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1 **MreB5 is a determinant of rod-to-helical transition in the cell wall-less**
2 **bacterium *Spiroplasma***

3

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17 Running title: Helical shape determination in *Spiroplasma*

18

19 Keywords: *Spiroplasma*, shape determination in cell wall-less bacterium, helical
20 shape, MreB, fibril, cytoskeletal protein

21

1 **Summary**

2 In most rod-shaped bacteria, the spatial coordination of cell wall synthesis machinery
3 and MreBs is the main theme for shape determination and maintenance in cell-
4 walled bacteria [1–9]. However, how rod or spiral shapes are achieved and
5 maintained in cell wall-less bacteria is currently unknown. *Spiroplasma*, a helical
6 *Mollicute* which lacks cell wall synthesis genes, encodes five MreB paralogs and a
7 unique cytoskeletal protein fibril [10,11]. Here we show that MreB5, one of the five
8 MreB paralogs, contributes to cell elongation and is essential for the transition from
9 rod-to-helical shape in *Spiroplasma*. Comparative genomic and proteomic
10 characterization of a helical and motile wildtype *Spiroplasma* strain and a non-
11 helical, non-motile natural variant helped delineate the specific roles of MreB5.
12 Moreover, complementation of the non-helical strain with MreB5 restored its helical
13 shape and motility by a kink-based mechanism described for *Spiroplasma* [12].
14 Earlier studies had proposed that length changes in fibril filaments are responsible
15 for the change in handedness of the helical cell and kink propagation during motility
16 [13]. Through structural and biochemical characterization, we identify that MreB5
17 exists as antiparallel double protofilaments which interact with fibril and the
18 membrane, and thus potentially assists in kink propagation. In summary, our study
19 provides direct experimental evidence for MreB in maintaining cell length, helical
20 shape and motility, thus revealing the role of MreB in sculpting the cell in the
21 absence of a cell wall.

22

1 **Results and discussion**

2 Early studies proposed a role for the cytoskeletal filaments fibril and MreB in
3 morphogenesis and kinking motility of *Spiroplasma* [12,14,15]. The fibril protein does
4 not have a homolog and is unique to the genus *Spiroplasma*, which also possess 5-7
5 paralogs of MreB [10,11]. A cytoskeletal ribbon, consisting of MreB and fibril
6 filaments, positioned along the shortest helical path was proposed to drive changes
7 in handedness of the helical cell of *Spiroplasma*, leading to kinking motility [15–17].
8 However, experimental evidence demonstrating functions of fibril and the multiple
9 MreB paralogs in *Spiroplasma* physiology is unavailable, mainly due to limited tools
10 for disruption of target genes.

11 Our pursuit towards understanding fundamental mechanisms of achieving cell
12 shape led us to a comparative genomic and proteomic characterization of the
13 wildtype helical and motile strain *Spiroplasma citri* GII-3 (hereafter referred to as GII-
14 3) and a natural variant, ASP-I, which is non-helical and non-motile [18]. The pair
15 serves as an ideal model system to understand the molecular basis of helicity in
16 Spiroplasmas. A previous study on comparison of semi-purified membrane fractions
17 of *S. citri* SP-A (wildtype; helical and motile) and ASP-I cells revealed an unidentified
18 protein of about 39 kilodalton (kDa) of unknown function to be missing from the latter
19 [18].

20 Motile GII-3 cells exhibit translational motility on soft agar and form diffuse
21 colonies, in contrast to compact colonies formed by the non-motile ASP-I [19], which
22 can be clearly differentiated using Dienes' stain (Figure 1A) [20]. In electron
23 micrographs, the GII-3 cells were helical while ASP-I cells were rod shaped (Figure
24 1B). To quantify cell helicity, we measured the ratio of the cell contour (L1) and the

1 shortest distance between two points along its body length (L2). For a perfect rod,
2 the L1/L2 ratio will be 1 and deviation from this value (> 1) indicates
3 bent/curved/helical shapes. We observed the mean L1/L2 ratio for GII-3 and ASP-I
4 cells to be 1.25 ± 0.12 and 1.07 ± 0.05 , respectively [mean \pm SD (standard
5 deviation); Figure 1C]. Additionally, we differentiated between bent and helical cells
6 based on the number of crossovers between the paths of L1 and L2. For a helix, the
7 path L1 will cross L2 (shortest path) at least once whereas, the path L1 of a curved
8 or a bent cell need not cross L2. GII-3 and ASP-I cells showed mean values of 1.85
9 ± 0.42 and 0.06 ± 0.18 crossover/ μm of L2 (mean \pm SD; Figure 1D), respectively.
10 The two strains possessed similar widths (108.9 ± 1.3 nm and 100.6 ± 1.1 nm for
11 GII-3 and ASP-I respectively; Figure 1E). Some of the ASP-I cells exhibited a
12 branched morphology (marked by *, Figure 1B), while some appeared bulged at the
13 tip (marked by #, Figure 1B), both features were not observed in the GII-3 images.
14 Thus, we established that the two strains used in our study were morphologically
15 different, with GII-3 exhibiting a characteristic helical shape and ASP-I cells being
16 rod shaped.

17 To investigate if fibril and/or MreB proteins have been affected in the ASP-I
18 strain, corresponding genes from GII-3 and ASP-I genomes were sequenced. The
19 pair-wise sequence alignment of *fibril* and *mreB* sequences revealed that the
20 proteins encoded by fibril and MreB1 to MreB4 were intact in both cell types.
21 However, a point mutation was observed in *mreB5* gene of ASP-I cells at the 400th
22 nucleotide (Figure 1F, Figure S1). The mutation (G \rightarrow T) results in a stop codon
23 (TAG) after 133 amino acids, resulting in a truncated MreB5 protein in ASP-I cells
24 instead of the 352-amino-acid wildtype MreB5 protein (38.5 kDa). Whole genome
25 sequencing of ASP-I strain (reference number PRJNA635252) confirmed the point

1 mutation in *mreB5* and the absence of additional *mreB* sequences other than *mreB1-*
2 *5* in the genome.

3 Further, we performed mass spectrometry analysis of trypsinized samples of
4 the cytosolic and membrane/Triton X-114 solubilized fractions from the two cell
5 types. The peptide fragments of all MreBs, including MreB5 were found in both
6 cytosolic and membrane fractions of wildtype (GII-3) cells (Figure 1G). However, no
7 MreB5 peptides were detected in the cytosolic fractions of ASP-I cells while two
8 peptides corresponding to a 4.6 % coverage of MreB5 were detected at very low
9 abundance in the membrane fraction (Figure 1G; bar corresponding to MreB5 is not
10 seen because it lies below the displayed y-axis range).

11 To demonstrate that the absence of a functional MreB5 is responsible for the
12 altered phenotype of ASP-I cells, we performed vector-based expression of wildtype
13 *mreB5* under *ef-Tu* (*tuf*) promoter in the ASP-I cells. Polymerase chain reaction
14 (PCR)-based controls proved that the transformants were indeed ASP-I cells
15 carrying pSD4 vector (Figure S2A). Consistent with the colony morphology of non-
16 motile ASP-I (Figure 1A, right) [19], the ASP-I cells transformed with empty pSD4
17 vector (*S. citri* ASP-I^{pSD4}) formed compact colonies (Figure 2A, top). The ASP-I cells
18 expressing wildtype *mreB5* (*S. citri* ASP-I^{MreB5}) displayed a diffuse colony
19 morphology (Figure 2A, bottom) similar to motile GII-3 cells (Figure 1A, left) [19].
20 This indicated that the expression of wildtype *mreB5* enabled translational motility in
21 *S. citri* ASP-I cells.

