Supplementary Information

Polar stalk synthesis in the *A. excentricus spmX⁻* mutant.

Phosphate starvation of the *A. excentricus spmX* mutant causes the synthesis of polar stalks whereas it does not in wild-type cells. The most likely explanation for this effect is that, while the ancestral site remains and can be recognized, the native SpmX preferentially localizes to the new, subpolar, position. The preferential localization of the subpolar over the polar position in wild-type cells is probably due to the fact that the SpmX sequences mediating the interaction with the ancestral, polar, target have diverged and cannot recognize it as efficiently as the subpolar target, whose recognition is under selective pressure. This model is supported by the fact that *A. biprosthecum* SpmX can recognize both the polar and the subpolar targets in *A. excentricus*, presumably because the selective pressure maintaining the preferential subpolar target recognition in *A. excentricus* has been removed in *A. biprosthecum*, which synthesizes lateral stalks.

Evolution of the SpmX C-terminal region

To determine what region of SpmX evolved to specify the location of stalk synthesis, we constructed chimeric SpmX proteins by mixing and matching the muramidase and the C-terminal domains (intermediate region and transmembrane domains) of different SpmX proteins. First, we tested whether mutations in the C-terminal region of SpmX after the divergence of *Asticcacaulis* and *Caulobacter* resulted in its ability to drive stalk synthesis in *A. excentricus* (Fig. 1e). We expressed SpmX$_{CC(P)}$ in the *A. excentricus spmX*$^{-}$ mutant, which localized to the pole but failed to rescue stalk synthesis (Fig. 3b). Similarly, SpmX$_{CC(P)}$-EGFP expressed in the *A. biprosthecum spmX*$^{-}$ mutant localized to the pole but failed to rescue stalk synthesis (Extended Data Fig. 9f). We then fused the *C. crescentus* SpmX muramidase domain with the C-terminal region of *A. excentricus* SpmX to generate SpmX$_{CC(P)\text{-}AE(S)}$ and expressed this chimera in wild-type and *spmX*$^{-}$ *A. excentricus*. The hybrid SpmX$_{CC(P)\text{-}AE(S)}$ was able to perform almost like SpmX$_{AE(S)}$, being both able to induce multiple sub-polar stalk formation in the wild-type (Extended Data Fig. 4d) and rescue the synthesis of sub-polar stalks in the *spmX*$^{-}$ mutant (Fig. 3c, d). These results indicate that the evolution of the C-terminal region of SpmX is critical for its ability to drive sub-polar stalk synthesis in *A. excentricus*.

Next, we tested whether evolution of the C-terminal region led to the transition from sub-polar to bi-lateral stalk synthesis. We generated and expressed SpmX$_{AB(L)\text{-}AE(S)}$ and SpmX$_{AE(S)\text{-}AB(L)}$ in wild-type and *spmX*$^{-}$ *A. biprosthecum* and *A. excentricus*. Most notably, SpmX$_{AB(L)\text{-}AE(S)}$ functioned like SpmX$_{AE(S)}$: it induced multiple sub-polar stalk synthesis in wild-type *A. excentricus* (Extended Data Fig. 4e), and rescued sub-polar stalk synthesis in the *A. excentricus spmX*$^{-}$ mutant (Fig. 3e, g). Strikingly, SpmX$_{AB(L)\text{-}AE(S)}$ induced sub-polar stalk synthesis in the *A. biprosthecum spmX*$^{-}$ mutant (Fig. 3h, j). Similarly, SpmX$_{AE(S)\text{-}AB(L)}$ functioned like SpmX$_{AB(L)}$: it induced bi-lateral stalk synthesis in the *A. biprosthecum spmX*$^{-}$ mutant (Fig. 3i, j), and partially induced stalk synthesis at mostly polar positions in the *A. excentricus* *spmX*⁻ mutant (Fig. 3f, g).

In summary, the phenotype of the *spmX*⁻ mutants expressing the various chimeras correlated with the source of their C-terminal region. We conclude that mutations in the SpmX C-terminal region are responsible for the evolution of SpmX's ability to drive stalk synthesis from polar to sub-polar to bi-lateral positions.

