Supporting Information

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SI Experimental Procedures

Growth Conditions. *C. crescentus* NA1000, CB15, and derivatives were grown in PYE, PYEX (PYE supplemented with 0.3% xylose), M2G, or Hutner base–imidazole-buffered–glucose–glutamate (HIGG) medium (1) at 30 °C. Unless otherwise stated cells were grown in PYE. *E. coli* strains were grown at 37 °C in LB with the appropriate antibiotics (2, 3).

***StpX* Gene Annotation.** The *stpX* gene was identified through BLAST searches. StpX is encoded by a 1,929-nt ORF on the *Caulobacter crescentus* chromosome (4) (spanning the CC1238 locus) at coordinates 1396070 (5′) and 1394142 (3′) and consists of 642 aa.

Microscopic Techniques. ***Standard wide-field fluorescence imaging.*** An upright Nikon 90i fitted with a 100× TIRF objective (na 1.45) and a Photometrics QuantEM 512SC CCD camera operated through Metamorph 7.2 was used. For photobleaching experiments, a Leica TCS SP5 scanning confocal microscope equipped with a HCX PL APO Lambda Blue 63× 1.4 oil objective (Leica Microsystems) was used.

FLIP/FRAP experiments. An argon laser was used for excitation at 488 nm and GFP emission was measured between 500 and 600 nm. Cells expressing WT or mutant StpX-sEGFP derivatives were grown for 40 h in HIGG minimal medium containing 30 μM phosphate (low phosphate content) and mounted on 0.6% agarose pads with HIGG (30 μM phosphate). Fluorescence excitation was at 4% of the maximum laser power for nonbleaching frames. For bleaching frames, 100% laser power was used in user-specified regions of bleaching while fluorescence in the rest of the field was simultaneously monitored using 4% power. For FLIP experiments, prebleach images were acquired, followed by fluorescence bleaching for 52 sec and capturing postbleach images. For FRAP experiments comparing full-length StpX-GFP and its truncated variants, prebleach images were acquired, followed by bleaching for 13 sec and capturing of a postbleach image immediately after the conclusion of the bleaching interval. Fluorescence recovery was then monitored over 100–200 sec during the recovery period by images in 10-sec intervals.

Comparison of StpX(Δper)-GFP and cytoplasmic GFP diffusion. Cells expressing cytoplasmic GFP and StpX(Δ*per*)-GFP were cultured in HIGG (30 μM phosphate) for 48 h and mounted on HIGG (30 μM phosphate) pads containing 1% wt/vol agarose. GFP was imaged using the Leica SP5 as described in *Experimental Procedures* in the main text. For FRAP experiments, prebleach images were acquired, after which a tip of the cell body was bleached for 1.3 sec. A postbleach image was acquired immediately and recovery was monitored at 1.3-sec intervals over a period of 90 sec. Data from these experiments were quantified for every time point by normalizing the fluorescence intensity in the bleached region to its prebleach intensity. These normalized fluorescence intensities were averaged across four different cells for each strain at each time point to generate fluorescence recovery curves.

Quantification of differential diffusion along the length of the stalk. For FLIP experiments examining diffusion of StpX-GFP, StpX (Δ*per*)-GFP, and StpX(Δ*cd*)-GFP within the stalk (Fig. 4.), fluorescence intensities were quantified at each point along the stalks tested. For each point, denoted by the distance of its position on the stalk from the cell body/stalk junction, fluorescence intensities were averaged across two prebleach frames and two postbleach frames to generate plots of the positional intensity spectra of stalks before and after bleaching.

Quantification of StpX(Δper)-GFP recovery in bleached stalks. For the FRAP experiment examining recovery of StpX(Δ*per*)-GFP in a bleached stalk (Fig. 4*F*), the average stalk fluorescence was measured at each time point during the prebleach, postbleach, and recovery periods. These stalk-fluorescence values were calculated as a percentage of the average prebleach stalk fluorescence and are plotted as a function of time to demonstrate recovery.

Quantification of cell-body fluorescence recovery. For FRAP experiments examining fluorescence recovery in bleached cell bodies of cells expressing StpX-GFP, StpX(Δ*per*)-GFP, and StpX(Δ*cd*)-GFP quantification was done as follows. Average cell body fluorescence estimates were obtained for each strain by averaging the fluorescence intensities across arc-shaped regions of interest drawn within each cell body. Fluorescence intensities during prebleach, immediate postbleach, and 200-sec recovery imaging were computed for each strain by averaging its cell body fluorescence across four frames at each of the above time points.

