glass microscope slide, and allowing the solution to cool for 20 min before use. Fluorescence microscopy was performed with a Nikon 80i microscope with a phase-contrast objective Nikon Plan Apo 100× and an Excite 120 metal halide lamp. GFP was visualized using a C-FL HYQ FITC Filter Cube (FITC excitation filter 460–500 nm, barrier filter 515–550 nm). Images were captured with a Photometrics CoolSNAP HQ2 camera in black and white, false-colored and averaged using ImageJ software.

Tethering Studies. For preparing slides for tethering studies, glass coverslips were treated with 3-aminopropyltriethoxysilane (Thermo Scientific catalog no. 80370) according to the manufacturer's protocol. These coverslips were then treated with Sulfo-SMCC (Thermo Scientific catalog no. 22322) dissolved in coupling buffer PBS with EDTA (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 10 mM EDTA, pH 7.5) at 2 mg/mL in a humid chamber for 1 h (Sulfo-SMC was first dissolved in 10 mM EDTA, and 10× PBS was added). The coverslips were then washed with PBS and placed with the treated side facing down on top of two coverslips (with a gap of ∼1–2 cm between the coverslips) attached to a glass slide with vacuum grease. Strains used for tethering studies lack the *swrA* gene, which results in fewer than 10 flagella per cell (36). Cells were grown at 23 °C for 12–13 h in LB medium supplemented with appropriate antibiotics to OD_{600} ∼0.6–1.0 (IPTG was added for the last 3 h of culture growth for appropriate strains), and 10 mL of cells were washed once in chemotaxis buffer [100 mM Tris (pH 7.0), 0.14 mM $CaCl_2$, 0.3 mM $(NH4)_2SO4$, 0.10 mM EDTA, 5 mM sodium lactate, and 0.05% glycerol] (55) and were resuspended in 1 mL of chemotaxis buffer. Flagella were sheared by passing the resuspended cells ∼100 times back and forth between two syringes connected via two 23-gauge needles (BD catalog no. 305145) and tubing (BD Intramedic catalog no. 427410). The sheared cells were then resuspended to a volume of 10 mL in chemotaxis buffer and were washed once with chemotaxis buffer. The cells were then finally resuspended to a volume of 4 mL and were introduced into the tunnel slides. The cells were allowed to settle on the treated side of the coverslip by gravity for 1 h to achieve cross-linking of cells to the slide. The tunnel slide was washed with chemotaxis buffer to remove any non–cross-linked cells. The cells were then imaged using a Nikon 80i microscope with a phase-contrast objective Nikon Plan Apo 100×, and movies were recorded using a Photometrics CoolSNAP HQ2 camera in black and white for 60 s at 10 frames/s. The movies were then analyzed by MicrobeJ (35) tracking software, and the angle traversed by individual cells (100 cells for each strain) was calculated using the SHAPE.orientation (https://www.youtube.com/watch?v=zx2HLEjEjD0) function in the MicrobeJ tracking software.

Swimming Velocity Analysis. Cells were grown to an OD_{600} of ∼0.6 and then were resuspended to an OD_{600} of 0.2 in LB medium. Tunnel slides were prepared by placing two coverslips with an ∼2-cm gap between them on a glass slide, and a third coverslip was placed over them. All the coverslips were secured using nail polish strengthener. The cells were then introduced into the tunnel slides and imaged using a Nikon 80i microscope with a phase-contrast objective Nikon Plan Apo 40×, and movies were recorded using a Photometrics CoolSNAP HQ2 camera in black and white for 30 s at 5 frames/s. The videos were then analyzed by MicrobeJ (35) tracking software, and the velocity was determined using the MOTION.Velocity function (100 cells for each strain).