Supplementary Table 1. Non-parametric statistical analysis of the SpmX localization profiles and information about primers, plasmids, strains used in the study. P-values are highlighted in red if <0.05.

Supplementary Table 1a. Comparison of native SpmX localization in *C. crescentus* (CC), *A. excentricus* (AE), and *A. biprosthecum* (AB) by the KS-test

	$SpmX_{AB}$	$SpmX_{CC}$	$SpmX_{AE}$
$SpmX_{AB}$	NA	0.00E+00	0.00E+00
$SpmX_{CC}$	0.00E+00	NA	0.00E+00
$SpmX_{AE}$	0.00E+00	0.00E+00	NA

Supplementary Table 1b. Comparison of native SpmX localization in C. crescentus (CC), *A. excentricus* (AE), and *A. biprosthecum* (AB) by the MW-test

	$SpmX_{AB}$	$SpmX_{CC}$	$SpmX_{AE}$
$SpmX_{AB}$	NA	2.83E-193	4.13E-161

SpmX$_{\text{CC}}$	2.83E-193	NA	4.13E-23
SpmX$_{\text{AE}}$	4.13E-161	4.13E-23	NA

Supplementary Table 1c. Comparison of the localization of various SpmX expressed in the *A. biprosthecum spmX*$^{-}$ mutant by the KS-test

	SpmX$_{\text{AB}}$	SpmX$_{\text{AE-AB}}$	SpmX$_{\text{AE}}$	SpmX$_{\text{AB-AE}}$
SpmX$_{\text{AB}}$	NA	1.00E+00	0.00E+00	0.00E+00
SpmX$_{\text{AE-AB}}$	1.00E+00	NA	0.00E+00	0.00E+00
SpmX$_{\text{AE}}$	0.00E+00	0.00E+00	NA	1.00E+00
SpmX$_{\text{AB-AE}}$	0.00E+00	0.00E+00	1.00E+00	NA

Supplementary Table 1d. Comparison of the localization of various SpmX expressed in the *A. biprosthecum spmX*$^{-}$ mutant by the MW-test

	SpmX$_{\text{AB}}$	SpmX$_{\text{AE-AB}}$	SpmX$_{\text{AE}}$	SpmX$_{\text{AB-AE}}$
SpmX$_{\text{AB}}$	NA	5.58E-01	5.81E-58	3.09E-50
SpmX$_{\text{AE-AB}}$	5.58E-01	NA	4.85E-38	1.32E-33
SpmX$_{\text{AE}}$	5.81E-58	4.85E-38	NA	1.00E+00
SpmX$_{\text{AB-AE}}$	3.09E-50	1.32E-33	1.00E+00	NA

Supplementary Table 1e. Comparison of the localization of various SpmX expressed in the *A. excentricus spmX*$^{-}$ mutant by the KS-test

	SpmX$_{\text{AB}}$	SpmX$_{\text{AE-AB}}$	SpmX$_{\text{AE}}$	SpmX$_{\text{AB-AE}}$	SpmX$_{\text{CC-AE}}$	SpmX$_{\text{CC}}$

SpmX$_{AB}$	NA	1.00E+00	0.00E+00	0.00E+00	1.19E-13	1.97E-02
SpmX$_{AE-AB}$	1.00E+00	NA	4.90E-14	5.82E-10	1.91E-08	6.86E-03
SpmX$_{AE}$	0.00E+00	4.90E-14	NA	1.00E+00	1.00E+00	0.00E+00
SpmX$_{AB-AE}$	0.00E+00	5.82E-10	1.00E+00	NA	1.00E+00	0.00E+00
SpmX$_{CC-AE}$	1.19E-13	1.91E-08	1.00E+00	1.00E+00	NA	0.00E+00
SpmX$_{CC}$	1.97E-02	6.86E-03	0.00E+00	0.00E+00	0.00E+00	NA