22 Dark field microscopy observations revealed that the GII-3 cells showed
23 helical morphology and kinking motility (Video S1; Figure 2B). ASP-I cells were rod-
24 shaped and non-motile (Video S2), while ASP-I^{MreB5} cells were helical and motile

1 (Video S3), similar to the wildtype (GII-3) cells (Figure 2B). We found that the ASP-
2 I^{MreB5} cells moved with a mean velocity of $4.16 \pm 0.78 \mu\text{m}/\text{sec}$ analogous to GII-3
3 cells ($3.59 \pm 0.99 \mu\text{m}/\text{sec}$) (Figure 2C). The ASP-I cells were non-motile (0.02 ± 0.04
4 $\mu\text{m}/\text{sec}$; Figure 2C; mean \pm SD) and exhibited erratic twitching/flexing movements
5 only (Video S2).

6 Expression of a full-length MreB5 protein in ASP-I cells resulted in a helical
7 shape, similar to that of wildtype (GII-3) cells. Mean ratio of cell contour length (L1)
8 to the shortest distance between cell poles (L2) for the three cell types in exponential
9 phase were 1.35 ± 0.13 (GII-3), 1.26 ± 0.12 (ASP- I^{MreB5}) and 1.07 ± 0.06 (ASP-I)
10 (Figure 2D), consistent with the observed helical cells of GII-3 and ASP- I^{MreB5} . In late
11 exponential phase, mean L1/L2 ratio of GII-3 (1.09 ± 0.10) as well as ASP- I^{MreB5}
12 (1.15 ± 0.15) cells decreased to ~ 1 , indicating a loss of helicity for both cell types
13 (Figure 2D).

14 Typically, an MreB is known for its role in cell elongation. To check if the
15 absence of MreB5 affects cell length in *S. citri*, we estimated the contour length L1 of
16 the cell types at equivalent stages of exponential and late exponential growth
17 phases. In exponential phase, the mean length of ASP-I cells ($2.35 \pm 0.62 \mu\text{m}$) was
18 shorter than that of GII-3 ($6.21 \pm 2.02 \mu\text{m}$) and ASP- I^{MreB5} ($5.26 \pm 2.37 \mu\text{m}$) (Figure
19 2E). In late exponential phase, the mean cell lengths observed were $2.77 \pm 0.81 \mu\text{m}$
20 (ASP-I), $7.92 \pm 2.34 \mu\text{m}$ (GII-3) and $5.24 \pm 2.61 \mu\text{m}$ (ASP- I^{MreB5}) (Figure 2E),
21 demonstrating that ASP-I cells, in the absence of MreB5, grew to shorter lengths
22 compared to GII-3 and ASP- I^{MreB5} .

23 It is noteworthy that none of the other four MreBs could compensate for the
24 absence of MreB5 for retaining helicity and motility in ASP-I cells. This shows that at

1 least a few of the MreBs may not be redundant in function and demonstrates the
2 plausible existence of 'division of labour' among the multiple MreB paralogs in
3 *Spiroplasma*. Analysis of all *Spiroplasma* genomes in the database revealed that
4 they encode a minimum of 5 and maximum of 8 paralogs of *mreB* gene (Figure 3A).
5 Based on the phylogenetic tree which showed 5 major MreB clusters (Figure 3A), we
6 classified the MreBs per species into MreB1 – MreB5 (Table S1). The different
7 paralogs of MreBs in each organism share pairwise average sequence identities in
8 the range of 33 – 74 % (Figure 3B; Table S1), many of which are as divergent as
9 MreB sequences from phylogenetically distant bacteria. An equivalent of MreB1 is
10 absent in some of the species, and a few, including *S. citri* [11], have a duplicated
11 MreB4 instead of MreB1 (M4* and M1/M4 clusters in Figure 3A; Table S1). We
12 observed that Spiroplasmas possessing > 5 *mreBs* have duplicated or triplicated
13 copies of either *mreB1*, *mreB4* or *mreB5* or combinations thereof (Figure 3C, Table
14 S1). Despite an evolutionary pressure for a minimum genome, all *Spiroplasma*
15 species maintain at least 4 different MreB types according to the above phylogenetic
16 classification (Table S1). This is again suggestive of the requirement of multiple
17 MreBs with distinct properties and functions.

18 In cell-walled bacteria, the proteins MreC, MreD (expressed as the *mreBCD*
19 operon including MreB) and RodZ are essential for communication with the cell wall
20 synthesis machinery during rod shape determination [21–24]. In the absence of cell
21 wall in *Spiroplasma*, an alternative protein that moulds cell shape could be functional
22 equivalents of MreC, MreD and RodZ. BLAST searches [25] using MreC, MreD and
23 RodZ as queries against genomes of 26 species of *Spiroplasma* did not yield any
24 hits. Analysis of neighboring genes of MreBs in *Spiroplasma* genomes revealed that
25 there are no conserved neighbouring genes for *mreBs* across Spiroplasmas (Figure

1 3C). Further, investigation of the DNA sequences upstream of *mreBs* suggested that
2 each *mreB* has its own promoter sequence (Figure 3D). These findings indicate that
3 each MreB might be expressed independently and at different levels.

4 To understand the structural features of MreB from a cell wall-deficient
5 bacteria, we determined the crystal structure of *Spiroplasma citri* MreB5 (a construct
6 with a C-terminal hexahistidine tag denoted as MreB5-His₆; PDB ID: 7BVY; Figure
7 4A; Table S2). The crystal packing showed a single protofilament interface for
8 MreB5-His₆, conserved among other structurally characterized MreBs from cell-
9 walled organisms (eg. PDB ID: 1JCE of *Thermatoga maritima* MreB). Electron
10 microscopy observations suggest that MreB5-His₆ forms antiparallel double
11 protofilaments, a characteristic of MreBs [26,27] (Figure 4B, C). A double
12 protofilament structure generated by superposition of MreB5 monomers on
13 *Caulobacter crescentus* MreB (PDB ID: 4CZE) (Figure 4C) clearly shows that the
14 projection in the 2D class averages corresponds to the antiparallel double
15 protofilament organization.

16 Earlier reports suggest that fibril and MreB filaments are present near the cell
17 membrane [15,16]. Also, in *C. crescentus* and *H. pylori*, MreB functions along with
18 constitutive protein filaments such as crescentin and Ccm proteins for generating
19 curved shapes [28–30]. We hypothesized that MreB5 should bind to the membrane
20 and interact with fibril, a constitutive filament in *Spiroplasma*, for facilitating helical
21 shape and motility. We therefore performed *in vitro* sedimentation assays to verify if
22 MreB5-His₆ and fibril interact with each other. Our sedimentation assays showed that
23 MreB5-His₆ does not pellet down whereas fibril is obtained in the pellet upon
24 spinning at high speed (159,000 xg) (Figure 4D). However, in the presence of fibril, a
25 fraction of MreB5-His₆ is also obtained in the pellet (Figure 4D, E). A control

1 experiment with hexahistidine-tagged mCherry instead of MreB5-His₆ did not co-
2 pellet with fibril (Figure S2B). Hence, we concluded that MreB5-His₆ interacts with
3 fibril. Further, when mixed with liposomes of composition mimicking *Spiroplasma*
4 membrane and spun at high speeds (100,000 xg), MreB5-His₆ co-sedimented in the
5 pellet fraction along with the liposomes indicating that it is capable of binding to the
6 membrane (Figure 4F, G). A control experiment with RNaseA, a protein without lipid-
7 binding properties, did not co-sediment with liposomes (Figure S2C). Hence, we
8 speculate that MreB5 contributes to the cytoskeletal ribbon assembly in *Spiroplasma*
9 by interacting with both fibril and the membrane.