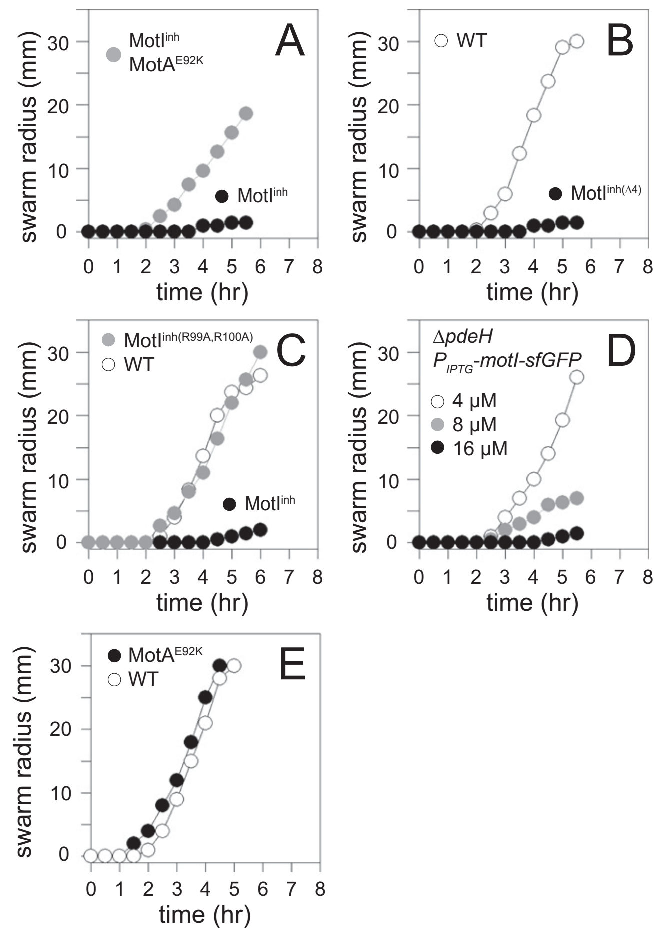


Fig. S1. MotI inhibition is relieved by mutations in MotA or mutations in the MotI c-di-GMP–binding site. (*A*) Quantitative swarm expansion assay for $MotI^{Inh}$ strain NPS351 (black symbols) and $MotI^{Inh}$ *soi* strain NPS358 expressing the $MotA^{E92K}$ allele (gray symbols). (*B*) Quantitative swarm expansion assay for wild-type strain 3610 (open symbols) and $MotI^{Inh}$ strain NPS911 expressing the $MotI^{\Delta4}$ allele (black symbols). (*C*) Quantitative swarm expansion assay for wild-type strain 3610 (open symbols), $MotI^{Inh}$ strain NPS351 (black symbols), and the $MotI^{Inh}$ strain that had been mutated to express the $MotI^{R99A,R100A}$ allele (gray symbols). (*D*) Quantitative swarm expansion assay for strain NPS913 mutated for PdeH and containing an IPTG-inducible MotI-sfGfP construct in the presence of 4 μM (open symbols), 8 μM (gray symbols), and 16 μM (black symbols) IPTG. (*E*) Quantitative swarm expansion assay for strain NPS363 expressing $MotA^{E92K}$ (black symbols) and for wild-type strain 3610 (open symbols). Each point is the average of three replicates.

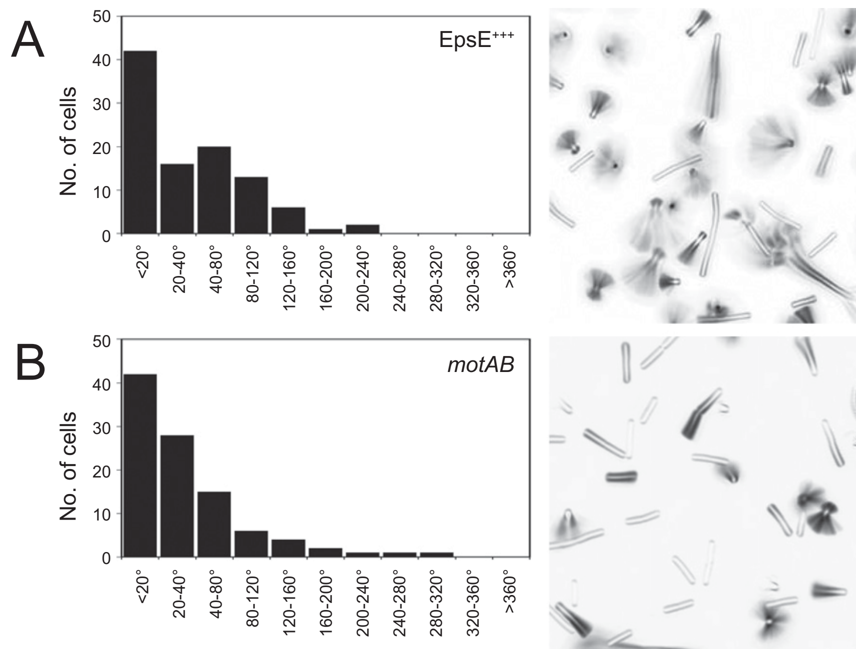


Fig. S2. EpsE inhibits flagellar rotation like a clutch. (*A*) EpsE-induced cells (strain DS3317) were tethered by flagellar stubs and monitored for 60 s in time-lapse microscopy. (*B*) Cells without the stator proteins MotAB (DS3318) were tethered by flagellar stubs and monitored for 60 s in time-lapse microscopy. (*Left*) The angles of rotation of 100 cells were binned and expressed as frequency histograms. (*Right*) Time-lapse composite images of sample fields.