Cell Fractionations. To separate soluble and insoluble fractions strains were grown in 5 mL of HIGG (30 μM phosphate) to an OD_{600} of ~1.0. All procedures were carried out on ice or at 4 °C. Cells were pelleted by centrifugation at 8,000 × *g* for 15 min. Cell pellets were resuspended in 500 μL of 10 mM Tris HCl (pH 8) containing a protease inhibitor mixture (Complete Mini, EDTA-free; Roche Pharmaceuticals) and sonicated in three 20-sec bursts to achieve cell lysis. Unbroken cells were removed by centrifugation at 16,000 × *g* for 2 min. Supernatants containing whole-cell lysates were transferred to fresh tubes and centrifuged at 16,000 × *g* for 30 min to pellet total membranes. Supernatants contained soluble fractions and pellets were resuspended in 500 μL of 10 mM Tris HCl (pH 8, with protease inhibitors) to yield total membrane fractions.

Immunoblotting. An N-terminally His6-tagged C-terminal fragment of StpX (MSAVAHHDDHHDHGHGHDDHHGHDDHGHG-HDDHGHDAHGHGGHGHDDHGHHEDHGHGHHDAPK-KELEHAHH) was overexpressed from pET28 in *E. coli* Rosetta (Novagen), purified under native conditions, and used to immunize two New Zealand White rabbits (Josman, LLC). The resulting serum was used at a dilution of 1:30,000 and immunoblotting was as described previously (2, 3). A custom-made rabbit polyclonal antiserum to GFP (Josman, LLC) or a commercially available monoclonal antibody (JL-8; Clontech) was used to generate the immunoblots shown in Fig. 3 and Fig. S6, respectively.

Library and Strain Constructions. *C. crescentus* NA1000, CB15, and derivatives were grown in PYE, PYEX (PYE supplemented with 0.3% xylose), M2G, or HIGG (1) at 30 °C. *E. coli* strains were grown at 37 °C in LB with the appropriate antibiotics as previously described (2, 3). Libraries of EGFP-fusion strains were made through the following procedures. A mini-Tn*5* transposon carrying a start-codon-less EGFP and a p15A-derived origin of replication was delivered from plasmid pKK881 by intergeneric conjugation from *E. coli* strain S17-1 to NA1000. The Tn*5* insertion sites were determined by sequencing of plasmids harboring genomic sequences adjacent to the Tn*5*. Such plasmids were obtained by HinP1I partial digest, religation, and electroporation into *E. coli*.

In addition, plasmid libraries constructed in suicide vectors pCWR302 and pCWR304 (described below) were prepared and used to electroporate NA1000 cells directly (5). Antibiotic-resistant transposon mutants and single recombinants were grown on the

appropriate selective plates. For the plasmid-based localization screen, vectors pCWR288, pCWR293, pCWR302, and pCWR304 were digested with either ClaI or BamHI and ligated to *C. crescentus* genomic DNA that was partially digested with AciI, HinP1I, TaqI, or Sau3AI. Ligations were electroporated into *E. coli* and resulting transformants were grown in liquid media before plasmid purification to generate random GFP-gene fusion libraries. Electroporation of NA1000, selection of transformants, and growth of resulting strains were performed successively in selective media as part of the screening scheme.

The Δ*stpX*, *stpX(*Δ*per)*, and *stpX(*Δ*per*Δ*tm)* strains were constructed by creating in-frame deletions of the sequence encoding residues 16–629, 16–363, and 16–387, respectively. The two-step recombination sucrose-counterselection procedure was subsequently used to generate mutant *stpX* derivatives by exchanging endogenous *stpX* with mutant versions using pNPTS138. Next, the 3′ ends of wild-type or mutant *stpX* were replaced with *EGFP* (pCWR320) and *sEGFP* tagged versions (pCWR325). To this end, pCWR320 or pCWR325 that harbors this fragment and confers resistance to kanamycin was integrated by a one-step homologous recombination into NA1000 and *stpX* deletion mutant strains. To construct *stpX-EGFP* reporter strains in different mutants, lysates from the StpX-sEGFP (full-length, NA1000:: pCWR325) or the *stpX*-Tn*5-gfp* (partial-length) strains were prepared and used to transduce each respective mutant strain to kanamycin resistance. Subsequent selection on PYE-Kan plates was performed in each case to isolate strains with EGFP-tagged *stpX*. A chromosomal GFP-tagged *stpX*Δ*cd* allele was generated directly by exchanging a partial fragment of *stpX* fused to sEGFP (lacking a start codon) at residue 386 with its endogenous counterpart. In this strategy, a single recombination event gives rise to a single truncated ORF fused to the *sEGFP* gene. Swarmer cell isolation (synchronization) and localization experiments were performed as described previously (2, 3).

