Sequential evolution of bacterial morphology by co-option of a developmental regulator

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What mechanisms underlie the transitions responsible for the diverse shapes observed in the living world? Although bacteria exhibit a myriad of morphologies1, the mechanisms responsible for the evolution of bacterial cell shape are not understood. We investigated morphological diversity in a group of bacteria that synthesize an appendage-like extension of the cell envelope called the stalk2,3. The location and number of stalks varies among species, as exemplified by three distinct subcellular positions of stalks within a rod-shaped cell body: polar in the genus Caulobacter and subpolar or bilateral in the genus Asticcacaulis4. Here we show that a developmental regulator of Caulobacter crescentus, SpmX, is co-opted in the genus Asticcacaulis to specify stalk synthesis either at the subpolar or bilateral positions. We also show that stepwise evolution of a specific region of SpmX led to the gain of a new function and localization of this protein, which drove the sequential transition in stalk positioning. Our results indicate that changes in protein function, co-option and modularity are key elements in the evolution of bacterial morphology. Therefore, similar evolutionary principles of morphological transitions apply to both single-celled prokaryotes and multicellular eukaryotes.

Stalks are a common feature in aquatic bacterial species living in oligotrophic environments5,6. When these species are subjected to nutrient limitation, stalks elongate to increase the effective length and surface area of the cells7, thereby increasing the rate of nutrient uptake8,9. The thin cylindrical stalk is composed of inner and outer membranes separated by peptidoglycan10, and compartmentalized by proteinaceous structures called ‘cross-bands’9,11 (Fig. 1a). In the Caulobacteraceae family, stalk synthesis occurs at a specific stage of a dimorphic life cycle in which a non-replicating motile swimmer cell differentiates into a sessile stalked cell11 (Fig. 1b). In C. crescentus, the stalk is positioned at a single cell pole; in Asticcacaulis excentricus, the stalk is synthesized at a subpolar position off-centre from a cell pole; and in Asticcacaulis biprosthecum, two stalks are positioned bilaterally on the cell body4 (Fig. 1a).

The natural variation in stalk location provides an opportunity to study the mechanisms underlying the precise targeting of cell envelope growth zones to generate different morphologies. Stalks in C. crescentus are synthesized from their base12 by insertion of peptidoglycan within a small area of the cell body13,14. To test whether this mechanism is conserved in the genus Asticcacaulis, we used pulse-chase labelling with Texas Red succinimidyl ester (TRSE)15,16 to study cell envelope growth and a fluorescent D-amino acid to label regions of peptidoglycan synthesis13. The stalks of A. excentricus and A. biprosthecum are also synthesized by insertion of peptidoglycan at their base (Extended Data Fig. 1a, b), suggesting that all three species share the same stalk synthesis mechanism. In light of the above results, we considered that if a conserved stalk morphogen exists, it must localize to the base of stalks. Because many evolutionary trajectory of stalk positioning with the predicted origin of morphology calibrated by 16S rRNA identity26. Colours of shading, branches and SpmX (filled circles) denote the polar (red), subpolar (purple) and bilateral (yellow) stalk positioning, respectively. The size of SpmX is indicated in amino acids (aa). NP, orthologues not present. Bracket indicates subpolar stalked species that share the last common ancestor with the A. biprosthecum clade. Scale bar, number of substitutions per site. Myr, million years.

c, Domain organization of SpmX; transmembrane domains (TM) are shown as grey bars.

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Figure 1 | Natural variation and evolution of stalk positioning correlates with SpmX localization. a, Left, schematic of the stalk. CB, cross-band; IM, inner membrane; OM, outer membrane; PG, peptidoglycan. Right, transmission electron micrographs of representative species. b, Dimorphic life cycle of C. crescentus. SpmX (green) and holdfast (red). c, Structured illumination microscopy images of cells with outer membrane protein stained with Pacific Blue succinimidyl ester (blue), SpmX–eGFP (green) and fluorescent-lectin-bound holdfast (red). Data are representative of three biological repetitions. Scale bars, 1 μm. d, Phylogenetic tree and inferred evolutionary of stalk positioning with the predicted origin of morphology calibrated by 16S rRNA identity26. Colours of shading, branches and SpmX (filled circles) denote the polar (red), subpolar (purple) and bilateral (yellow) stalk positioning, respectively. The size of SpmX is indicated in amino acids (aa). NP, orthologues not present. Bracket indicates subpolar stalked species that share the last common ancestor with the A. biprosthecum clade. Scale bar, number of substitutions per site. Myr, million years.

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proteins localize at the pole in *C. crescentus*<sup>17</sup>, we took advantage of the non-polar localization of the stalks in *Asticcacaulis* to identify stalk morphogen candidates. We constructed fluorescent protein fusions to orthologues of the pole-localized proteins from *C. crescentus* DivJ, PleC, PopZ and SpmX and analysed their localization in *A. biprosthecum*. Strikingly, only the regulatory histidine kinase DivJ<sup>18</sup> (Extended Data Fig. 1a) localized at the base of the stalks in *A. biprosthecum*. During the cell cycle, *A. biprosthecum* DivJ–enhanced green fluorescent protein (eGFP) localized at the base of stalks only after cytokinesis, during swarmer to stalked cell differentiation (Extended Data Fig. 2b). In stark contrast, SpmX–eGFP localized to bilateral positions in the incipient swarmer half of the pre-divisional cell before cytokinesis and subsequent stalk synthesis (Extended Data Fig. 1c, e). Therefore SpmX localization precedes both DivJ localization and stalk synthesis, potentially marking the future site of stalk synthesis.