10 A unique feature of *Spiroplasma* is the change in handedness of the cell body
11 during motility. A bacterial flagellum achieves different helical conformations by
12 varying lengths of constituent protofilaments [31]. An analogous model based on
13 differential length changes of fibril filaments, required for generation of helicity and
14 change in handedness, has been proposed for *Spiroplasma* [15,32,33]. Our study
15 shows that MreB5 is an essential component for driving the proposed length
16 changes. Since our results show that MreB5 interacts with the membrane lipids and
17 fibril filaments, it is possible that MreB5 and fibril work in conjunction for a rod-shape
18 to a helix transition. It is currently unknown if MreB5 contributes to the differential
19 length changes of the filaments by driving conformational changes or promoting
20 sliding between fibril filaments. Another possibility is that MreB5 undergoes
21 conformational transitions followed by polymerization or de-polymerization, leading
22 to length changes in itself.

23 Although helical shape seems to be one of the pre-requisites for kinking
24 motility of *Spiroplasma*, helical shape does not ensure cell motility, for instance, the
25 *scm1* mutant of *S. citri* was helical but non-motile [19]. The loss of helicity in late

1 exponential phase observed in both the helical strains, GII-3 and ASP-I^{MreB5} (Figure
2 2D), is presumably due to the dissipation of transmembrane potential [34].

3 Localization of MreB is also dependent on the membrane potential [35]. Inhibitors of
4 rotary motors such as CCCP or DCCD, reportedly affect kinking motility [36,37]. The
5 loss of helicity in the late exponential phase emphasizes that factors such as pH
6 might contribute to maintain helicity. This further implies that components other than
7 MreB5 and fibril are required for *Spiroplasma* helicity and motility.

8 MreB plays a role in DNA segregation [38,39], cell length regulation [38] and
9 in *Myxococcus xanthus* motility [40], in addition to morphogenesis of cell-walled
10 bacteria. *Bacillus subtilis* possesses three paralogs of MreB namely MreB, Mbl and
11 MreBH. Each MreB paralog has been shown to polymerize independently, but their
12 co-localizing assemblies are necessary for rod-shape in *B. subtilis* [41,42]. As
13 demonstrated by our dissection of the role of MreB5 using the *S. citri* ASP-I strain, it
14 will be exciting to investigate the individual roles of MreBs 1, 2, 3 and 4. The
15 elongated, but shorter cells of ASP-I strain demonstrate that MreB1, 2, 3 or 4 or fibril
16 might suffice to attain rod shape. The decreased length with no apparent increase in
17 width of ASP-I cells (Figure 1E) or change in growth rate (inferred based on colony
18 size) indicate that there may be additional mechanisms to maintain the cell width for
19 a growing cell volume. One such mechanism is the Y-shaped division observed in
20 *Spiroplasma* species [43], which might explain the instances of branched cells in
21 ASP-I. MreB5 could regulate cell length through regulation of cell division events.
22 Suppression of a cross-sectional division by a functional MreB5 could lead to longer
23 cells while trigger of Y-shaped division in the absence of MreB5 maintains width.

24 We have experimentally demonstrated the requirement of MreB5 for
25 maintaining cell length, helical shape and motility of a cell wall deficient bacterium, *S.*

1 *citri*. Thus, MreB5 serves as a bona fide cytoskeletal protein for shaping the cell.
2 *Spiroplasma* MreBs perform their function independent of the cell wall synthesis
3 machinery. The conserved structural features of MreB5 filaments compared to
4 MreBs from cell-walled bacteria indicate that the fundamental mechanism of MreB to
5 sculpt the cell might remain conserved across cell-walled and cell wall-deficient
6 organisms. Thus cell wall-less organisms, such as the helical Spiroplasmas, provide
7 us with multi-pronged approaches to study basic principles of cell shape
8 determination.

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6

7 **Author contributions**

8 SH designed, conceptualised, performed all experiments other than mentioned
9 below, carried out data and sequence analyses and wrote the manuscript; SD
10 performed and supervised the *Spiroplasma* molecular biology experiments; VP
11 determined the crystal structure and performed electron microscopy experiments of
12 MreB5, designed and performed liposome binding assays; MB assisted in
13 representation of sequence analysis data, gene sequencing of *fibril* and *mreBs* of the
14 two strains and in cloning of the complementation construct; LB designed, performed
15 and supervised *Spiroplasma* genetics, biochemistry and microscopy experiments;
16 PG designed, conceptualised and supervised *in vitro* experiments and sequence and
17 structure analyses and wrote the manuscript. All authors reviewed and provided
18 inputs for the manuscript.

19

20 **Declaration of interests**

21 The authors declare no competing interests.

22

23 **Figure legends**

24 **Figure 1. Comparative characterization of *S. citri* GII-3 and ASP-I cells.**

1 **A.** Light microscopy images of colonies of *S. citri* GII-3 cells (green box) and *S. citri*
2 ASP-I cells (purple box). Within each box, the images on the left and right show
3 unstained colonies and colonies stained with Dienes' stain, respectively. Red arrows
4 point to diffused colonies. Scale bar represents 100 μm . **B.** Transmission electron
5 microscopy (TEM) images showing morphology of *S. citri* GII-3 (wildtype; left) and
6 ASP-I cells (right). The cell contour (L1) is marked in blue and the shortest distance
7 between two points along its length (L2) is marked in red. Scale bar represents 0.5
8 μm . '*' symbols highlight branching points in cells, while '#' symbols mark a bulged
9 tip. **C.** Distribution of ratios of cell length to the shortest distance between two points
10 along its length (L1/L2) for *S. citri* GII-3 (green; n = 94) and *S. citri* ASP-I (purple; n =
11 94) cells. Each data point represents a cell. Mean and standard deviation is shown
12 with long and short black lines respectively (unpaired t-test, *** $p \leq 0.001$). **D.**
13 Number of crossovers of each cell per μm of L2 for *S. citri* GII-3 (green; n = 94) and
14 *S. citri* ASP-I (purple; n = 94) cells is plotted. Each data point represents a cell. Mean
15 and standard deviation is shown with long and short black lines respectively
16 (unpaired t-test, *** $p \leq 0.001$). **E.** Cell width measurements for *S. citri* GII-3 (green; n
17 = 94) and ASP-I (purple; n = 108). Each data point represents a cell. Mean and
18 standard deviation is shown with long and short black lines respectively (unpaired t-
19 test, *** $p \leq 0.001$). **F.** DNA sequences of region in *mreB5* gene from *S. citri* GII-3
20 (top) and *S. citri* ASP-I (bottom). Amino acids coded by respective codons are written
21 above (*S. citri* GII-3) and below (*S. citri* ASP-I) the DNA sequences. Highlighted in
22 grey background is the codon in *S. citri* ASP-I with point mutation (refer Figure S1 for
23 chromatogram) and the corresponding codon in *S. citri* GII-3. Numbers in superscript
24 indicate the position of the nucleotide/amino acid from the start codon/first amino
25 acid in the sequence. **G.** Plot showing relative abundance (in log scale) of the 5