Plasmids pP_{xyl}-*stpX* and pP_{xyl}-*staR* were introduced into the resulting strains by electroporation as described and subsequent selection was performed on PYE-Tet plates supplemented with glucose (0.2%).

Plasmid Construction and Manipulations. KOD thermostable DNA polymerase (EMD Biosciences) was used for amplifications using PCR. The Δ*stpX, stpX*Δ*per* and *stpX*Δ*per*/Δ*tm* deletion constructs were made in pNPTS138. To make the full in-frame deletion construct, a fragment 700 bp upstream of the *stpX* start codon and extending 45 bp into the predicted ORF (coordinates 1396771–1396026) and a 3′ region containing the last 42 bp of *stpX* and extending 720 bp downstream of the gene (coordinates 1394183–1393421) were amplified while introducing KpnI and EcoRI sites in the 5′ and KpnI and HindIII sites in the 3′ fragments, respectively. Both fragments were ligated into the EcoRI- and HindIII-digested pNPTS138 vector to yield vector pNPTΔ*stpX*. To create in-frame deletions of the regions corresponding to the periplasmic (*per*) and the TM (*per*/*tm*) domains, the 3′ fragment of pNPTΔ*stpX* was replaced with an amplified fragment of 735 bp (coordinates 1394912–1394178) that flanked the *per* domain (creating a *stpX*Δ*per* deletion construct) to yield plasmid pNPT$stpX^{\Delta per}$ and an amplified 807-bp fragment flanking the *per*/*tm* domain (coordinates 1394984–1394178, creating a *stpX*Δ*per*Δ*tm* construct) named pNPT$stpX^{\Delta per/\Delta tm}$.

Plasmids pCWR320 and pCWR325 were made by amplifying and cloning the 3′ end of *stpX* as an XbaI/BamHI fragment into NheI/BamHI-digested pXGFP4 or pCWR302, respectively.

To create pP_{xyl}-*stpX* a 1,200-bp 5′ portion and an overlapping 1,352-bp 3′ region of the *stpX* ORF were amplified while creating a 5′ NdeI site (that includes the start codon) in the 5′ fragment and creating a 3′ EcoRI site in the 3′ fragment. Both fragments were then digested with HindIII to cleave at an endogenous HindIII site located at base pairs 598–603 of the *stpX* ORF. Finally, resulting fragments (598 and 1,332 bp, respectively) were ligated into NdeI/EcoRI-digested vector pMT375 (6).

To create pP_{xyl}-*staR* (also known as pCWR383), the *staR* ORF was amplified by PCR using primers that incorporate an NdeI restriction site overlapping the start codon and an EcoRI site after the stop codon. The fragment was cleaved with NdeI/EcoRI and cloned into pMT375.

Plasmid pCWR302 was made by releasing the EGFP gene from pXGFP4 and replacing it with an sEGFP derivative (7) synthesized as a codon-optimized version for *Caulobacter* by Dna 2.0 Inc. that was isolated as an EcoRI/XbaI fragment.

To make pCWR304, the *sEGFP* gene was cloned as a ClaI/XbaI fragment into pBBR-MCS-gent (8).

To make pCWR288, the *EGFP* gene was released from pXGFP4 using EcoRI/XbaI and cloned into pJS14.

Plasmid pCWR293 was made by cloning *EGFP* from pXGFP4 as an EcoRI/XbaI fragment into pBBR-MCS-gent.

To make pCWR435, a DNA fragment encoding a truncated 54-residue StpX(Δ*per*) polypeptide starting from the start codon and ending after the TM domain (residue 400 in wild-type StpX) was amplified from genomic DNA of the *stpX*(Δ*per*) mutant strain. Oligos were used that introduced an NdeI site overlapping the start codon and a 6-bp EcoRI linker sequence after the TM domain. The PCR fragment was cleaved with NdeI/EcoRI and triple ligated along with an EcoRI/XbaI-prepared *sEGFP* fragment into pMT374 (6) that had been cleaved with NdeI and NheI.

To construct pCWR437, the same strategy was employed as that used to make pCWR435 except that the PCR amplification step was performed on genomic DNA from the *stpX*(Δ*per*Δ*tm*) mutant strain.

Plasmid p2060-TM-long-510 was engineered by cleaving a PCR fragment of *pflI* encompassing the start codon and the sequence for the TM domain with NdeI/EcoRI along with an EcoRI/XbaI-restricted *sEGFP* fragment into NdeI/XbaI-prepared pMT335 (6).