Interestingly, although the *A. biprosthecum* div<sup>−</sup> mutant still synthesized bilateral stalks (Extended Data Fig. 2a), the *A. biprosthecum* spmX<sup>−</sup> mutant was stalkless (Fig. 2a). Moreover, although newly synthesized peptidoglycan material co-localized with SpmX–eGFP in wild-type cells, no bilateral foci of fluorescent n-ε-amino acid staining were observed in the absence of SpmX (Extended Data Fig. 1h, j), demonstrating that SpmX is required for stalk peptidoglycan synthesis in *A. biprosthecum*. Finally, stalk elongation only occurred when SpmX was expressed (Extended Data Fig. 1f), suggesting that SpmX is required both for the initiation and the elongation of stalk synthesis in *A. biprosthecum*. Similar results were obtained for *A. excentricus* (Fig. 1c, middle; Extended Data Fig. 1d, e, g, i, k), suggesting that the role of SpmX is conserved in both *Asticcacaulis* species. Notably, SpmX is not required for stalk synthesis in *C. crescentus*. Because the *Caulobacter* genus diverged earlier than *Asticcacaulis* (Fig. 1d), we conclude that SpmX has been co-opted for stalk synthesis in *Asticcacaulis*. However, despite its newly acquired role in stalk synthesis, the ancestral function of SpmX in DivJ localization has been retained in *A. biprosthecum* (Extended Data Fig. 2c).

![Figure 2](image)

**Figure 2** SpmX specifies the location of stalk synthesis in *Asticcacaulis*. a, SpmX is required for stalk synthesis in *Asticcacaulis*. Transmission electron microscopy images of *Asticcacaulis* species and their respective spmX<sup>−</sup> mutants. Data are representative of five biological repetitions. b, Heat maps of the localization patterns of SpmX–eGFP in three species with differentially positioned stalks. The number (n) of foci quantified is shown at the bottom of each map. c, d, Microscopy images and heat maps of the *A. biprosthecum* and *A. excentricus* spmX<sup>−</sup> mutant expressing SpmX<sub>AB(L−)</sub>–eGFP or SpmX<sub>AE(S−)</sub>–eGFP. The percentage represents the stalk synthesis ability for each strain compared with the control spmX<sup>+</sup> mutant expressing native SpmX–eGFP.

To test the hypothesis that SpmX has a pivotal role in the evolutionary transitions in stalk positioning, we performed cross-complementation experiments by expressing heterologous SpmX and SpmX fusions in wild-type or spmX mutant strains of the two *Asticcacaulis* species and quantitatively analysed SpmX localization. (Fig. 2 and Extended Data Figs 3–6). When we expressed SpmX–eGFP in either the homologous or heterologous wild-type backgrounds, SpmX both localized and drove stalk synthesis at its host-specific location, suggesting that the endogenous SpmX may be able to recruit the heterologous SpmX (Extended Data Fig. 4b, c, h, i). To test this possibility, we expressed heterologous SpmX in the absence of the native spmX gene. Strikingly, when SpmX from the subpolar stalked species *A. excentricus* (SpmX<sub>AE(S−)</sub>–eGFP) was expressed in the bilateral stalked species *A. biprosthecum* spmX<sup>−</sup> mutant, it localized to and drove stalk synthesis at a subpolar position (Fig. 2c and Extended Data Fig. 7). (For simplicity throughout the paper, we use SpmX<sub>CC(P−)</sub>, SpmX<sub>AE(S−)</sub> and SpmX<sub>AB(L−)</sub> to denote SpmX from *C. crescentus* (CC), *A. excentricus* (AE) and *A. biprosthecum* (AB), with the subscripts P, S and L indicating their native polar, subpolar and lateral positioning, respectively.) Therefore, *A. excentricus* SpmX can recruit the heterologous stalk synthesis machinery of *A. biprosthecum* to synthesize a stalk at an ectopic subpolar position. In contrast, when SpmX from the bilateral stalked species *A. biprosthecum* (SpmX<sub>AB(L−)</sub>–eGFP) was expressed in the subpolar stalked species *A. excentricus* spmX<sup>−</sup> mutant, it localized mostly to poles where it induced stalk synthesis (Fig. 2d and Extended Data Fig. 7). These results indicate that although the subpolar positional information exists in *A. biprosthecum* and can be recognized by SpmX<sub>AE(S−)</sub>–eGFP, the specific bilateral positional information present in *A. biprosthecum* is absent or not recognizable in *A. excentricus*. Remarkably, these observations also suggest that *A. excentricus* possesses the ability to synthesize polar stalks in the absence of its endogenous
SpmX. Indeed, phosphate starvation, which stimulating stalk synthesis in wild-type strains of all three species (Extended Data Fig. 2f, g), rescued stalk synthesis in *A. excentricus* *spmX*^-^ cells, but stalks were located at the pole (Extended Data Fig. 2e, g, h). Using holdfast polysaccharide adhesion as a polar marker (Fig. 1c), we found that stalks from phosphate-starved *A. excentricus* *spmX*^-^ cells were tipped by a holdfast (Extended Data Fig. 2e), confirming that they were synthesized polarly. We infer that *A. excentricus* possesses an alternative polar stalk synthesis mechanism that is normally masked by the endogenous SpmX-driven subpolar stalk synthesis mechanism (as detailed in Supplementary Information).