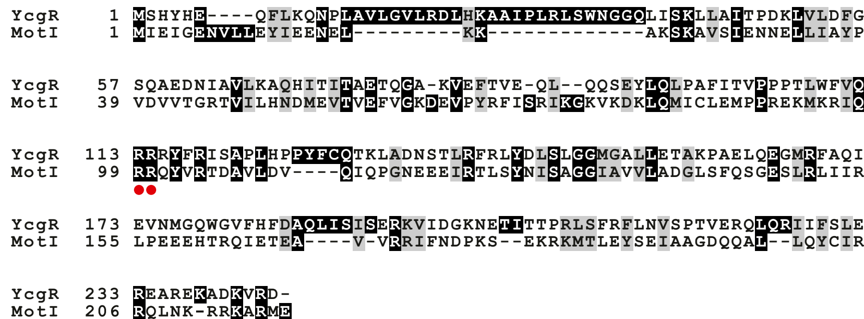


Fig. S3. MotI is a distant relative of YcgR but contains highly conserved c-di-GMP binding residues. Multiple sequence alignment comparing YcgR from *E. coli* and MotI from *B. subtilis*. *B. subtilis* MotI residues R99 and R100, shown to make contact with c-di-GMP in Fig. 4*B*, are indicated by red circles below the alignment.

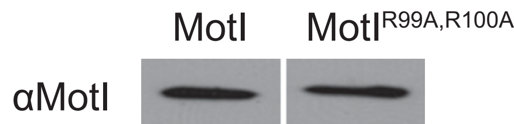


Fig. S4. MotI^{R99A,R100A} is not defective for motility inhibition due to a reduction in protein level. Western blot analysis using anti-MotI primary antibody against lysates of a MotI^{inh} strain (NPS235) and a MotI$^{inh(R99A,R100A)}$ strain (NPS928). Lanes were loaded and normalized according to culture OD_{600}. Both lanes were cropped from the same gel.

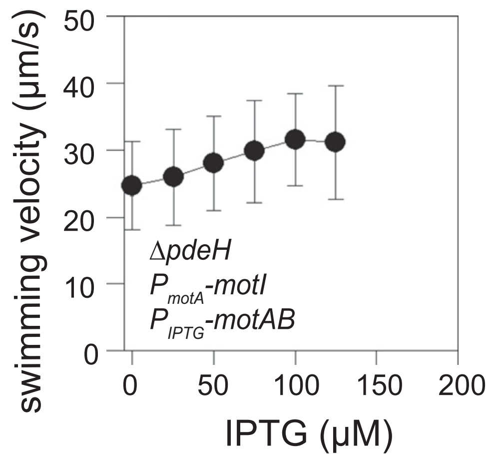


Fig. S5. Overexpression of MotA and MotB overcomes subinhibitory clutch inhibition of the flagellar rotor. Wet-mount tunnel microscopy measurement of swimming speed in cells with subinhibitory MotI concentrations and an IPTG-inducible MotA and MotB construct (*pdeH* P_{motA}*-motI* P_{IPTG}*-motAB*, NPS1030) shown as a function of IPTG concentration. Cell speeds of 100 motile cells were measured using MicrobeJ.