1. Ely B (1991) Genetics of Caulobacter crescentus. *Methods Enzymol* 204:372–384.
2. Viollier PH, Shapiro L (2003) A lytic transglycosylase homologue, PleA, is required for the assembly of pili and the flagellum at the Caulobacter crescentus cell pole. *Mol Microbiol* 49:331–345.
3. Chen JC, Viollier PH, Shapiro L (2005) A membrane metalloprotease participates in the sequential degradation of a Caulobacter polarity determinant. *Mol Microbiol* 55: 1085–1103.
4. Nierman WC, et al. (2001) Complete genome sequence of Caulobacter crescentus. *Proc Natl Acad Sci USA* 98:4136–4141.
5. Viollier PH, et al. (2004) Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proc Natl Acad Sci USA* 101:9257–9262.
6. Thanbichler M, Iniesta AA, Shapiro L (2007) A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in Caulobacter crescentus. *Nucleic Acids Res* 35:e137.
7. Pédelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol* 24:79–88.
8. Kovach ME, et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176.

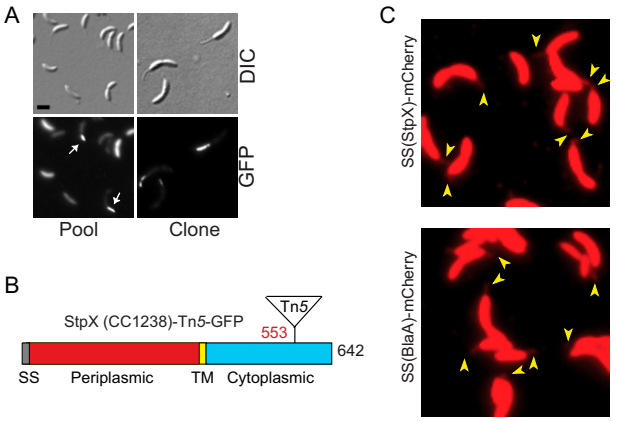

Fig. S1. Identification of *stpX* (CC1238). (*A*) Strain BR-E7 (StpX-Tn*5*-GFP) was isolated from a pool of strains containing chromosomal Tn*5*-GFP insertions that were imaged by differential interference contrast (DIC) and GFP-mediated fluorescence microscopy. (*B*) Schematic of the StpX domain architecture and the Tn*5*-GFP fusion site (residue 553) within StpX. (*C*) Expression of mCherry chimeras harboring N-terminal polypeptides with sequences resembling the SecA-dependent signal sequence (SS) in wild-type *C. crescentus* (NA1000) yields fluorescent cell bodies as well as dimly fluorescent stalks.

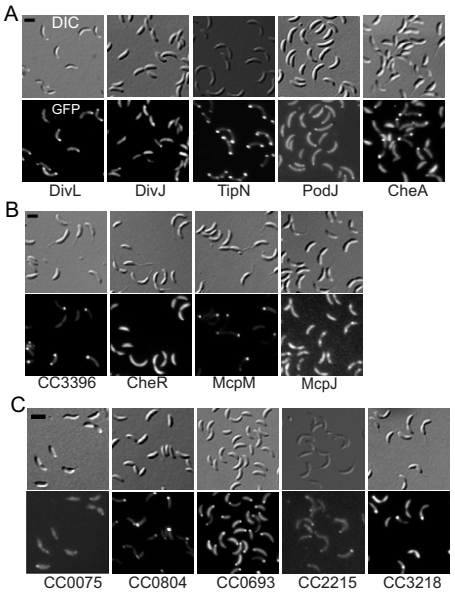

Fig. S2. Identification of previously known (*A*), unknown (*B* and *C*), and hypothetical (*C*) localized proteins from the GFP-imaging screen (see *Experimental Procedures* and Tables S1 and S2 for details). (Scale bar, 2 μm.)