1 MreBs from *S. citri* GII-3 (green) and ASP-I (purple) cells in the membrane and
2 cytosolic fractions.

3 **Figure 2. MreB5 confers helicity and motility in *S. citri* ASP-I cells.**

4 **A.** Light microscopy images of colonies of *S. citri* ASP-I cells transformed with empty
5 pSD4 vector (*S. citri* ASP-I^{pSD4}; top) and *mreB5* cloned in pSD4 (*S. citri* ASP-I^{MreB5};
6 bottom). Within each box, the images on the left and right show unstained colonies
7 and colonies stained with Dienes' stain, respectively. Red arrows point to diffused
8 colonies. **B.** Snapshots from videos (see also Videos S1- S3) of *S. citri* strains GII-3
9 (left, green box), ASP-I (middle, purple box), and ASP-I^{MreB5} (right, orange box)
10 showing cell morphologies at periodic time points. Time range lies between the
11 values mentioned on first and last frames for a given cell and with equal time
12 intervals between subsequent frames. Coloured arrows point to the kinks in the cell
13 body. Scale bar represents 3 μ m. **C.** Distribution of velocities of *S. citri* strains GII-3
14 (green, n = 32), ASP-I (purple, n = 39) and ASP-I^{MreB5} (orange, n = 40). Each data
15 point represents the velocity of a cell. Mean and standard deviation is shown with
16 long and short black lines respectively (unpaired t-test, *** p \leq 0.001; ** p \leq 0.01; ns
17 (non-significant) p > 0.05). **D.** Distribution of ratios of cell length to the shortest
18 distance between two points along the cell length (L1/L2) for *S. citri* GII-3 (green),
19 ASP-I (purple) and ASP-I^{MreB5} (orange) cells. The data in exponential and late
20 exponential phases of cell growth are represented by solid circles and crosses
21 respectively (n = 50 each for all cases). Mean and standard deviation is shown with
22 long and short black lines respectively (unpaired t-test, *** p < 0.0001; * p \leq 0.05; ns
23 (non-significant) p > 0.05). **E.** Distribution of cell lengths of *S. citri* GII-3 (green), ASP-
24 I (purple), and ASP-I^{MreB5} (orange) in exponential (solid circles) and late exponential

1 (cross) phases of growth. Mean and standard deviation is shown with long and short
2 black lines respectively (unpaired t-test, *** $p < 0.0001$; * $p \leq 0.05$).

3 See also Figure S2 for PCR tests for confirmation of strains and Videos S1 – S3.

4 **Figure 3. Classification of *Spiroplasma mreBs***

5 **A.** Rooted phylogenetic tree [44,45] showing distribution and classification of MreBs
6 from 26 species (27 strains including two strains of *S. citri* ScR8A2 and ScGII3 are
7 included) of *Spiroplasma* into 5 clusters. Sequences of MreBs and FtsA from *E. coli*,
8 *B. subtilis*, *C. botulinum* and *C. crescentus* as well as Mbl and MreBH from *B. subtilis*
9 were also included in the tree. MreBs are color coded according to the clusters
10 [MreB1 (M1, orange), MreB2 (M2, green), MreB3 (M3, purple), MreB4 (M4, dark
11 pink), duplicated MreB4 (M4*, light pink), MreB1/MreB4 (M1/M4; red), MreB5 (M5,
12 dark cyan), MreBs, Mbl and MreBH from cell walled bacteria (grey), FtsA from cell
13 walled bacteria (black)]. M1/M4 is a branch in the phylogenetic tree which has not
14 been classified into either M1 or M4 and hence mentioned as M1/M4. **B.** Percentage
15 sequence identity matrix among *Spiroplasma* MreBs belonging to 5 clusters [46].
16 The percentage identity among *S. citri* GII-3 MreBs is shown in black color (squares
17 to the right of the diagonal) while average percentage identities among MreBs
18 belonging to the 5 different clusters of each species (averaged across 26 species of
19 *Spiroplasma* according to the classification in Table S1) are shown in grey color
20 (squares to the left of the diagonal). Sequences belonging to the class M1/M4 were
21 excluded from the averaging, while M4* (duplicated M4) were considered as M4 in
22 the pairwise matrix. **C.** Schematic representation of genes neighboring *Spiroplasma*
23 *mreBs*. Neighbors (+3 and -3 positions) of *mreB* genes from *Spiroplasma* species
24 have been aligned and depicted as arrows. The arrows with black borders represent

1 MreBs (color coded as in Figure 3A), while arrows with grey borders represent genes
2 neighboring *mreBs* and colour coded according to their annotation (shown by the
3 colour key in the right box). The direction of the arrows indicates gene orientation 5'
4 → 3'. Asterisk and white arrow with black border represents a *mreB* pseudogene that
5 translates into a truncated MreB. **D.** Weblogos of consensus sequences predicted to
6 be -35 and -10 sequences for the 5 clusters of *mreBs* across 26 *Spiroplasma*
7 species [47]. These sequences can act as promoter elements and facilitate
8 expression of each *mreB* independent of other *mreBs* in the genome.
9 See Table S1 for further details of MreB classification.

10 **Figure 4. MreB5-His₆ possesses anti-parallel double protofilament assembly *in***
11 ***vitro* and interacts with fibril and liposomes.**

12 **A.** Crystal structure of *S. citri* MreB5 (with a C-terminal hexahistidine tag; MreB5-
13 His₆; PDB ID: 7BVY; see Table S2 for data collection and refinement statistics)
14 shows a single protofilament organization according to crystal packing. Three
15 adjacent monomers are shown and the subdomains are color coded and labelled as
16 1A, 2A, 1B and 2B. The black arrow corresponds to a direction of the protofilament
17 with subdomains 1A and 2A at the base (barbed end according to actin
18 nomenclature). **B.** Electron cryomicroscopy images of MreB5-His₆ filaments *in vitro*.
19 Insets highlight the double protofilaments in a zoomed section of the image (bottom
20 left) and show the top four 2D class averages of segment of filaments (bottom right).
21 **C.** A view of the double protofilament structure of MreB5-His₆, modeled based on
22 *Caulobacter crescentus* MreB (PDB ID 4CZE), matches with the projection view of
23 the 2D class averages in panel (B). The subdomains are color-coded as in panel (A).
24 The view corresponds to an orientation with 1A and 1B sub-domains behind the

1 plane of 2A and 2B sub-domains. The orientation of the protofilaments is highlighted
2 by the black arrows, marked according to the convention in panel (A). **D.** A
3 representative 12 % SDS-PAGE gel showing sedimentation assays of untagged
4 fibril, MreB5-His₆ and MreB5-His₆ in presence of fibril (untagged). I, P and S
5 respectively represent input, pellet and supernatant fractions upon sedimentation at
6 159,000 xg. Red and blue '*' symbols show the full length bands of fibril and MreB5,
7 respectively, while the lower bands are the impurity or degradation bands obtained
8 during fibril purification. **E.** A plot showing relative mean intensities (expressed in
9 percentage) of bands corresponding to MreB5-His₆ position (marked by blue * and
10 region enclosed by dashed blue box) in the SDS-PAGE gels from 3 independent
11 experiments, as described in panel (D). The relative intensities of pellet (or
12 supernatant) fractions at the position corresponding to MreB5-His₆ band were
13 calculated as intensity of pellet (or supernatant) divided by sum of the two intensities
14 and represented in the graph as a percentage. Pel and Sup represents the pellet and
15 supernatant fractions, respectively, upon spinning at 159,000 xg. **F.** A representative
16 12 % SDS-PAGE gel showing sedimentation of MreB5-His₆ upon adding increasing
17 concentrations of liposomes. P and S respectively represent pellet and supernatant
18 fractions upon sedimentation at 100,000 xg. Lanes 2 and 3 are repeats of the
19 samples in lanes 4 and 5 along with a protein molecular weight marker (note that the
20 band intensities differ for the same concentrations due to use of a 10-well gel). **G.** A
21 plot showing relative mean intensities (expressed in percentage) of bands
22 corresponding to MreB5-His₆ position in the SDS-PAGE gels from 3 independent
23 experiments, as shown in panel (F). The relative intensities of pellet (or supernatant)
24 fractions at the position corresponding to MreB5-His₆ band were calculated as
25 intensity of pellet (or supernatant) at the position corresponding to MreB5-His₆ band

1 divided by sum of the two intensities and represented in the graph as a percentage.
2 See Figure S2 for control experiments for sedimentation and liposome binding
3 assays, and Table S3 for the list of strains and plasmids used.
4

5 **STAR Methods**

6 **RESOURCE AVAILABILITY**

7

8 **LEAD CONTACT**

9 Further information and requests for resources and reagents should be directed to
10 and fulfilled by the Lead Contact, Pananghat Gayathri (gayathri@iiserpune.ac.in).