Supplementary Table 1f. Comparison of the localization of various SpmX expressed in the *A. excentricus spmX*⁻ mutant by the MW-test

	SpmX$_{AB}$	SpmX$_{AE-AB}$	SpmX$_{AE}$	SpmX$_{AB-AE}$	SpmX$_{CC-AE}$	SpmX$_{CC}$
SpmX$_{AB}$	NA	1.00E+00	3.63E-25	5.60E-21	6.72E-14	2.20E-03
SpmX$_{AE-AB}$	1.00E+00	NA	2.90E-11	1.57E-09	1.59E-06	3.74E-03
SpmX$_{AE}$	3.63E-25	2.90E-11	NA	1.00E+00	1.00E+00	5.77E-29
SpmX$_{AB-AE}$	5.60E-21	1.57E-09	1.00E+00	NA	1.00E+00	3.19E-26
SpmX$_{CC-AE}$	6.72E-14	1.59E-06	1.00E+00	1.00E+00	NA	4.95E-20
SpmX$_{CC}$	2.20E-03	3.74E-03	5.77E-29	3.19E-26	4.95E-20	NA

Supplementary Table 1g. Plasmids

YB#	Plasmid name (resistance)	Reference
5899	pRXGFPC-5(TetR)	This study
5902	pRXCHYC-5(TetR)	This study

5900	pRXCFPC-5(TetR)	This study
5901	pRXYFPC-5(TetR)	This study
5895	pRXGFPC-2(KanR)	This study
5898	pRXCHYC-2(KanR)	This study
5896	pRXCFPC-2(KanR)	This study
5897	pRXYFPC-2(KanR)	This study
5877	pBGST18-$spmX_{AB}$ (KanR)	This study
5878	pBGST18-$spmX_{AE}$ (KanR)	This study
5881	pBGST18-$divJ_{AB}$ (KanR)	This study
5681	pGFPC-1-$spmX_{AB}$ (GentR)	This study
5682	pCHYC-1-$spmX_{AB}$ (GentR)	This study
5683	pCFPC-1-$spmX_{AB}$ (GentR)	This study
5684	pGFPC-2-$spmX_{AB}$ (KanR)	This study
5685	pCHYC-2-$spmX_{AB}$ (KanR)	This study
5686	pGFPC-1-$spmX_{AE}$ (GentR)	This study
5687	pCHYC-1-$spmX_{AE}$ (GentR)	This study
5688	pGFPC-1-$divJ_{AB}$ (GentR)	This study
5689	pCHYC-1-$divJ_{AB}$ (GentR)	This study
5690	pCHYC-2-$divJ_{AB}$ (KanR)	This study
5691	pGFPC-2-$divJ_{AB}$ (KanR)	This study
5882	pCHYC-2-*1-750aa*-$spmX_{AB}$ (KanR)	This study
5883	pCHYC-2-*1-150aa*-$spmX_{AB}$ (KanR)	This study
5884	pRXGFPC-5-$spmX_{AB}$ (TetR)	This study

5885	pRXGFPC-5-$spmX_{CC-AB}$ (TetR)	This study
5886	pRXGFPC-5-$spmX_{AE}$ (TetR)	This study
5888	pRXGFPC-5-$spmX_{AB-AE}$ (TetR)	This study
5889	pRXGFPC-5-$spmX_{AE-AB}$ (TetR)	This study
5890	pRXGFPC-5-$spmX_{CC}$ (TetR)	This study
5891	pRXGFPC-5-$spmX_{CC-AE}$ (TetR)	This study
5892	pRXCHYC-5-$spmX_{AB}$ (TetR)	This study
5893	pRXCHYC-5-$spmX_{AE}$ (TetR)	This study
5894	pRXGFPC-5-*150-835aa*-$spmX_{AB}$ (TetR)	This study
7129	pRXCHYC-5-*150-835aa*-$spmX_{AB}$ (TetR)	This study
7142	pRXMCS-5-$spmX_{AB}$ (TetR)	This study
7143	pRXMCS-5-$spmX_{AE}$ (TetR)	This study
7678	pGFPC-2- $spmX_{CC}$ (GentR)	This study