In contrast, the *A. biprosthecum* *spmX*^-^ mutant remained stalkless when starved for phosphate (Extended Data Fig. 2f), indicating that the *spmX*-independent pathway for stalk biosynthesis has been lost in *A. biprosthecum*, or is no longer regulated by phosphate starvation. We conclude that both *Asticcacaulis* species possess the ability to synthesize stalks at exogenous positions, which is masked by the effects of the endogenous SpmX in wild-type cells. We reasoned that the exogenous positions of stalk synthesis are phylogenetically ancestral, and we next sought to infer the evolutionary trajectory of stalk positioning.

To improve the phylogenetic resolution of stalk positioning, we sequenced the genomes of several extra species from two sister caulobacteriaceae families (*Maricaulis maris* and *Oceanicaulis alexandrii*) and inferred their phylogeny (see Methods and Fig. 1d). Based on parsimony, the emergence of the polar stalk morphology occurred very early. In addition, two subpolar stalked strains, *Asticcacaulis benevestitus* and *Asticcacaulis* sp. AC466 (Fig. 1d, bracket), diverged from the same ancestor that led to the sub-clade containing *A. biprosthecum*, indicating that subpolar stalk synthesis is ancestral to bilateral stalk synthesis. In conclusion, stalk positioning evolved from an ancestral single polar stalk to a single subpolar stalk, and subsequently to bilateral stalks.

We next sought to understand how SpmX has evolved at the protein level by testing the requirement of its main domains for localization and stalk synthesis (Fig. 1e and Extended Data Fig. 8). We constructed a set of truncated alleles removing various domains of *A. biprosthecum* SpmX, which failed to localize or rescue the stalkless phenotype of the *A. biprosthecum* *spmX*^-^ mutant (Extended Data Fig. 7d, e). The muramidase domain and the carboxy (C)-terminal region (intermediate region and transmembrane domains) of SpmX are indispensable for its localization and function. To determine what region of SpmX evolved to specify the location of stalk synthesis, we constructed chimaeric SpmX proteins by mixing and matching the muramidase and the C-terminal regions of different SpmX proteins. In each case, the phenotype of the *spmX*^-^ mutants expressing the various chimaeras correlated with the source of their C-terminal region (Fig. 3, Extended Data Fig. 4 and Supplementary Information). We conclude that mutations in the SpmX C-terminal region are responsible for the evolution of the ability of SpmX to drive stalk synthesis from polar to subpolar to bilateral positions.

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**Figure 3 | Evolution of the C-terminal region of SpmX drives the morphological transition in stalk positioning.** a, Schematic of inferred evolutionary trajectory of stalk positioning. b-g, Microscopy images, heat maps and angle profile analysis for the *A. excentricus* *spmX*^-^ mutant expressing SpmXCC(P) (b), the chimaera SpmXCC(P)-AE(S) (c), SpmXAB(L)-AE(S) (e) and SpmXAB(L)-AB(S) (f). h-j, Microscopy images, heat maps and angle profile analysis for the *A. biprosthecum* *spmX*^-^ mutant expressing the chimaera SpmXAB(L)-AE(S) (h) and SpmXAB(L)-AB(S) (i). Percentages indicate stalk synthesis ability compared with the respective controls as in Fig. 2. Error bars in the angle profiles denote standard deviation of the sample evaluated by the Jackknifing method (see Methods). All profiles of absolute angles (θ) were generated by measuring the data points in associated heatmaps (same n) and were analysed by non-parametric statistical methods, as detailed in Supplementary Table 1 and Methods. ***p < 0.001. Scale bars, 1 μm.
Morphological transitions generate the diversity of biological forms. A few cases have been studied in eukaryotes, highlighting the importance both of changes in regulatory sequences and of functional protein evolution19–23. Our study has begun to unravel the elusive mechanisms of morphological transitions in bacteria by showing that evolution of the SpmX morphogen underlies the evolutionary trajectory of stalk positioning in the Caulobacteraceae family. Polar stalk synthesis arose from non-stalked species before the divergence of the Caulobacteraceae and Hyphomicrobiumadaceae families, but C. crescentus SpmX is not required for stalk synthesis, probably representing the ancestral state. Through differential protein evolution, changes in the SpmX C-terminal region led to stalk synthesis and positioning functions in the Asticcacaulis clade. Interestingly, the ancient polar targeting mechanism is conserved in Asticcacaulis because SpmXCC(P) can localize to the pole in both A. eccentricus and A. biprosthecum (Fig. 3b and Extended Data Figs 4a, g and h). Conversely, both SpmXARIS and SpmXARIS can still localize to the polar target in the C. crescentus strains, suggesting the lack of recognizable alternative targets (Extended Data Fig. 9). During the transition from polar to subpolar stalk positioning, the C-terminal region of SpmX evolved to position and coordinate the synthesis of stalks, coupled with its co-option at the subpolar target in A. eccentricus (Fig. 4). Further divergence of the C-terminal region of SpmX led to its ability to recognize new targets, combined with its co-option at the bilateral targets in A. biprosthecum (Fig. 4).

Our results highlight the modular nature of the positioning mechanism that directs the zonal peptidoglycan synthesis responsible for stalk synthesis. This modularity is evident in both Asticcacaulis species, because SpmX always localizes at the base of ectopically synthesized stalks in several genetically engineered strains (Figs 2 and 3 and Extended Data Fig. 2d). In addition, the fact that changes in the abundance of SpmX alone can alter the number of stalks in A. eccentricus (Extended Data Fig. 2d) suggests that simple changes in the regulation of SpmX expression could drive the evolution of a species with several subpolar stalks. Conceptually, to position the stalk around the cell body, the cells only need to evolve the ability to localize SpmX to a new subcellular position, where it recruits the stalk synthesis module. This morphogenetic modularity could be exploited in synthetic biology to generate the optimal cell shape for a given process.