11 **MATERIALS AVAILABILITY**

12 *Spiroplasma* strains and the plasmids generated in the study are available with
13 Laure Béven (laure.beven@inrae.fr), while the plasmids for MreB5-His₆ and fibril
14 proteins in *E. coli* are available with Pananghat Gayathri (gayathri@iiserpune.ac.in).

15 **DATA AND CODE AVAILABILITY**

16 The whole genome sequencing read of *S. citri* ASP-I is deposited in the Sequence
17 Read Archive at NCBI, under accession number (Bioproject PRJNA635252). The
18 accession number for the crystal structure of MreB5-His₆ complexed with AMPPNP
19 reported in this paper is PDB ID: 7BVY.

20 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

21 **Cultures**

22 *S. citri* GII-3 [48] and *S. citri* ASP-I [18] cultures available in the lab of Dr. Laure
23 Béven were used in the present study. All *Spiroplasma* cells in the present study
24 were grown in modified SP4 medium [Composition per 300 mL of media: A)

1 Components to be autoclaved: 1 g Mycoplasma base broth, 1.6 g peptone, 3 g
2 tryptone, 1.2 mL phenol red (9 mg/mL 1M NaOH), adjust pH to 7.6, make up the
3 volume to 205 mL with MilliQ grade water. B) Components to be filter sterilized and
4 added to autoclaved components: 50 mL heat-inactivated foetal calf serum, 15 mL
5 CMRL 1066 (10X), 25.5 mL Yeastolate (stock concentration 4 % w/v), 3 mL D-
6 glucose (50 % w/v) and 1.5 mL Penicillin G (stock concentration 200,000 U/mL)]
7 [49].

8 *E. coli* cell strains used in this study for cloning, Stellar™ (Takara Bio) electro-
9 competent *E. coli*, and for protein expression *E. coli* BL21 (AI), were grown in LB
10 (Luria Bertani) broth supplemented with ampicillin (final concentration 100 µg/mL) at
11 37 °C.

12 **METHOD DETAILS**

13 **Electron microscopy imaging of *Spiroplasma* and cell length analysis**

14 Growing cells (80 µL) of *S. citri* GII-3 or ASP-I were mixed with 20 µL Protein A-
15 conjugated colloidal gold nanoparticles (10 nm, Cytodiagnosics). 5 µL of this mixture
16 was applied to a glow-discharged EM grid (R2/2, 200 mesh, copper, Quantifoil) and
17 left at room temperature (25 °C) for 60 seconds. The grids were transferred to
18 Vitrobot™ (Marc IV, FEI; chamber at 32 °C with 95 % humidity) and excess media
19 absorbed by back-blotting manually using a blotting paper (TED PELLA, INC.). The
20 grid was plunge frozen in a mixture of ethane and propane (37 % : 63 % v/v) and
21 then transferred to labelled grid storage box. Boxes containing frozen grids were
22 stored in liquid nitrogen for at least 24 hours before imaging. The samples were
23 observed and images acquired using Titan Krios (300 KeV; FEI) electron microscope
24 with K2 Summit direct electron detector and Quantum LS imaging filter.

1 ImageJ 1.52p (USA) [50] was used for the measurement of cell contour (L1) and the
2 shortest distance between two points along cell body length (L2) from electron
3 microscopy images. The cell length refers to the contour length L1 at all instances in
4 the text, while cell width is the extent of cells along a cross section approximately
5 perpendicular to the cell contour line. The data is expressed as the mean \pm standard
6 deviation (SD). Statistical significance was estimated by the unpaired t test, two
7 tailed. GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego California
8 USA, www.graphpad.com) was used for statistical analysis of the data and plotting
9 the graphs.

10 **DNA isolation and sequencing**

11 With 100 ng of *S. citri* GII-3 and ASP-I genomic DNA as template, the 5 *mreB* and
12 *fibril* genes were PCR amplified using the flanking primers given in Table S3,
13 followed by gene sequencing.

14 **Whole genome sequencing**

15 Whole genome sequencing of *S. citri* ASP-I was performed at the Genome
16 Transcriptome facility of Bordeaux (<https://pgtb.cgfb.u-bordeaux.fr/>) using a
17 combination of Oxford Nanopore (GridION sequencer) and paired-end 250 bp
18 Illumina (MiSeq sequencer) reads. The genome was assembled into 4 main contigs
19 using both short and long reads and the Unicycler v0.4.6 pipeline (PMID: 28594827).
20 One contig of 7999 bp corresponded to the previously described plasmid pSciA
21 (GenBank accession AJ966734.1). The three chromosomal contigs came up to a
22 total of 1493377 bp.

23 **Protein extraction and proteomic analysis**

1 *Spiroplasma* proteins were extracted, using the protocol as reported earlier [51].
2 Briefly, a 50 mL culture pellet of *Spiroplasma* cells (ASP-I and GII-3) was re-
3 suspended into 1 mL Tris-buffered saline [TBS; 10 mM Tris-HCl (pH 7.4), 150 mM
4 NaCl] and lysed by sonication. 100 μ L extraction buffer [10 mM Tris (pH 7.4), 154
5 mM NaCl] and 100 μ L Triton X-114 (10 % w/v) were added to 800 μ L lysate on ice,
6 to facilitate extraction of membrane proteins by Triton X-114. This was followed by
7 separation of the mixture into insoluble fraction, Triton X-114 soluble fraction and
8 supernatant, as described in the reference. Fractions containing total proteins,
9 aqueous soluble proteins and Triton X-114 solubilized proteins were checked on a
10 12 % SDS-PAGE gel. Proteins were identified by peptide mass fingerprint and
11 tandem-mass spectrometry (MS/MS) sequencing from protease-digested protein at
12 the Proteome Platform, Functional Genomic Center of Bordeaux, University of
13 Bordeaux as follows.

14 Sample preparation and protein digestion

15 Protein samples were solubilized in Laemmli buffer [2X; SDS (4 % w/v),
16 Bromophenol blue (0.2 % w/v), glycerol (20 % v/v), DTT (200 mM), and 100 mM
17 Tris-HCl, pH 6.8] and 5 μ g were deposited onto preparative SDS-PAGE for
18 concentration and cleaning purpose. Separation was stopped once proteins entered
19 the resolving gel. After colloidal blue staining, each lane was cut in 1 mm x 1 mm gel
20 pieces. Gel pieces were de-stained in 50 % acetonitrile (ACN) containing 25 mM
21 ammonium bicarbonate (ABC), rinsed twice in ultrapure water and shrunk in ACN for
22 10 min. After ACN removal, gel pieces were dried at room temperature, covered with
23 the trypsin solution (10 ng/ μ L in 50 mM ABC), rehydrated at 4 °C for 10 min, and
24 finally incubated overnight at 37 °C. Spots were then incubated for 15 min in 50 mM
25 ABC at room temperature with rotary shaking. The supernatant was collected, and

1 an H₂O/ACN/formic acid (47.5 : 47.5 : 5) extraction solution was added onto gel
2 slices for 15 min. The extraction step was repeated twice. Supernatants were pooled
3 and dried in a vacuum centrifuge. Digests were finally solubilized in 0.1 % formic
4 acid.