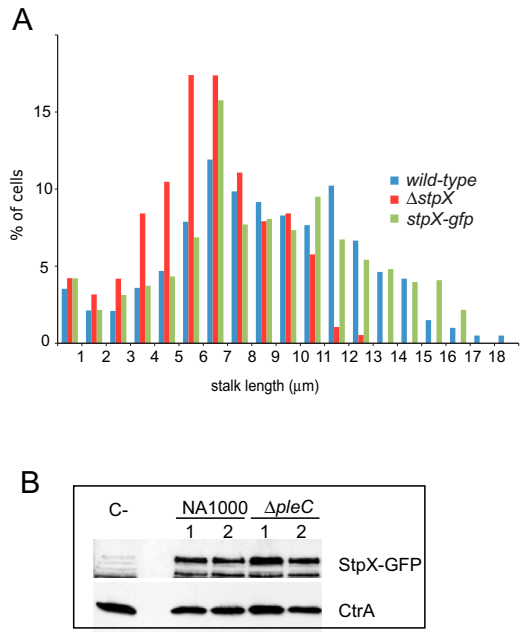

Fig. S3. Frequency distribution of stalk lengths of WT (NA1000), Δ*stpX*, and *stpX-gfp* cells grown in HIGG (30 μM phosphate) (>180 cells per strain) (*A*) and immunoblot of StpX-GFP and CtrA (control) in WT and Δ*pleC* cells (*B*). (*A*) Cells were grown overnight in PYE, washed three times in water, and resuspended to an OD_{600} of 0.1 in HIGG containing 30 μM phosphate. At OD_{600} of 1.5, they were imaged on HIGG (30 μM phosphate) pads containing 0.6% agarose. *StpX-GFP* stalks are similar in mean length (8.5 ± 4.0 μm, $n = 187$) to WT stalks and significantly [$t(375) = 6.9$, $P < 0.0001$] elongated over Δ*stpX* stalks. (*B*) Extracts from WT and Δ*pleC* cells expressing StpX-GFP in place of native StpX were separated by SDS/PAGE, blotted, and probed with a polyclonal anti-GFP antibody (*Upper*) or anti-CtrA antibody (*Lower*). C−, control WT cells expressing untagged (native) StpX.

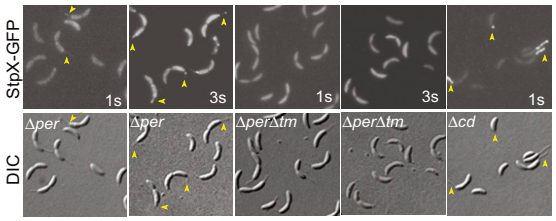

Fig. S4. Microscopic analyses of StpX(Δ*per*)-GFP without the periplasmic domain, StpX(Δ*per*Δ*tm*)-GFP without both periplasmic and TM domains, and StpX (Δ*cd*)-GFP without the predicted C-terminal domain. StpX-GFP and derivatives were expressed from the *stpX* chromosomal locus under the control of its endogenous promoter. Exposure times for fluorescence images were either for 1 (1s) or 3 sec (3s). The 3-sec exposure panels reveal a low level of fluorescence in the stalk for StpX(Δ*per*)-GFP, but not for StpX(Δ*per*Δ*tm*)-GFP. Yellow arrowheads point to fluorescent stalks. Note that the 3-sec panels are reproduced from Fig. 3*B* in the main text.

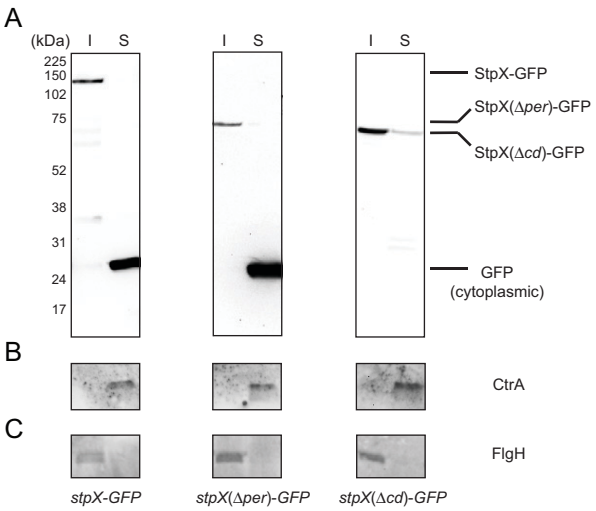

Fig. S5. Immunoblots of insoluble (I) and soluble (S) fractions of extracts from *stpX-GFP*, *stpX*(Δ*per*)*-GFP*, and *stpX*(Δ*cd*)*-GFP* cells grown in HIGG. Blots were probed with antibodies to GFP (*A*), CtrA (a transcription factor, *B*) and FlgH (the flagellar L-ring protein, *C*). The blots in *B* and *C* serve as controls for the fractionation as CtrA is known to reside in the cytoplasm and FlgH is associated with the membrane. Note that the fractionation procedure resulted in enhanced proteolysis as StpX-(Δ*per*Δ*tm*)-GFP proved too unstable to be detected and a significant amount of free (cytoplasmic) GFP was liberated. Note that there is a reduction StpX-GFP isoforms in HIGG compared to growth in PYE (Fig. 3 in the main text).