Finally, this study has demonstrated that functional evolution of a regulatory protein into a morphogenetic module made the evolution of stalk positioning possible, which in turn generated distinct cellular morphologies. Therefore, protein evolution, co-option and modularity can drive morphological transitions both in single-celled prokaryotes and in multicellular eukaryotes, contributing to the diversity of Darwin’s endless forms most beautiful24 from the microscopic to the macroscopic world.

METHODS SUMMARY

C. crescentus, A. eccentricus and A. biprosthecum strains were used in this study. Strains were grown in liquid peptone yeast extract (PYE) medium at 30 °C for C. crescentus and 26 °C for the Asticcacaulis strains. A detailed list of strains and plasmids and their methods of construction is provided in the Supplementary Information and Methods. For the quantitative analysis of fluorescent protein fusion localization, cells were incubated for 18 h in the presence of inducer, mounted on a 1% (w/v) agarose pad and imaged. Quantitative subcellular localization of fluorescent protein fusions was performed at sub-pixel resolution using a specifically developed plug-in for ImageJ25. For bioinformatics analysis, orthologs of SpmX were identified using the BLAST suite hosted by the National Center for Biotechnology Information (NCBI) and Integrated Microbial Genomes (IMG). Phylogenetic trees were generated using the maximum likelihood method, and a concatenation of the products of six housekeeping genes was used to infer the phylogeny of species involved. All procedures are detailed in Methods.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions C.J., P.J.B.B. and Y.V.B. designed the experiments. C.J. performed the experiments and A.D. developed the automated image analysis tools. C.J., P.J.B.B., A.D. and Y.V.B. analysed and interpreted the data. C.J. and Y.V.B. wrote the paper. C.J., P.J.B.B., A.D. and Y.V.B. edited the paper.

Author Information Genomic data of strains sequenced in this study are deposited in GenBank/EMBL/DDBJ under accession numbers AWGD00000000 (Asticcacaulis sp. AC460), AWGE00000000 (Asticcacaulis sp. AC466), AWGC00000000 (Asticcacaulis sp. AC402), AWGF00000000 (Asticcacaulis sp. YBE204) and AWGB00000000 (Asticcacaulis benevestitus DSM16100). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Y.V.B. (ybrun@indiana.edu).
METHODS

Bacterial strains and growth conditions. All C. crescentus, A. excentricus and A. biprosthecum strains used in this study were grown in liquid PYE medium at 30 °C for C. crescentus and 26 °C for the rest to early stationary phase. Strains were maintained on PYE plates supplemented with antibiotics as necessary (kanamycin 20 μg ml⁻¹, tetracycline 2 μg ml⁻¹ and gentamicin 5 μg ml⁻¹). For phosphate starvation, cells were grown in Hutner base-imidazole-buffered-glucose (HIGG) medium containing 30 mM phosphate (phosphate-limited), supplemented with biotin at 40 ng ml⁻¹. For induction, 0.05% (w/v) xylose was used (0.1% for overexpression). To quantify the localization of fluorescent protein fusions in various strains, cells were incubated for 18 h in the presence of inducer, mounted on a 1% (w/v) agarose pad and imaged. To evaluate stalk synthesis ability, these cells were further sub-cultured by diluting 30-fold, grown for another 24 h under induction to maximize stalk synthesis potential, mounted on a 1% (w/v) agarose pad and imaged. A detailed list of strains and plasmids and their methods of construction is included in the plasmid/strain construction sections (Supplementary Table 1).

Recombinant DNA methods. DNA ligation, restriction digests and PCR were performed according to manufacturers’ recommendations. Primers (Eurolinos MWG Operon Technologies) used in this study are listed in Supplementary Table 1. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. Plasmids were purified from Escherichia coli (α-selected competent cells, Bioline) using Zapp plasmid mini-prep kit (Zymo Research Corporation). Plasmids were introduced into E. coli (α-selected competent cells, Bioline) by chemical transformation. Plasmids were introduced into C. crescentus, A. excentricus and A. biprosthecum by electroporation using protocols based on previously published studies. Insertional mutations (gene disruption) in respective organisms were generated using homologous recombination with the integrating plasmid pBGST18 (ref. 28) containing a 500–600 base pair internal fragment from the gene of interest. Sequencing was performed by the Indiana Molecular Biology Institute, Indiana University, Bloomington, Indiana, using diodeoxynucleotide chain termination and ABI BigDye (Applied Biosystems) with double stranded plasmid or PCR templates, which were purified with Qiaquick gel extraction columns (Qiagen). Chromosomal DNA was purified using the Promega Magic MiniPrep DNA purification system (Promega).

Microscopy. Fluorescence imaging used an upright Nikon 90i with a Plan Apo ×100/1.40 Oil Ph3 DM objective, with 51017 CFP/YFP and 83700 DAPI/FITC/Texas Red filter cubes from Chroma, and a Photometrics Cascade 1K EMCCD camera. In addition, fluorescence imaging and time-lapse imaging were done on an inverted Nikon Ti-E, using a Plan Apo ×60/1.40 Oil Ph3 DM or Plan Apo VC ×60/1.40 Oil DIC N2 objective, with a GFP/Red filter cube, and an Andor DU885 EM CCD camera. Images were captured using NIS Elements (Nikon).