5 nLC-MS/MS analysis

6 Peptide mixture was analyzed on a Ultimate 3000 nanoLC system (Dionex,
7 Amsterdam, The Netherlands) coupled to a Electrospray Orbitrap Fusion™ Lumos™
8 Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). 10 μL of
9 peptide digests were loaded onto a 300 μm (inner diameter) x 5 mm C18 PepMap™
10 trap column (LC Packings) at a flow rate of 10 μL/min. The peptides were eluted
11 from the trap column onto an analytical 75 mm (inner diameter) x 50 cm C18 Pep-
12 Map column (LC Packings) with a 4 – 40 % linear gradient of solvent B in 45 min
13 (solvent A was 0.1 % formic acid and solvent B was 0.1 % formic acid in 80 % ACN).
14 The separation flow rate was set to 300 nL/min. The mass spectrometer operated in
15 positive ion mode at a 1.9 kV needle voltage. Data were acquired using Xcalibur 4.1
16 software in a data-dependent mode. MS scans (m/z 375-1500) were recorded at a
17 resolution of R = 120 000 (@ m/z 200) and an AGC target of 4 x 10⁵ ions collected
18 within 50 ms. Dynamic exclusion was set to 60 s and top speed fragmentation in
19 Higher-energy collisional dissociation (HCD) mode was performed over a 3 s cycle.
20 MS/MS scans with a target value of 3 x 10³ ions were collected in ion trap with a
21 maximum fill time of 300 ms. Additionally, only + 2 to + 7 charged ions were selected
22 for fragmentation. Others settings were as follows: no sheath, nor auxiliary gas flow,
23 heated capillary temperature, 275 °C; normalized HCD collision energy of 30 % and
24 an isolation width of 1.6 m/z. Monoisotopic precursor selection (MIPS) was set to
25 Peptide and an intensity threshold was set to 5 x 10³.

1 Database search and results processing

2 Data were searched by SEQUEST through Proteome Discoverer 2.3 (Thermo Fisher
3 Scientific Inc.) against homemade protein databases consisting of products of *S. citri*
4 ASP-I and GII-3 CDS (Chromatography Data System). Spectra from peptides higher
5 than 5000 dalton (Da) or lower than 350 Da were rejected. The search parameters
6 were as follows: mass accuracy of the monoisotopic peptide precursor and peptide
7 fragments was set to 10 parts per million (ppm) and 0.6 Da respectively. Only b- and
8 y-ions were considered for mass calculation. Oxidation of methionines (+ 16 Da) and
9 protein N-terminal modifications (Acetylation + 42 Da) were considered as variable
10 modifications and carbamidomethylation of cysteines (+ 57 Da) as fixed
11 modifications. Two missed trypsin cleavages were allowed. Peptide validation was
12 performed using Percolator algorithm [52] and only “high confidence” peptides were
13 retained corresponding to a 1 % false-positive rate at peptide level. Peaks were
14 detected and integrated using the Minora algorithm embedded in Proteome
15 Discoverer. Proteins were quantified based on unique peptides intensities.
16 Normalization was performed based on total protein amount.

17 **Cloning, transformation and colony characteristics**

18 The *ef-tu* (*tuf*) promoter region from pSTP1 vector [53] was amplified using primers
19 P1 and P2 (Table S3). The *mreB5* gene was amplified from genomic DNA of *S. citri*
20 GII-3 cells using primers P3 and P4. The amplified products of these two PCRs were
21 purified using a PCR clean-up kit (Qiagen) and eluted in 30 μ L MilliQ water
22 separately. An overlap-extension PCR was set up using the purified PCR products to
23 obtain the *tuf* promoter-*mreB5* DNA fragment. The product DNA was extracted from
24 the gel using a gel extraction kit (Qiagen) and re-amplified using end primers. The

1 amplified product was cleaned up, digested with restriction enzyme EcoRI and
2 cloned into the pSD4 vector [54] by restriction digestion-ligation method. The ligation
3 reaction was transformed into Stellar™ (Takara Bio) electro-competent *E. coli* cells
4 by electroporation. The obtained transformants were grown in LB (Luria Bertani)
5 broth supplemented with ampicillin (final concentration 100 µg/mL) and plasmid
6 extracted using a QIAprep miniprep kit (Qiagen). DNA sequence inserted into the
7 plasmid was sequenced using M13 forward and EV13 primers (Table S4) to confirm
8 the sequence.

9 Transformation

10 In two different reactions, the confirmed clone and empty pSD4 vector (control) was
11 transformed into growing *S. citri* ASP-I cells using the previously described protocol
12 [55]. 500 ng of *mreB5* clone or empty pSD4 vector was transformed into *S. citri* ASP-
13 I cells by electroporation using MicroPulser Electroporator (Bio-Rad) and following
14 parameters- 2500 V voltage, 3 µF capacitance, 1000 Ω resistance, and spread on
15 SP4 agar plates supplemented with tetracycline (final concentration of 2 µg/mL). The
16 plates were incubated at 32 °C until the development of colonies. Appearance of
17 colonies was monitored by observation of plates, using a binocular loupe until
18 colonies were visible.

19 Diene's staining

20 Colonies were stained with Dienes's stain using the method described [20].
21 Spiroplasmas grown in liquid SP4 were diluted with fresh SP4 to a concentration of
22 about 10³ cells/mL. The diluted suspensions were plated on SP4 containing 1 %
23 Difco™ Agar Noble (Becton, Dickinson and Company, USA) in petri dishes (5 cm

1 diameter) and incubated at 32 °C for 14 days. Dienes' stain was prepared by
2 dissolving 2.5 g methylene blue, 10 g maltose, 1.25 g azure II and 0.25 g sodium
3 carbonate in 100 mL ultrapure water and filtered through Whatman filter paper No.1.
4 The colonies were stained in Dienes' stain diluted to 3 % (v/v) (5 mL/Petri dish) for 5
5 min. The stained colonies were then extensively rinsed in distilled water, and
6 examined using a microscope Nikon SMZ1270, equipped with the camera Nikon DS-
7 Fi2. Images were acquired using the software NIS-Elements D.

8 PCR-based confirmatory tests for clones

9 Total DNA (including genomic DNA and plasmids) was extracted from *S. citri* GII-3
10 and ASP-I cells transformed with either *mreB5* gene cloned in pSD4 vector (*S. citri*
11 ASP-I^{pSD4}) or empty pSD4 vector (*S. citri* ASP-I^{MreB5}) using the Wizard® Genomic
12 DNA purification kit (Promega). Three sets of PCRs were performed to confirm that
13 the transformants obtained by electroporation of *S. citri* ASP-I cells with the pSD4
14 vector containing *tuf* promoter-*mreB5* gene were indeed transformants. The first
15 PCR was performed using *sr14* and *sr16* primers [56] (Table S3) as positive control
16 for amplifying a fragment of the *spirallin* gene. The second PCR was carried out
17 using *tet1* and *tet2* primers [57] (Table S3) to confirm the presence of the
18 tetracycline-resistance gene carried on the pSD4 vector. The third PCR was
19 performed using *Scarp* 235 primers [58] (Table S3) to check for the presence of
20 genes coding for *S. citri* adhesion-related proteins (SCARPs) carried on pSci vectors
21 that are present in *S. citri* GII-3 cells but absent in *S. citri* ASP-I^{pSD4} and *S. citri* ASP-
22 I^{MreB5} cells. The PCRs against total DNA of *S. citri* GII-3 cells and *S. citri* ASP-I^{pSD4}
23 were set up as controls. All the PCR products were migrated on agarose gels (2 %
24 w/v) and visualized by ethidium bromide staining (Figure S2A).