Supplementary Table 1h. Strains

YB#	Genotype (resistance)	Reference
	***E. coli* strains**	
-----	Alpha Select - Silver Efficiency	Bioline USA, Inc.
	Wild type strains	
642	*Asticcacaulis biprosthecum* C19	[1]
258	*Asticcacaulis excentricus* AC48	[2]

5874	*Asticcacaulis benevestitus DSM16100*	[3]
135	*Caulobacter cresentus CB15*	[2]
3927	*Asticcacaulis sp. AC402,* isolated from Gull Lake, Michigan	Jeanne Poindexter
3929	*Asticcacaulis sp. AC460,* isolated from School Street Marsh, Woods	Jeanne Poindexter
3932	*Asticcacaulis sp. AC466,* isolated from School Street Marsh, Woods	Jeanne Poindexter
5875	*Asticcacaulis sp. YBE204,* isolated from a construction pond.	This study
	***A. biprosthecum* strains**	
5692	YB642 *spmX::spmX-egfp* (GentR)	This study
5693	YB642 *spmX::spmX-egfp* (KanR)	This study
5694	YB642 *spmX::spmX-mcherry* (GentR)	This study
5695	YB642 *spmX::spmX-mcherry* (KanR)	This study
6606	YB642 $spmX^{-}$ (KanR) insertional gene disruption by pBGTS18	This study
6607	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{AB}$(TetR KanR)	This study
6608	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{AE}$ (TetR KanR)	This study
6610	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{AB\text{-}AE}$ (TetR KanR)	This study
6611	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{AE\text{-}AB}$ (TetR KanR)	This study
6612	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{CC}$(TetR KanR)	This study
6613	YB6606 $spmX^{-}$ pRXGFPC-5-$spmX_{CC\text{-}AE}$ (TetR KanR)	This study
6615	YB642 pRXGFPC-5-$spmX_{AE}$ (TetR)	This study
6616	YB642 pRXGFPC-5-$spmX_{AB}$ (TetR)	This study

6618	YB642 pRXGFPC-5-*spmX*$_{AB\text{-}AE}$ (TetR)	This study
6619	YB642 pRXGFPC-5-*spmX*$_{AE\text{-}AB}$ (TetR)	This study
6620	YB642 pRXGFPC-5-*spmX*$_{CC}$ (TetR)	This study
6621	YB642 pRXGFPC-5-*spmX*$_{CC\text{-}AE}$ (TetR)	This study
6623	YB642 *divJ*$^{-}$ (KanR) insertional gene disruption by pBGTS18	This study
6624	YB642 *divJ::divJ-egfp* (KanR)	This study
6625	YB642 *divJ::divJ-egfp* (GentR)	This study
6626	YB6623 *divJ*$^{-}$ *spmX::spmX-egfp* (KanR GentR)	This study
6656	YB6606 *spmX*$^{-}$ *divJ::divJ-egfp* (KanR GentR)	This study
6657	YB642 *spmX::1-750aa-spmX-mcherry* (KanR)	This study
6658	YB642 *spmX::1-150aa-spmX-mcherry* (KanR)	This study
6659	YB6606 *spmX*$^{-}$ pRXCHYC-5-*150-835aa-spmX*$_{AB}$(KanR TetR)	This study
6660	YB6606 *spmX*$^{-}$ pRXGFPC-5-*150-835aa-spmX*$_{AB}$(KanR TetR)	This study
7144	YB6606 *spmX*$^{-}$ pRXCHYC-5-*spmX*$_{AB}$(TetR KanR)	This study
7145	YB6606 *spmX*$^{-}$ pRXCHYC-5-*spmX*$_{AE}$ (TetR KanR)	This study
7146	YB6606 *spmX*$^{-}$ pRXMCS-5-*spmX*$_{AB}$(TetR KanR)	This study
7147	YB6606 *spmX*$^{-}$ pRXMCS-5-*spmX*$_{AE}$ (TetR KanR)	This study
7148	YB5692 *spmX::spmX-egfp* pRXCHYC-5-*spmX*$_{AB}$(GentR TetR)	This study
	***A. excentricus* strains**	
6662	YB258 *spmX::spmX-egfp* (GentR)	This study
6663	YB258 *spmX::spmX-mcherry* (GentR)	This study
6664	YB258 *spmX*$^{-}$(KanR) insertional gene disruption by pBGTS18	This study
6665	YB6664 *spmX*$^{-}$ pRXGFPC-5-*spmX*$_{AB}$ (TetR KanR)	This study