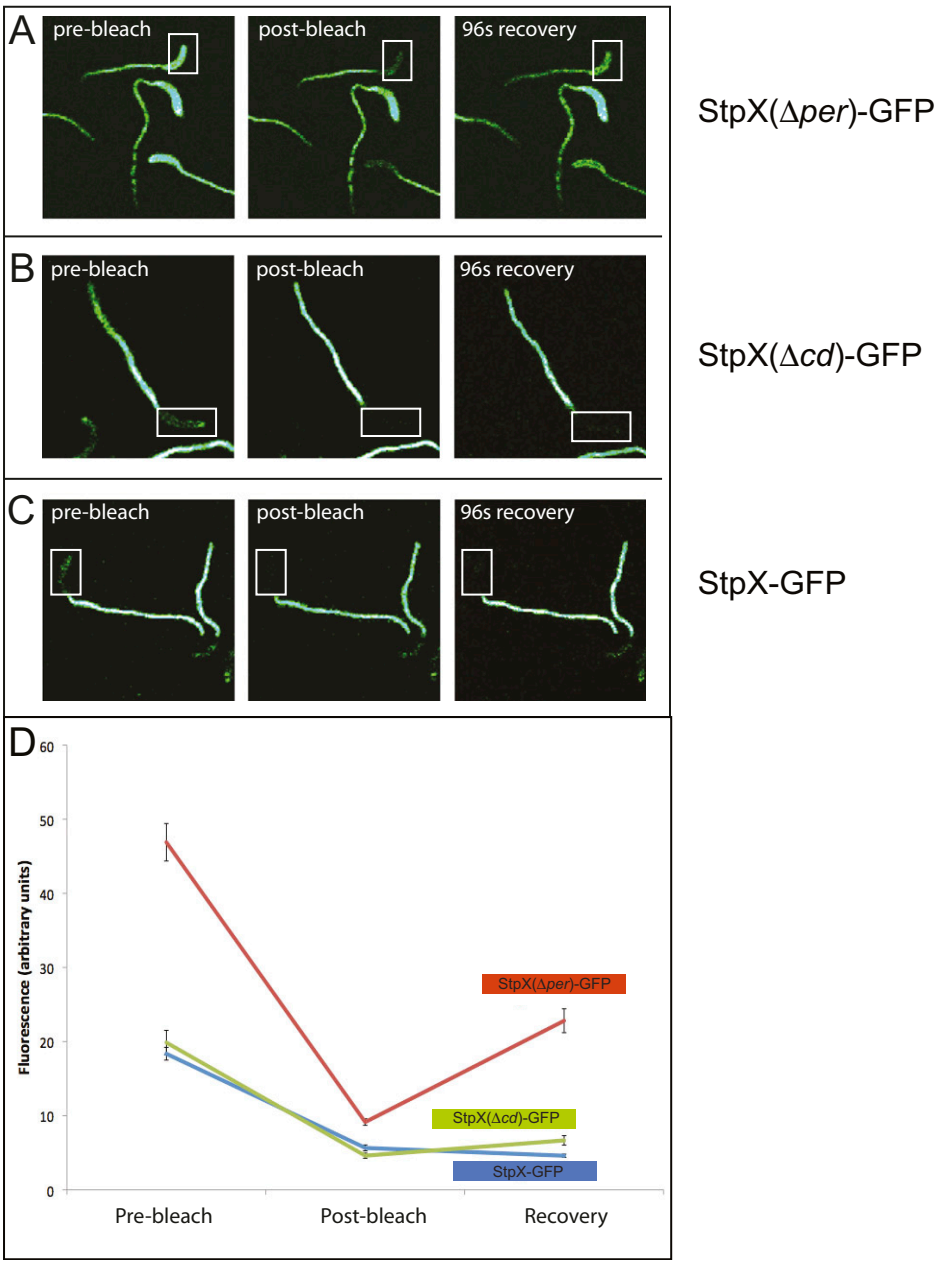

Fig. S6. FRAP analysis of StpX diffusion between stalk and cell body. Cell bodies of cells expressing (*A*) StpX(Δ*per*)-GFP, (*B*) StpX(Δ*cd*)-GFP, and (*C*) wild-type StpX-GFP were bleached for 13 sec (white rectangles). Following the immediate acquisition of a postbleach image, recovery in the cell bodies was monitored and quantified (*D*) over a period of 200 sec.