Table S1. Strains used in this work

Strain	Genotype
3610	Wild type
DK1042	*comI*^{Q12L}
DK1995	*swrA::tet hag*^{T209C} *pdeH::kan amyE::*$P_{hyspank}$*-motI spec motI::mls*
DK5142	*amyE::*$P_{hyspank}$*-MotI-mNeonGreen spec pdeH::kan motI::erm*
DS1855	*amyE::*$P_{hyspank}$*-epsE spec*
DS2569	*ΔpBS32*
DS3317	*thrC::*$P_{hyspank}$*-epsE mls swrA::kan lacA::*P_{hag}*-hag*^{T209C} *spec*
DS3318	*motAB::tet swrA::kan lacA::*P_{hag}*-hag*^{T209C} *spec*
DS8022	*ΔswrA lacA::*P_{hag}*-hag*^{T209C} *mls*
DS8062	*lacA::*P_{hag}*-hag*^{T209C} *mls*
DS9294	*lacA::*P_{hag}*-GFP mls*
NPS235	*amyE::*$P_{constitutive}$*-motI spec, pdeH::kan*
NPS309	*pdeH::kan amyE::*$P_{constitutive}$*-motI spec lacA::*P_{hag}*-hag*^{T209C} *mls*
NPS350	*amyE::*$P_{hyspank}$*-motI spec pdeH::kan motI::erm mls*
NPS351	*pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS357	*motA*^{E92K} *(sup21) pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS358	*motA*^{E92K} *(sup22) pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS359	*motA*^{E92K} *(sup23) pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS360	*motA*^{E92K} *(sup24) pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS361	*motA*^{E92K} *(sup25) pdeH::tet amyE::*$P_{constitutive}$*-motI cat thrC::*$P_{constitutive}$*-MotI spec, lacA::*$P_{hyspank}$*- CD1420 erm remA::TnYLB kan*
NPS363	*motA*^{E92K}
NPS394	*ΔpdeH amyE::*$P_{constitutive}$*-motI spec lacA::*P_{hag}*-GFP mls*
NPS911	*amyE::*$P_{hyspank}$*-motI*$^{\Delta 4}$ *spec pdeH::kan motI::erm*
NPS913	*amyE::*$P_{hyspank}$*-MotI-sfGFP spec pdeH::kan motI::erm*
NPS915	*motA*^{E92K} *amyE::*$P_{hyspank}$*-motI-sfGFP spec pdeH::kan motI::erm*
NPS928	*amyE::*$P_{hyspank}$*-motI*^{R99A,R100A} *spec pdeH::kan motI::erm*
NPS1030	*thrC::*P_{motA}*-motI spec pdeH::tet amyE::*$P_{hyspank}$*-motAB kan*
PY79	*swrA*^{FS} *sfp*^{0} *trpC2*

Table S2. Primers used in this work

Primer	Sequence
3250	acgactcactatagggcgaattg
3251	ctcactaaagggaacaaaagctgg
3428	ggaaagcgctttaatatcccg
3429	caattcgccctatagtgagtcgtacaaacaccctcattgatttatc
3430	ccagcttttgttccctttagtgagatggacgcaaaatgattgcgg
3431	ggacatttaaaggacagcagg
GXH482	gggccatgggaatagagattggagaaaatgtacttttag
GXH512	gggggaattcttactttcttctcttatttaactggcgc
GXH620	gaacgtcccggggagctcatgaattcgactctctagcttgag
GXH621	gagtgcggccgcccgcggtaggatcctaactcacattaattg
GXH624	gagcggataacaattaagcttgtgagtatattgaatgcgaaagagtg
GXH631	ccaccgaattagcttgcatgcggaagtgtttgccgatgtttattcc
SS466	ccaccgaattagcttgcatgcttactttcttctcttatttaactggcg
SS471	cttcacctttactcaccatgccgctggaccctcccggctgaatttgcacatc
SS472	gatgtgcaaattcagccgggagggtccagcggcatggtgagtaaaggtgaag
SS473	gtgcggatctcttcttcattaccagaacttcctttgtagagttcatccatgccg
SS474	cggcatggatgaactctacaaaggaagttctggtaatgaagaagagatccgcac
SS602	gccattcgccagggctgcaggaattcaaccatccaagaatgggttgaggatgaaatg
SS605	ggccaaaaaactgctgccttcggatccgtgtttgccgatgtttattcc
SS608	ccatagacaagctagtaaaatgcgaaagagtgaagtcaatgatagag
SS609	ctctatcattgacttcactctttcgcattttactagcttgtctatgg
SS641	tagatgtgcaaattcagccgggagggtccagcggcatggtttcgaaaggagaggagg
SS642	cctcctctcctttcgaaaccatgccgctggaccctcccggctgaatttgcacatcta
SS643	gtatggatgaactctataagggaagttctggtaatgaagaagagatccgcacactat
SS644	atagtgtgcggatctcttcttcattaccagaacttcccttatagagttcatccatac