The structured illumination microscopy used a prototype of the Deltavision(OM) XM V2.0 (Applied Precision) with ×100 1.40 NA oil objective (UplanSapo, Olympus) and 1.514 index immersion oil. Fluorescence excitation was performed using 405/ 488/561 nm lasers for DAPI/green/red fluorophores. Fluorescence images were captured by a dedicated Cascade II 512 EM-CCD camera (Photometrics). Acquisition was controlled by the DV-OMX controller software (Applied Precision). Reconstructions were made with the OMX specific SoftWorx v4.5.0 software package (Applied Precision).

For transmission electron microscopy, droplets of exponentially growing cultures were deposited onto electron microscopy carbon-formvar-coated copper grids (200 mesh) and cells were allowed to settle for 30 min. Excess liquid was removed with filter paper, and the grids were quickly washed four times by floating onto (200 mesh) and cells were allowed to settle for 30 min. Excess liquid was removed with filter paper, and the grids were quickly washed four times by floating onto

Image analysis. For each experiment, stacks of images were first normalized to correct for background fluctuations over time or over different fields. If required, the background intensity of phase contrast images was subtracted to optimize auto-thresholding operations. Cells boundary, centrel ine and pole position were detected with a specifically developed plug-in for ImageJ12. Briefly, cells were detected with an auto-thresholding function, and sub-pixel-resolution refined cell contours were obtained using a cubic spline fitting algorithm. Cells in a cluster (cells touching each other) were resolved using an iterative auto-thresholding function. Centrelines of cells were deducted from the skeleton and expanded in both directions to the most probable point maximizing the cell-boundary curvature and minimizing the angle between the centrel ine and the cell boundary. Fluorescent foci were detected using local and sub-pixel-resolution maxima detection algorithms. For each focus, the distance from the pole (radial coordinate, r) and the angle (angular coordinate, Θ) were determined and used to plot foci localization on a normalized heat map. By identifying a point means that the angle Θ is measured clockwise from the polar axis formed by the cell centrel ine. A null angular coordinate means that the focus is localised at the tip of the cell pole, whereas 90° means that the focus is localized on cell sides. Because cells are axially symmetrical, the position of each focus in the cell was oriented to the upper left corner of the heat map. For cells with more than one focus, the predominant position of foci localization was oriented to the upper left corner, with its relative position to the other foci conserved on the heat map. For angle profiles, only the angular coordinate Θ was used as it reflects the positioning of SpmX images, as shown in this study. Pearson’s correlation coefficient was calculated between the intensities of pixels above the background value and inside each cell boundary from the channel corresponding to SpmX–eGFP images and the channel corresponding to SpmX–mCherry images.

Statistical analysis. For all experiments, data were obtained from biological replicates (n ≥ 2) and technical replicates (n ≥ 2). Statistical analysis used the R software

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PCR fragments were treated with KpnI/SacI or NdeI/KpnI and ligated into equally treated pGFC/PCHYC-1/2.

For spmXAB truncation studies, nucleotide fragments coding for protein sequences ending at 150 amino acids and 750 amino acids were amplified using their respective primer pairs (ABspmXMTmdElfKpnI/ABspmXMTmdElRsaI for 1–750 amino acids and AspXCTeRdelfKpnI/AspXCTeRdelaSacI for 1–150 amino acids), treated with KpnI/SacI and ligated into equally treated pCHYC-2 to create pCHYC-2-1-750aa-spmA-KpnI and pCHYC-2-1-150aa-spmA-KpnI.

Insertion mutagenesis of the C-terminal internal fragments of respective genes (spmAAB, spmAEB, spmXAB, and divLAB) were amplified using their corresponding primer pairs (ABspmXIKOFPl/ABspmXIKORSacPl and AspXmIKOFKPl/AspXmIKORSacPl), treated with PstI/SacI or KpnI/SacI and ligated into equally treated pBGST18 plasmid to create pBGST18-spmAAB, pBGST18-spmAEB, and pBGST18-divLAB.

Strain construction. Strains expressing various spmX alleles in different backgrounds of different species: strains YB6607, 6616, 6665, 6674, 6917, 6920 and 7134 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 6664, 258, 6913, 5873 and 135, respectively.

Strains YB6608, 6615, 6663, 6918, 7131 and 7133 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 6664, 258, 6913, 5873 and 135, respectively.

Strains YB6610, 6618, 6663, 6667 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 6664 and 258, respectively.

Strains YB6611, 6619, 6667, 6677 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 6664 and 258, respectively.

Strains YB6612, 6620, 6670 and 6908 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 6664 and 258, respectively.

Strains YB6613, 6621, 6671, 6909, 7156 and 7157 were constructed by electroporating pRXGFPC-5-spmAEB into YB 6606, 642, 258, 5873 and 135, respectively.

Strains YB6659 and 6660 were constructed by electroporating pRXGFPC/PCHYC-1-150-835aa-spmXAB into YB6606, respectively.

Strains YB7144, 7148 and 7149 were constructed by electroporating pRXCHYC-5-spmAEB into YB6606, 6682 and 6664, respectively.

Strains YB 7145, 7150, 7153 and 7155 were constructed by electroporating pRXCHYC-5-spmAEB into YB6606, 6642, 258, 6664, and 258, respectively.

Strains YB7146 and 7151 were constructed by electroporating pRXMCS-5-spmAEB into YB6606 and 6664, respectively.

Strains YB7147, 7152 and 7154 were constructed by electroporating pRXMCS-5-spmAEB into YB6606 and 6664, respectively.

C-terminal fusion strains: YB5692, 5693, 5694 and 5695 were constructed by electroporating pPGFC-1-spmAAB, pPGFC-2-spmAAB, pCHYC-1-spmAEB, and PCHYC-2-spmAEB into YB642, respectively.