6666	YB6664 *spmX*⁻ pRXGFPC-5-*spmX*$_{AE}$ (TetR KanR)	This study
6668	YB6664 *spmX*⁻ pRXGFPC-5-*spmX*$_{AB\text{-}AE}$(TetR KanR)	This study
6669	YB6664 *spmX*⁻ pRXGFPC-5-*spmX*$_{AE\text{-}AB}$ (TetR KanR)	This study
6670	YB6664 *spmX*⁻ pRXGFPC-5-*spmX*$_{CC}$ (TetR KanR)	This study
6671	YB6664 *spmX*⁻ pRXGFPC-5-*spmX*$_{CC\text{-}AE}$(TetR KanR)	This study
6673	YB258 pRXGFPC-5-*spmX*$_{AE}$ (TetR)	This study
6674	YB258 pRXGFPC-5-*spmX*$_{AB}$(TetR)	This study
6676	YB258 pRXGFPC-5-*spmX*$_{AB\text{-}AE}$ (TetR)	This study
6677	YB258 pRXGFPC-5-*spmX*$_{AE\text{-}AB}$(TetR)	This study
6908	YB258 pRXGFPC-5-*spmX*$_{CC}$ (TetR)	This study
6909	YB258 pRXGFPC-5-*spmX*$_{CC\text{-}AE}$ (TetR)	This study
7149	YB6664 *spmX*⁻ pRXCHYC-5-*spmX*$_{AB}$ (TetR KanR)	This study
7150	YB6664 *spmX*⁻ pRXCHYC-5-*spmX*$_{AE}$ (TetR KanR)	This study
7151	YB6664 *spmX*⁻ pRXMCS-5-*spmX*$_{AB}$ (TetR KanR)	This study
7152	YB6664 *spmX*⁻ pRXMCS-5-*spmX*$_{AE}$ (TetR KanR)	This study
7153	YB258 pRXCHYC-5-*spmX*$_{AE}$ (TetR)	This study
7154	YB258 pRXMCS-5-*spmX*$_{AE}$ (TetR)	This study
7155	YB6662 *spmX::spmX-egfp* pRXCHYC-5-*spmX*$_{AE}$ (TetR GentR)	This study
	***C. crescentus* strain**	
6556	*spmX::spmX-mcherry*	[4]
5873	*ΔspmX*	[4]
7677	*spmX::spmX-egfp* (GentR)	This study
6920	YB5873 *ΔspmX* pRXGFPC-5-*spmX*$_{AB}$ (TetR)	This study

7131	YB5873 *ΔspmX* pRXGFPC-5-$spmX_{AE}$ (TetR)	This study
7156	YB5873 *ΔspmX* pRXGFPC-5-$spmX_{CC\text{-}AE}$ (TetR)	This study
7134	YB135 pRXGFPC-5-$spmX_{AB}$ (TetR)	This study
7133	YB135 pRXGFPC-5-$spmX_{AE}$ (TetR)	This study
7157	YB135 pRXGFPC-5-$spmX_{CC\text{-}AE}$ (TetR)	This study
7158	YB5873 *ΔspmX* pRXGFPC-5-$spmX_{CC}$ (TetR)	This study