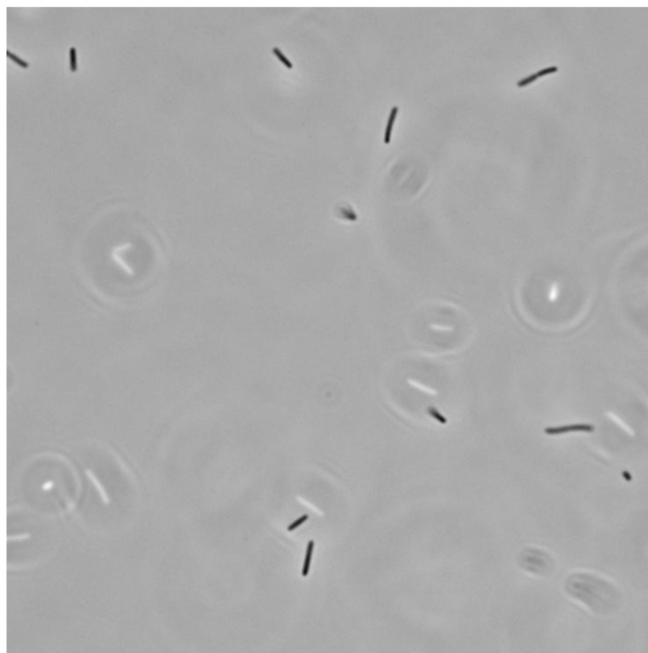
Table S3. Plasmids used in this work

Plasmid	Genotype
pDR111	*amyE::*$P_{hyspank}$*, spec amp*
pDR183	*lacA::mls amp*
pDG1731	*amyE::spec mls amp*
pDG1515	*tet amp*
pEC6	*amyE::*P_{motA}*-motAB spec amp*
pXG095	*amyE::*$P_{constitutive}$*-motI, spec amp*
pSRK-sfgfp-K_m	sfgfp kan
pHIS-parallel	*6-His amp*
pXG104	*thrC::*$P_{hyspank}$*-cd1420 mls amp*
pXG126	*thrC::*$P_{constitutive}$*-motI,spec amp*
pXG128	*lacA::*$P_{hyspank}$*-cd1420 mls amp*
pSS889	*amyE_*$P_{hyspank}$*-*$motI^{\Delta 4}$ *spec amp*
pSS891	*amyE_*$P_{hyspank}$*-motI-sfGFP spec*
pXG093	pHIS-parallel-$motI^{\Delta 4}$ *amp*
pSS907	*thrC_*P_{motA}*-motI, spec*
pSS913	*amyE_*$P_{hyspank}$*-motI-mNeon spec*

Table S4. X-ray diffraction data and refinement statistics

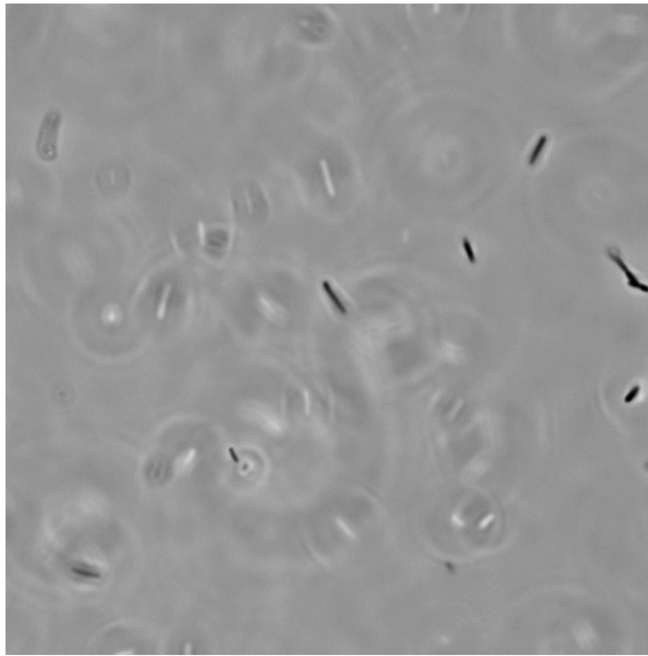
	MotI (Protein Data Bank ID code 5VX6)	SeMet MotI
Data collection		
Space group	$P6_522$	$P6_522$
Cell dimensions		
A = *b*, *c*, Å	75.45, 404.05	75.41, 402.47
Wavelength	0.9763	0.9763
Resolution, Å	50–3.2 (3.26–3.20)	50–3.45 (3.51–3.45)
R_{sym} or R_{merge}	0.138 (0.388)	0.169 (0.399)
R_{pim}	0.036 (0.152)	0.033 (0.117)
$I/\sigma I$*	17 (2.4)	20.5 (2.6)
Completeness*, %	98.9 (93.5)	99 (89.5)
Redundancy*	15.4 (5.9)	24.7 (10.5)
CC^*_1	(0.980)	(0.990)
Refinement		
Resolution, Å	50–3.2	
No. reflections	11,077	
R_{work}/R_{free}	0.236/0.277	
No. of molecules per asymmetric unit	2	
No. of atoms		
Protein	3,195	
Ligand/peptide	184	
B-factors		
Protein	61.6	
Ligand/peptide	55.9	
Rmsd		
Bond lengths, Å	0.004	
Bond angles, °	0.861	