YB6624 and 6625 were constructed by electroporating pGFC-2-divLAB and pGFC-1-divLAB into YB642, respectively. YB7677 was constructed by electroporating pGFC-2-pspmAEB into YB135.

YB6657 and 6658 were generated by electroporating pCHYC-2-1-750aa-spmAEB and pCHYC-2-1-150aa-spmAEB into YB642, respectively.

YB6662 and 6663 were generated by electroporating pGFC-1-spmAEB and pGFC-1-1-spmAEB into YB642, respectively.

Insertional mutant strains: Strains YB 6606, 6623 and 6664 were constructed by electroporating pBGST18-spmAAB, pBGST18-divLAB and pBGST18-spmAEB into YB642 (first two) and YB258, respectively. YB6662 and 6656 were constructed by electroporating pGFC-1-spmAEB and pGFC-1-divLAB into YB6623 and 6666, respectively.

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Extended Data Figure 1 | SpmX localization precedes, and is required for, stalk synthesis in Asticcacaulis. a, Stalk synthesis occurs at the junction between the cell body and stalk in A. biprosthecum and A. excentricus. Cell surface proteins were pulse-labelled with TRSE, excess TRSE was removed and cells were allowed to grow for seven to eight doublings. The cell body and cell body proximal stalk label were diluted by cell elongation, whereas the cell body distal part of the stalk remained stained, indicating that new material is incorporated at the base of the stalk both in A. biprosthecum (left) and in A. excentricus (right). b, Fluorescent D-amino acids track stalk peptidoglycan synthesis to its base. Cells were stained with the fluorescent D-amino acid 7-hydroxycoumarin-amino-D-alanine (HADA) (blue), which labels regions of peptidoglycan synthesis, in A. biprosthecum (left) and A. excentricus (right). c, d, SpmX localization precedes stalk synthesis in Asticcacaulis. Time-lapse microscopy tracks the dynamics of native SpmX–eGFP localization in A. biprosthecum (c) and A. excentricus (d), images taken every 50 (c) and 30 (d) minutes, respectively. Arrows indicate the early localization of SpmX–eGFP in the swarmer compartment of pre-divisional cells. Schematics of dynamic localization patterns are shown on top. e, Structured illumination micrographs of the localization of SpmX–eGFP at different stages of the life cycle in A. biprosthecum (top) and in A. excentricus (bottom). Cells were stained with the red outer membrane protein stain TRSE and SpmX–eGFP was expressed from its native chromosomal locus. SpmX–eGFP localizes at the future position of stalk synthesis in the daughter swarmer-cell compartment of the pre-divisional cell. f, g, SpmX is required for stalk elongation in A. biprosthecum (f) and A. excentricus (g). Cells expressing a xylose-inducible spmX allele were depleted of SpmX and the cell body and stalks were stained with the red outer membrane protein stain TRSE. Cells were then grown in the presence or absence of the xylose inducer. Schematics of the pulse-chase results are shown below each group. h, i, SpmX is required for the initiation of stalk peptidoglycan synthesis. Cells were stained for 5 min with HADA. The blue HADA staining correlates with SpmX–eGFP at the base of the stalks in A. biprosthecum (h) and A. excentricus (i). Dashed arrows indicate where peptidoglycan synthesis co-localizes with the SpmX–eGFP foci at the base of the stalks. Arrows indicate the localization of SpmX–eGFP in the swarmer cell or the swarmer-cell compartment. j, k, HADA staining of the spmX mutant in A. biprosthecum (j) and A. excentricus (k). HADA staining no longer produces the foci seen at the base of stalks in wild-type strains. All data are representative of at least two biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.
Extended Data Figure 2 | DivJ localizes to the base of the stalk, and the effects of SpmX overexpression and phosphate starvation on stalk synthesis. 

a, DivJ localizes to the base of the stalk (left) and the divJ^- mutant still synthesizes stalks (right) in A. biprosthecum. b, Localization dynamics of DivJ--eGFP tracked by time-lapse microscopy; images taken every 80 min. Note that DivJ--eGFP only localizes in the daughter cell after cytokinesis (arrow). 