1 **Dark-field microscopy, cell length measurements and velocity calculations**

2 The morphology of exponentially growing GII-3, ASP-I, ASP-I^{pSD4} and ASP-I^{MreB5}
3 cells of *S. citri* in modified SP4 media was observed using an Eclipse Ni (Nikon)
4 microscope working in reflection and equipped with a dark field condenser. The
5 Nikon oil immersion microscope objective was a 60X with a numerical aperture (N.
6 A.) of 0.80. Pictures were taken with a camera Nikon Digital Sight DS-Qi1Mc
7 (1280 × 1024 pixels). Motility was favoured by increasing the viscosity of the medium
8 following the protocol described in [59]: during the exponential growth phase of the
9 bacteria, one volume of the culture was diluted with one volume of methyl cellulose
10 dissolved in modified SP4. Here, 1 % (w/v) methyl cellulose [from Sigma (M7027),
11 Molecular weight 14000 g/mol] was used to adjust the viscosity of the solutions.
12 Bacterial solutions were prepared between two sealed microscope slides, with a
13 liquid thickness of 15 µm. Videos were recorded at a frame rate of 10 frames per
14 second (fps) using the software NIS-Elements Br (Nikon). *Spiroplasma* cell velocities
15 were estimated from videos using the 'Manual Tracking' plugin in ImageJ [50].
16 Similarly, cell lengths and widths were measured from images using ImageJ. The
17 data is expressed as the mean ± standard deviation (SD). Statistical significance
18 was estimated by the unpaired t test, two tailed. GraphPad Prism 5.00 for Windows
19 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for
20 statistical analysis of the data and plotting the graphs.

21 **MreB sequences analysis and classification**

22 *mreB* gene and protein sequences were obtained from NCBI
23 (<https://www.ncbi.nlm.nih.gov/>). The genes from each species were labelled in order
24 of their appearance in the genome with respect to *dnaA*, with the closest *mreB* at the

1 3' of *dnaA* being assigned number 1. All the MreB protein sequences were aligned
2 using Clustal Omega [46] with default settings. A phylogenetic tree was prepared
3 using all the aligned MreB sequences with the help of NGPhylogeny [44] using
4 default settings and visualized using IToL [45].

5 **Analysis of neighborhood genes**

6 To analyze the neighboring genes of *mreBs*, 3 genes each at the 5' and 3' ends of
7 respective *mreBs* in the genome were considered. The genes were labelled in
8 sequential order such that the gene preceding (at the 5' end of) *mreB* was labelled
9 as -1 position and the gene at the 3' end of *mreB* was assigned +1 position and so
10 on. In order to check if the hypothetical proteins at a given position are identical,
11 amino acid sequences of hypothetical proteins at that position were aligned using
12 Clustal Omega [46] and protein conservation checked. To check if the MreC, MreD
13 and RodA homologs are present in *Spiroplasma* species, sequence of corresponding
14 genes and proteins from *E. coli* K-12 (MG1655) was used. The sequence was
15 provided as a query for BLAST [25] against each of the 26 species of *Spiroplasma*
16 using default parameters.

17 **Identification of *mreB* promoter regions**

18 Nucleotide sequence corresponding to 100 basepairs (bp) region upstream (before
19 the 5' end) of each *mreB* gene of *Spiroplasma* species under consideration was
20 obtained from NCBI. The -35 and -10 elements from 100 bp regions of all *mreBs*
21 were identified using BPPROM software [47]. The identified -35 and -10 sequences for
22 all MreBs from a cluster (grouped on the basis of phylogenetic tree) across species
23 were used for generating a Weblogo using WebLogo 3 [60].

24 **Fibril expression and purification**

1 The *fibril* gene from *S. citri* (DSMZ 21846) genomic DNA was cloned into the pHIS17
2 vector (refer Addgene plasmid # 78201 for plasmid backbone) [61]. The cloning and
3 standardization of expression and purification is described in Harne, et al.,
4 (manuscript to be communicated). The protein was over-expressed using *E. coli*
5 BL21 (AI) cells by growing the transformants in Luria Bertani (LB) broth
6 supplemented with ampicillin (100 µg/mL final concentration) at 37 °C under shaking
7 conditions until OD₆₀₀ = 0.6. The cells were induced by addition of sterile L-arabinose
8 at final concentration of 0.2 % (w/v) and were grown at 25 °C for 6 hours post-
9 induction. The cells were harvested and lysed by sonication in 50 mM Tris pH 8.0.
10 The lysate was spun at 30,000 xg to get rid of cell debris and other macromolecules
11 as a pellet. The supernatant containing fibril was further spun at 159,000 xg, 4 °C for
12 30 minutes and fibril obtained in the pellet. The fibril containing pellet was re-
13 suspended in 1 mL of buffer containing 10 mM Tris pH 8.0 and 10 mM EDTA and
14 stirred at 4 °C overnight. The stirred fibril solution was loaded on top of a urografin
15 (76 % w/v; Cadila healthcare Ltd, Kundaim, India) linear gradient. The gradient was
16 prepared by layering equal volumes of 34 % [v/v in T₁₀E₁₀ (10 mM Tris pH 8.0, 10
17 mM EDTA) buffer] and 76 % urografin followed by overnight incubation at room
18 temperature. The fibril-loaded gradient was spun at 159,000 xg/ 4 °C / 120 minutes
19 and fractionated into 10 equal fractions and visualized on a 12 % SDS-PAGE gel.
20 Fractions containing fibril were pooled and washed with 50 mM Tris (pH 7.6) by 2
21 cycles of pelleting and re-suspension to remove urografin. The pellet containing
22 enriched fibril was re-suspended in minimum volume of reaction buffer [50 mM Tris
23 (pH 8.0), 300 mM KCl and 2 mM MgCl₂].

24 **MreB5-His₆ purification**

1 The *S. citri* (DSMZ 21846) *mreB5* gene with a hexa-histidine tag was cloned into a
2 pHIS17 vector [61] and over-expressed in *E. coli* BL21 (AI) cells. The standardization
3 of purification conditions and buffer optimization are described in Pande, et al.,
4 (manuscript to be communicated). The cells were grown in LB broth at 37 °C until
5 OD₆₀₀ was 0.8-1.0. The culture was induced with L-arabinose at a final concentration
6 of 0.05 % (w/v). Post-induction, the culture was grown at 20 °C for 12 hours. For
7 large scale protein purification, 2 litres of cell pellet was thawed and cells were
8 homogenized in lysis buffer (300 mM KCl, 50 mM Tris, pH 8.0 and 10 % glycerol)
9 and lysed by sonication. The lysate was centrifuged at 44,082 xg for 45 mins at 4 °C.
10 The supernatant was loaded on 5 mL Ni-NTA column (HisTrap, GE Healthcare) pre-
11 equilibrated with buffer A (50 mM Tris pH 8.0, 300 mM KCl). Hexahistidine tag
12 present in the C-terminus of protein facilitated binding to Ni-NTA column. Bound
13 protein was eluted using a step gradient of 5 %, 10 %, 20 %, 50 % and 100 % of
14 buffer B (50 mM Tris pH 8.0, 300 mM KCl and 500 mM Imidazole). Fractions
15 containing purest protein were identified by visualization on 12 % SDS-PAGE gel,
16 pooled and dialyzed against buffer containing 50 mM Tris pH 8.0, 300 mM KCl.
17 Protein purification and dialysis was carried out at 4 °C. Purified protein was further
18 concentrated using centricons with 10 kDa cutoff (Sartorius), flash frozen and stored
19 in -80 °C until further use.

20 For sedimentation experiments to study interaction with fibril, MreB5-His₆ was
21 expressed as described above. Purification was done using Ni-NTA His-bind
22 Superflow resin (Sigma) on the bench using a syringe column, eluted with buffer B,
23 fractions containing pure protein were pooled and concentrated using concentrators
24 with 3 kDa cut-off. The imidazole was removed by cycles of dilution during the
25 concentration step, resulting in a final buffer composition of 50 mM Tris pH 8.0, 300

1 mM KCl with negligible imidazole. The purified MreB5 was used for the
2 sedimentation assays the same day.