Supplementary Table 1i. Oligonucleotides

ABspmXXfFNdeI	GATACCATATGAGAGCGCGTCAAAAGGTTTC
ABspmXXfRKpnI	CTGGTACCTAAGTCCTTGAGGCCGC
AEspmXXfFNdeI	GATACCATATGAGAGCGCGCCATAAAATCTC
AEspmXXfRKpnI	CGTCGGTACCTAAGTCCTTCAAACCACCTA
CCspmXXfFNdeI	GACACCATATGAAACCGCGTCATCAGG
CCspmXXfRAflII	CAACTTAAGGTCAACTCTTCGTCGCTCACA
ABspmXXflRAflII	CAACTTAAGGTTAAGTCCTTGAGGCCGC
AEspmXXflRAflII	CAACTTAAGGTTAAGTCCTTCAAACCACCTA
ABspmXmuraR	ATC CGG TGT CAG GAA CAG GTT CTT TTC GGC GG
AEspmXmuraR	ATC CGG TGT CAG GAA CAG GCT CTT TTC AGA GG
ABspmXperiF	CTGTTCCTGACACCGGATGAAGCCTCCGGCAA
AEspmXperiF	CTGTTCCTGACACCGGATATGGAGGGGATGGA
CCspmXmuraR	ATCCGGTGTCAGGAACAGAGTCTTCTCGGC
CCspmXperiF	CTGTTCCTGACACCGGATAACGGCGAATGGGTCC

ABspmXCterfFKpnI	CGGGTACCAATCCCTATGCCAAGCCCGTT
ABspmXCterfRSacI	CGGAGCTCGACAGTCCTTGAGGCCGCCAA
ABdivJCterfFKpnI	GGGGTACCAACCTTCGACCTGCGCGAG
ABdivJCterfREcoRI	CGGAATTCCCACTCTTCGGCGGGCGGATG
AEspmXCterfFKpnI	CGGGTACCGGACGACGAGGTAGAGGATG
AEspmXCterfRSacI	GAGAGCTCGTAAGTCCTTCAAACCACCTA
ABspmXNterdelFNdeI	GATAGCATATGGACGAAGCCTCCGGCAATGC
ABspmXNterdelRKpnI	CGGGTACCTAAGTCCTTGAGGCCGCCA
ABSpmXTMdelFKpnI	CAGGTACCCGCCTGACACCGACTCCGAC
ABSpmXTMdelRSacI	GAGAGCTCGAGTCTCGTCCGCCGACAGGG
ABspmXCterdelFKpnI	CGGGTACCATGAGAGCGCGTCAAAAGGTTTC
ABspmXCterdelRSacI	GAGAGCTCGCGGCGTCAGAAACAGGTTCTTT
ABspmXIKOFPstI	AGACTGCAGGGGACGAAGCCCAGCAGGATA
ABspmXIKORSacI	GAGAGCTCTTACTCTTCGTCGCTGGGCTGGCT
AEspmXIKOFKpnI	CAGGTACCCCCAAAGCCCGTCATCACAC
AEspmXIKORSacI	GAGAGCTCCCAGAGCCTGAGCCTGACGC
AEspmXRKpnI	CGTCGGTACCTCAGTCCTTCAAACCACCTA
ABspmXRKpnI	GTCGGTACCTCAGTCCTTGAGGCCG

Sequencing primers are not included in this list.

References:

1 Pate, J. L. & Ordal, E. J. The fine structure of two unusual stalked bacteria. *J Cell Biol* **27**, 133-150 (1965).

2 Poindexter, J. S. Biological Properties and Classification of the Caulobacter Group. *Bacteriol Rev* **28**, 231-295 (1964).

3 Vasilyeva, L. V. *et al.* Asticcacaulis benevestitus sp. nov., a psychrotolerant, dimorphic, prosthecate bacterium from tundra wetland soil. *International journal of systematic and evolutionary microbiology* **56**, 2083-2088, doi:10.1099/ijs.0.64122-0 (2006).

4 Radhakrishnan, S. K., Thanbichler, M. & Viollier, P. H. The dynamic interplay between a cell fate determinant and a lysozyme homolog drives the asymmetric division cycle of Caulobacter crescentus. *Genes Dev* **22**, 212-225, doi:22/2/212 [pii]

10.1101/gad.1601808 (2008).