c, SpmX is required to localize DivJ (top) but DivJ is not required to localize SpmX (bottom) in A. biprosthecum. d, Micrographs of wild-type A. excentricus cells overexpressing SpmX--eGFP. e, Structured illumination micrographs of wild-type (left) and spmX^- (right) A. excentricus cells growing under low phosphate conditions. Cells (red) are stained with TRSE and the polar holdfast (green) is stained with a fluorescent wheat-germ agglutinin lectin. Phosphate starvation induces polar stalk synthesis in the A. excentricus spmX^- mutant, pushing the holdfast to its tip as shown in the right schematic. f, g, Micrographs of wild-type (left, with SpmX--eGFP) and spmX^- (right) A. biprosthecum (f) or A. excentricus (g) grown under phosphate starvation. h, Transmission electron micrographs of A. excentricus grown under phosphate starvation. The diffuse structure around the cell body and the stalk is a sheath whose synthesis is induced in response to environmental stress. All data are representative of at least two biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.
Extended Data Figure 3 | Expression and integrity of various SpmX–eGFP fusions. a, b, Expression of SpmX–eGFP in strains used in this study. All fusion proteins were expressed from a xylose-inducible promoter on a replicating plasmid in the presence of 0.05% (w/v) xylose. SpmX–eGFP expression was measured by quantifying the fluorescence intensity of the fusion proteins. Corrected fluorescence is calculated as (integrated fluorescence – integrated background)/area. Measurements for each strain were done in duplicate and at least 500 cells were quantified in each case. Error bars, standard error of the mean. c, Western blot of SpmX–eGFP fusion proteins expressed from either the chromosomal locus or a replicating plasmid in different species probed with anti-GFP antibody. Wild-type strains were used as controls. Note that only SpmX_{AB}–eGFP seems to have a clipping/degradation pattern. Data are representative of three biological repetitions. d, Western blot of various chimaeric/heterologous SpmX–eGFP fusions when expressed in the A. excentricus spmX^− mutant, probed by anti-GFP antibody. Note that the relative amounts of expression correlate with the results from quantitative fluorescence analysis (a). The size of SpmX–eGFP fusions ranges from 76 to 120 kDa, and free eGFP is expected to be around 31 kDa. Data are representative of three biological repetitions. e, f, SpmX–mCherry and SpmX–eGFP share the same localization pattern in A. biprosthecum (left, Pearson r = 0.79 ± 0.1) and A. excentricus (right, Pearson r = 0.81 ± 0.08) cells. Both strains are expressing SpmX–eGFP fusions from the chromosomal locus and the SpmX–mCherry fusions from a replicating plasmid. Scale bars, 1 μm. Data are representative of two biological repetitions. g, The elevated cytoplasmic fluorescence in A. biprosthecum may correlate with the clipping/degradation pattern of SpmX_{AB}–eGFP. Data are representative of two biological repetitions analysing at least 100 cells each. Error bars, standard deviation.
Extended Data Figure 4 | Localization of SpmX–eGFP fusions in various strain backgrounds. Full length or chimaeric SpmX–eGFPs were expressed in wild-type *A. biprosthecum* and *A. excentricus* as indicated on the left. Representative pictures are shown. All data are representative of three biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.
A. excentricus (AE)  
Subpolar (S)  
C. crescentus (CC)  
Polar (P)  
A. biprosthecum (AB)  
Lateral (L)  

| Organism             | status | Genome Size (Million bps) | Scaffolds | GC%  | Genes | Accession number |
|----------------------|--------|---------------------------|-----------|------|-------|-----------------|
| Asticcacaulis sp. AC460 | PD*    | 5.38                      | 26        | 62%  | 4767  | AWGD0000000000  |
| Asticcacaulis sp. AC466 | PD     | 4.65                      | 30        | 59%  | 4328  | AWGE0000000000  |
| Asticcacaulis sp. AC402 | PD     | 4.28                      | 42        | 61%  | 3848  | AWGC0000000000  |
| Asticcacaulis sp. YBE204 | PD     | 4.12                      | 16        | 61%  | 3915  | AWGF0000000000  |
| Asticcacaulis benevestitus DSM16100 | PD | 4.98                      | 112       | 58%  | 4674  | AWGB0000000000  |

*Permanent draft
Extended Data Figure 5 | Overview of the subcellular localization quantification method and characteristics of newly sequenced genomes.

a, Output images provided by the customized ImageJ-based software package. Fluorescent (left) and corresponding phase contrast (right) images are shown. The cell boundary is represented in green or orange according to the side of the cell. The major and the minor axes of the cell are represented in cyan. In each cell, fluorescent foci of SpmX–eGFP (white spot, left panel) were detected and outlined by a yellow circle centred to their respective sub-pixel resolution positions. The dark blue line links the focus coordinates to its relative position on the major axes.

b, Schematic representation of the polar coordinate system in which each point is defined by three coordinates: the distance from the pole or from the major axis ($r_1, r_2$), the angle relative to the major axis ($h_1, h_2$) and the distance from the mid-cell ($d_1, d_2$). A null angular coordinate means that the focus is localized at the tip of the cell pole, whereas 90° means that the focus is localized on cell sides. The symbol $d_P$ represents the distance between the pole (P or P') and the cell boundary (red dot). $F_1$ and $F_2$ represent subpolar and lateral localized objects with their respective coordinates.

c, Variation of the position of discrete points on the cell boundary relative to the pole (P or P') as determined by the polar coordinates ($n = 100$). Each point represents the normalized position on the major ($r_1/d_P \times \cos h_0$) and minor ($r_2/d_P \times \sin h_0$) axis of discrete positions along the cell boundary in the pole region. The shaded region indicates the interquartile range as a measure of the dispersion between the upper and lower quartiles of values observed for discrete values of $h_0$.

d, Schematic showing how localization of SpmX–eGFP is quantified by measuring the angle ($\theta$) and the radial distance ($r$) of each focus using the geometric centre of the pole as the origin of a polar coordinate system within a normalized cell body (see a–c). Red, purple and yellow coloured circles represent SpmX in C. crescentus (CC), A. excentricus (AE) and A. biprosthecum (AB), respectively. P, S and L in parentheses are shorthand to denote the native polar, subpolar and lateral stalk positioning of their respective SpmX proteins.

e, Density plot of the absolute values of measured angles ($|\theta|$) of heatmaps in Fig. 2b (same $n$). Measurements are binned for every $10^\circ$. Error bars in the angle profiles denote standard deviation of the sample evaluated by the Jackknifing method (see Methods).

f, The general characteristics of five Asticcacaulis genomes. PD denotes the permanent draft status of the genome.

g, Differential interference contrast micrographs of the sequenced strains in (f) except for A. benevestitus, which was previously published. Scale bars, 1 μm.

h, Phylogenetic tree inferred from SpmX sequences of different species. The alignment from Extended Data Fig. 8 was used to infer this tree based on the maximum likelihood method. Note that because E. coli and P. aeruginosa do not have spmX orthologues, species from the Hyphomonadaceae family serve as the outgroup (Maricaulis maris and Oceanicaulis alexandrii). We estimated the statistical support for each node by performing 500 bootstrap repetitions. The overall SpmX protein tree topology matches that of the species tree (Fig. 1d). The only exception is that Brevundimonas subvibriodes is placed differently, owing to the difficulty of resolving its phylogeny. Scale, number of substitutions per position.
Extended Data Figure 6 | SpmX fusion proteins are functionally equivalent to the native SpmXs.  