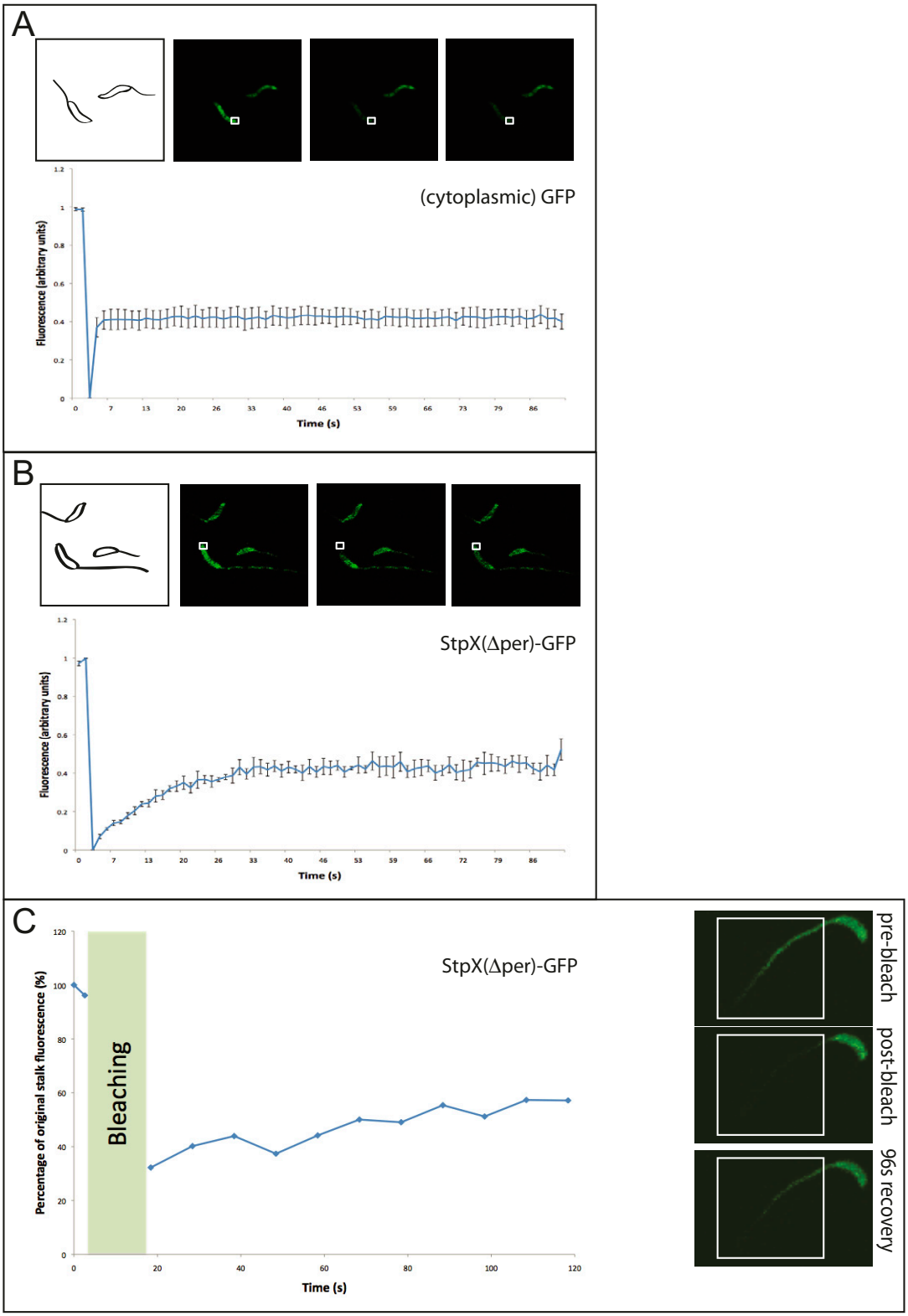

Fig. S7. Quantitative FRAP analysis of cytoplasmic GFP (*A*) and StpX(Δ*per)*-GFP (*B* and *C*) in cell bodies (*A* and *B*) and stalks (*C*). The areas defined in the white rectangles were bleached and quantified as described in *SI Experimental Procedures*.

Table S1. Genes of known function, identified in a genetic localization screen

Strain	Gene	Predicted function	Fusion	Local.	Coordinates
GQB8	CC0433	Chemotactic signaling kinase CheA	Tn*5*::*gfp*	FP	450523<
55D3	CC1063	Sensor histidine kinase DivJ	pCWR304	SP	1194893<
18C4	CC1485	Polar development protein TipN	pCWR304	BP/S	1642519<
134D6	CC2045	Polar development protein PodJ	pCWR302	BP	446109>
AOF2	CC3488	Polar development kinase DivL	Tn*5*::*gfp*	BP	3726583<

Blast searches using translated sequences were performed to identify the gene and determine the site of fusion. BP, bipolar; FP, flagellated pole; S, septal; SP, stalked pole.