*Values in parentheses are for the highest-resolution shell (56).



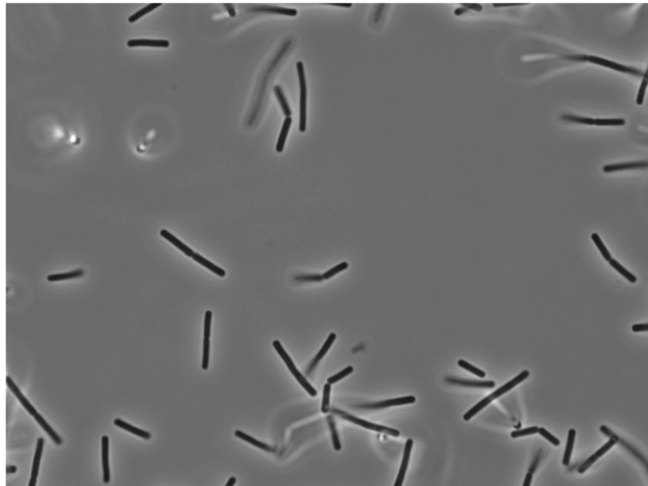
Movie S1. A sample field of wild-type cells in a wet-mount slide. The movie was captured over 10 s and was used to generate data in Fig. 1*B*.

Movie S1



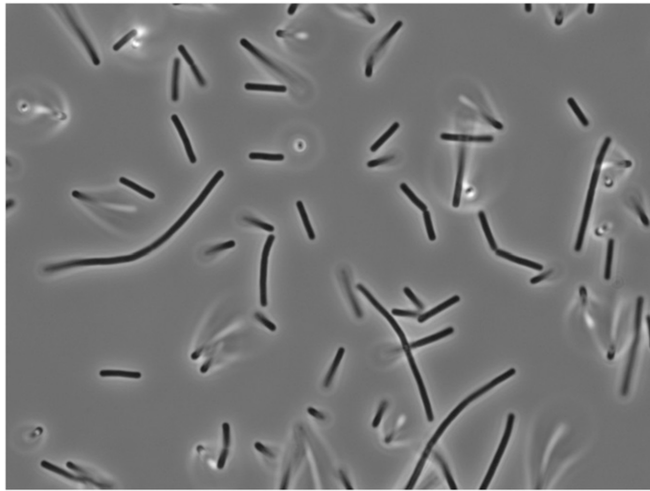
Movie S2. A sample field of MotI^{inh} cells in a wet-mount slide. The movie was captured over 10 s and was used to generate data in Fig. 1*C*.

Movie S2



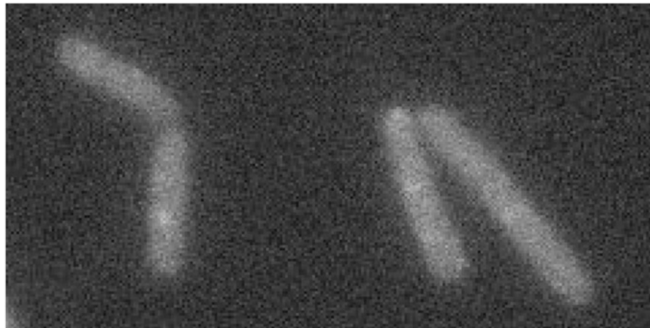
Movie S3. A sample field of wild-type cells tethered by flagellar stubs used to generate data in Fig. 2*A*. The movie was captured over 10 s.

Movie S3



Movie S4. A sample field of MotI^{inh} cells tethered by flagellar stubs used to generate data in Fig. 2*B*. The movie was captured over 10 s.

Movie S4



Movie S5. Time-lapse series of MotI^{inh}-mNeonGreen induced with 2 μM IPTG (DK5142) used to generate data in Fig. 4*E*. The movie was captured over 30 s.

Movie S5