**a**, Microscopic images and heatmaps of A. excentricus (left) or A. biprosthecum (right) expressing SpmX–mCherry fusions from the chromosomal locus. Note that the localization as well as the function in stalk synthesis is identical to that of the respective spmX::spmX-egfp and wild-type strains (Figs 1 and 2). **b, c**, Microscopic images of the A. excentricus spmX2 (left) and A. biprosthecum spmX2 (right) strains expressing non-tagged SpmX. Note that SpmXAB(L) induces mostly polar stalks in the A. excentricus spmX2 mutant (b) and SpmXAE(S) induces mostly subpolar stalks in the A. biprosthecum spmX2 mutant (c). Both SpmXs were able to complement the stalkless phenotype. **d, e**, Microscopic images and heatmaps of expression of SpmX–mCherry fusions in the A. excentricus spmX2 (left) and A. biprosthecum spmX2 (right) mutants. The phenotypes are identical to that of the non-tagged SpmX (as well as SpmX–eGFP in Fig. 3). **f**, Expression of SpmXAE(S)–mCherry (left) and SpmXAE(S) (right) in wild-type A. excentricus both induce synthesis of many stalks, as is the case for SpmX–eGFP (Extended Data Fig. 2). All data are representative of three biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.
Extended Data Figure 7 | SpmX is required for stalk positioning in Asticcacaulis and the integrity of SpmX is critical for its function in A. biprosthecum. a, Holdfast (red) localizes to the pole both in A. biprosthecum (left) and in A. excentricus (right) spmX mutants. b, Dual-labelling images of SpmX–eGFP (green) and holdfast (red) in the A. biprosthecum spmX mutant complemented by SpmX_<sub>AE(S)</sub> (left) and the A. excentricus spmX mutant complemented by SpmX_<sub>AB(L)</sub> (right). c, SpmX_<sub>AE(S)</sub> localizes and initiates stalk synthesis at the subpolar position in the A. biprosthecum spmX mutant (left). SpmX_<sub>AB(L)</sub> localizes to polar or subpolar locations before triggering either polar and subpolar stalk synthesis in the A. excentricus spmX mutant (right). We noticed that SpmX–eGFP sometimes localizes in the stalk, probably because of stalk outgrowth from its base because it should not be able to diffuse to positions in already synthesized stalks, where diffusion is constrained by cross-bands (Fig. 1a). d, From top to bottom: 1, SpmX–mCherry (825 amino acids + mCherry, 121 kDa) localizes to the base of stalks. This fusion protein was expressed from its native chromosomal locus (spmX::spmX-mCherry); 2–4, different truncated alleles of SpmX fail to complement the stalkless phenotype of the A. biprosthecum spmX mutant. Both C-ter SpmX–mCherry (1–150 amino acids + mCherry, 45 kDa) and TM SpmX–mCherry (1–750 amino acids + mCherry, 111 kDa) were expressed from the native spmX chromosomal locus replacing the natural allele. N-ter SpmX–mCherry (150–825 amino acids + mCherry, 104 kDa) was expressed from a replicating plasmid (YB7129) in the A. biprosthecum spmX mutant. e, Western blot of truncated SpmX–mCherry fusions detected with anti-mCherry polyclonal antibodies. Data are representative of two biological repetitions. Micrographs are representative of three biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.
Extended Data Figure 8 | Alignment of SpmX sequences from species/strains used in this study (as shown in Fig. 1d). Sequences were aligned in Jalview (http://www.jalview.org/). Coloured residues denote conservation across species. Vertical dashed lines denote the boundaries of the N-terminal muramidase domain (black) and the two transmembrane domains (red), respectively. Chimaeras were constructed using the end of the muramidase domains as the boundary between N- and C-terminal moieties (Fig. 3). Red arrows indicate the three main species used in this study: A. biprosthecum, A. excentricus and C. crescentus, respectively. From top to bottom, the order of the sequences reflects the tree topology as deduced from Fig. 1d. The coloured vertical lines next to the names of the species/strains denote their respective stalk positioning as shown in Fig. 1d (yellow, bilateral; purple, subpolar; red, polar). The numbers next to the names of the species/strains indicate the lengths of their respective SpmX proteins.
Extended Data Figure 9 | Expression of SpmXAB(E)–eGFP or SpmXAE(S)–eGFP in C. crescentus does not induce ectopic stalk synthesis. a, b. The localization of SpmXCC(P)–eGFP/SpmXCC(P)–mCherry and the phenotypes of the strains are identical. c–e, Asticcacaulis SpmX–eGFP variants localize mostly to the pole in wild-type C. crescentus (left) and C. crescentus spmX (right) strains. No ectopic stalks were observed, indicating that the alternative targets are lacking in C. crescentus. It should also be noted that the expression of variants of SpmXAE–eGFP can cause cell filamentation. f, SpmXCC–eGFP localizes to the pole in the A. biprosthecum spmX strain and cannot rescue stalk synthesis. All data are representative of three biological repetitions analysing at least 100 cells each. Scale bars, 1 μm.