Table S2. Genes identified in a genetic localization screen

Strain	Gene	Predicted function	Fusion	Local.	Coordinates
BD-E11	CC0010	DnaK, chaperone	Tn*5*::*gfp*	V	9901<
E-B2	CC0075	Hypothetical COG38987 (HemY)	Tn*5*::*gfp*	SP	75484<
C-D5	CC0482	5-Methyl-tetrahydropteroyltriglutamate-homocysteine methyltransferase	Tn*5*::*gfp*	V	504142<
AE-D8	CC0509	Hypothetical protein	Tn*5*::*gfp*	V	535814>
58D11	CC0532	Mandelate racemase	pCWR304	SP	556339>
9F4	CC0633	Lipopolysaccharide biosynthesis protein	pCWR293	V	700365>
76B4	CC0693	Hypothetical protein	pCWR304	SP	759449<
AM-K4	CC0804	Proline dehydrogenase	Tn*5*::*gfp*	SP	892159>
13H5	CC0804	Proline dehydrogenase	Tn*5*::*gfp*	SP	892159>
K-H4	CC0807	AcrB/AcrD/AcrF family protein	Tn*5*::*gfp*	BP	899408>
CE-C4	CC1007	RsaA, S-layer protein	Tn*5*::*gfp*	SP/V	1136110<
H-A3	CC1007	RsaA, S-layer protein	Tn*5*::*gfp*	SP/V	1135793<
124E2	CC1131	TonB-dependent receptor	pCWR304	BP/S	1282691<
BR-E7	CC1238	Hypothetical protein (StpX)	Tn*5*::*gfp*	ST	1394411>
9B2	CC1493	Phosphoenolpyruvate carboxylase	pCWR302	SP	1652884<
21H6	CC1580	Dna gyrase subunit A	pCWR304	V	1746350<
C-E4	CC1666	TonB-dependent receptor	Tn*5*::*gfp*	V	1842506>
15G12	CC1829	Urea amidolyase-related protein	pCWR293	SP/S	2023733<
24E4	CC1887	NADH-ubiquinone oxireductase	pCWR293	BP/V	2081709>
122A2	CC1969	Glutamine synthase	pCWR304	SP/S	2168073<
22C10	CC2068	1-Deoxy-D-xylulose-5-phosph. Synth	pCWR304	SP/S	2279990>
C-D2	CC2089	NA-dependent nucleoside transporter	Tn*5*::*gfp*	FP/S	2298177>
FJ-F7	CC2160	Hypothetical protein	Tn*5*::*gfp*	SP	2365205>
CA-G9	CC2215	Hypothetical protein	Tn*5*::*gfp*	SP/BP	2419608>
98H3	CC2226	EF-Hand domain protein	pCWR304	SP	2428799<
EL-D1	CC2317	McpM chemotaxis protein	Tn*5*::*gfp*	SP	2518694>
A-A6	CC2468	ClpA, protease subunit A	Tn*5*::*gfp*	SP/S	2675014<
H-F7	CC2481	Aminopeptidase N	Tn*5*::*gfp*	FP	2689397<
B12A	CC2545	MurB, cell envelope	pCWR304	SP/C	2752809>
10C6	CC2622	NADP-dependent malic enzyme	pCWR304	SP	2834628<
2C2	CC2711	Asparagine synthase	pCWR293	BP/V	2927533>
GR-F9	CC2969	PadR like protein	Tn*5*::*gfp*	SP/V	3190721>
I-I3	CC2981	Excinuclease ABC, subunit B	Tn*5*::*gfp*	V	3201345<
D-B6	CC3061	Hypothetical prot/abc transporter-like	Tn*5*::*gfp*	V	3287084<
I-H4	CC3145	McpJ, chemotaxis protein	Tn*5*::*gfp*	M	3390438<
GT-F3	CC3177	Transcriptional regulator	Tn*5*::*gfp*	SP	3425554<
4J8	CC3205	NusG, antitermination protein	pCWR293	SP/V	3460517>
AK-G4	CC3215	Phosphate dehydrogenase	Tn*5*::*gfp*	SP	3476717>
EB-G9	CC3215	Phosphate dehydrogenase	Tn*5*::*gfp*	SP/V	3476768>
GY-B4	CC3218	Hypothetical protein	Tn*5*::*gfp*	SP	3480242>
11E8	CC3226	FtsH protease	pCWR293	SP/BP	3489686>
77G8	CC3396	GGDEF family protein	pCWR304	SP	3639362>
9G2	CC3472	Chemotaxis protein CheR	pCWR288	FP	3715924<
10I10	CC3655	Malate dehydrogenase	pCWR304	SP	3912042>
4C6	CC3756	Thiophene and furan oxidation ThdF	pCWR304	V	4009469>

Blast searches using translated sequences were performed to identify the fused gene and determine the site of fusion. BP, bipolar; C, cell envelope; FP, flagellated pole; M, middle of chamber (quarter position in PD cells); S, septal; SP, stalked pole; ST, stalk; V, variable.