3 **mCherry expression and purification**

4 *mcherry* gene with a hexahistidine tag at its 5' end, cloned in pET15b vector was
5 obtained from Dr. Thomas Pucadyil, IISER, Pune, India. The clone was transformed
6 into *E. coli* BL21 (AI) cells by heat shock method. Single colony was inoculated in 20
7 mL LB broth supplemented with ampicillin at final concentration of 100 µg/mL and
8 incubated at 37 °C under shaking conditions for 12 hours. 5 mL of this culture was
9 inoculated into 1 Litre LB broth (supplemented with ampicillin at final concentration of
10 100 µg/mL) and grown under shaking condition at 37 °C until OD₆₀₀ reached 0.6.
11 The cells were induced by addition of sterile L-arabinose at final concentration of 0.2
12 % (w/v) and further were grown at 25 °C for 6 hours post-induction. The cells were
13 harvested and lysed by sonication in lysis buffer containing 50 mM Tris pH 8.0, 300
14 mM KCl and 10 % (v/v) glycerol. The lysate was spun at 159,000 xg to get rid of cell
15 debris as a pellet. The supernatant containing mCherry was used for purification on
16 bench using Ni-NTA His-bind Superflow resin (Sigma) in a syringe column and
17 concentrated using the protocol same as that used for MreB5 purification for
18 sedimentation assay (described above).

19 **Sedimentation assay**

20 Purified MreB5-His₆ was spun at 159,000 xg, 25 °C for 30 minutes to remove
21 denatured and/or aggregated MreB5-His₆ as pellet. Protein in the supernatant was
22 used for concentration estimation and sedimentation assay experiments. Similarly,
23 enriched fibril was also spun under the same conditions to pellet down fibril filaments
24 and then pellet re-suspended in reaction buffer. Since fibril was insoluble in the

1 buffer, its concentration was estimated by solubilizing small aliquot of fibril with
2 addition of sodium dodecyl sulphate (SDS) at a final concentration of 1 % (w/v) and
3 by measuring absorbance at 280 nm using NanoDrop (ThermoFisher Scientific).

4 Equimolar concentrations of purified MreB5-His₆ and enriched fibril were mixed in
5 the reaction buffer [50 mM Tris (pH 8.0), 300 mM KCl and 2 mM MgCl₂]. As controls,
6 reactions containing only MreB5-His₆ and only fibril samples were used. All the tubes
7 containing reactions were incubated at 25 °C for 30 minutes and then spun at
8 159,000 xg/ 25 °C for 30 minutes. The supernatant was transferred to a new tube
9 and the pellet was re-dissolved in reaction buffer with the final volume same as that
10 of the initial reaction volume (typically 50 μL). Equal volumes of supernatant were
11 mixed with 2X Laemmli buffer and visualized using 12 % SDS-PAGE gels.

12 The intensity of protein bands corresponding to the MreB5-His₆ band on SDS-PAGE
13 gels was analyzed using ImageJ 1.52p (USA) [50]. To calculate the fractional
14 intensity % of MreB in the pellet and supernatant, the intensities of the bands from
15 pellet or supernatant samples were multiplied by 100 and divided by the sum of the
16 intensities the bands in pellet and supernatant. The data in the graph is expressed
17 as the mean ± standard deviation (SD).

18 As a negative control, the sedimentation experiment was repeated by using
19 mCherry-His₆ instead of MreB5-His₆.

20 **Liposome preparation**

21 All the lipids used for liposome preparation were purchased from Avanti Polar Lipids,
22 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 850375C), 1,2-dioleoyl-sn-
23 glycero-3-phospho-(1'-rac-glycerol) (DOPG, 840475C), Brain SM (Sphingomyelin
24 (Brain, Porcine), 860062C), *E. coli* Cardiolipin (841199C). To obtain a final working

1 concentration of the liposome for the assay to 2 mM, 0.28 mM of DOPC, 0.76 mM of
2 DOPG, 0.66 mM of Brain SM and 0.30 mM of *E. coli* CL chloroform solutions were
3 aliquoted in a clean test tube and dehydrated to remove the chloroform. The
4 concentrations of these liposome were chosen based on the available molar ratio of
5 membrane lipids found in *S. citri* [62]. For preparing the liposome, dried lipid mixtures
6 were hydrated in buffer A (300 mM KCl, 50 mM Tris, pH 8.0) containing 1 mM MgCl₂
7 to a final concentration of 2 mM. This mixture was extruded through 100 nm
8 polycarbonate membranes (Avanti Polar Lipids).

9 **Liposome pelleting assay**

10 Protein aliquot of MreB5-His₆ was spun at 100,000 xg for 25 mins at 4 °C. 2 μM of
11 protein from the supernatant was added to the reaction mixture of 100 μL containing
12 liposomes in buffer A (300 mM KCl, 50 mM Tris pH8.0) and 1 mM MgCl₂. The
13 reaction was incubated at room temperature for 15 mins. Post incubation, the
14 reaction was spun at 100,000 xg for 25 mins at 25 °C. The supernatant was carefully
15 removed and the pellet was re-suspended in 50 μL of buffer A. Supernatant and
16 pellet were mixed with 2X Laemmli buffer and equal amounts of the pellet and
17 supernatant were loaded on 12 % SDS-PAGE gel. The intensity analysis of protein
18 bands were performed on ImageJ 1.52n [50]. The fractional intensity % calculation
19 was performed in the same way as described for sedimentation assay above. The
20 data in the graph is expressed as the mean ± standard deviation (SD). RNaseA
21 (Sigma-Aldrich) at equivalent concentrations was used instead of MreB5-His₆ in
22 negative control experiments.

23 **Crystallization and Structure determination of MreB5**

1 About 960 conditions of commercially available screens (Molecular dimensions,
2 Hampton Research) were screened, using drop sizes containing 100 nL of protein (4
3 mg/mL) and 100 nL of crystallization condition. Initial hits were seen for many of the
4 conditions and were further optimized to get a well diffracting crystal. Diffraction
5 quality crystals were obtained in the condition containing 4.1 mg/mL protein
6 crystallized with 2 mM AMPPNP and 2 mM Mg²⁺ in a condition containing 0.15 M
7 Na-K phosphate, 16 % PEG 3350, pH 7.8 by hanging drop method at 1:1 ratio. The
8 crystal was frozen in 20 % glycerol cryo-protectant contained in the parent condition
9 for diffraction. Data for the crystal was collected at ESRF. The crystal diffracted to
10 2.5 Å. Data reduction was performed using DIALS [63] and data scaling using
11 AIMLESS [64] in the CCP4i2 [65] package. Molecular replacement was done using
12 ScMreB5-ADP (PDB ID: 7BVZ) in PHASER [66] available in the CCP4 [67] package.
13 Refinement was performed using the PHENIX package [68] and model building
14 using Coot [69]. Data collection and refinement statistics are summarized in Table
15 S3.

16 **Electron cryomicroscopy and filament assembly model of MreB5**

17 For visualizing filaments of MreB5-His₆ electron cryomicroscopy was performed.
18 Quantifoil (Au 1.2/1.3) grids were used that were glow discharged for 90 sec. Protein
19 samples were centrifuged at 100,000 xg for 25 mins at 4 °C. For protein
20 polymerization, 50 μM of protein was incubated with 5 mM ATP and 5 mM MgCl₂ at
21 25 °C for 10-15 mins. The glow discharged grid was mounted on the VitrobotTM
22 (Mark IV, FEI) , 3 μL of the sample was put on the grid and incubated for 5-10 sec
23 before blotting for 3 sec followed by plunge freezing into liquid ethane for vitrification.
24 For image acquisition, grids were mounted on Triton-Krios 300 KeV with Falcon-3
25 direct detector and images were acquired at a magnification of 59,000X. Images of

1 the filaments were observed using ImageJ 1.52n [70]. 2-D class averages were
2 generated using default options in RELION software [71].
3 Double protofilament assembly of MreB5 was generated by superposing MreB5
4 coordinates sub-domainwise on the double protofilament assembly of CcMreB (PDB
5 ID- 4CZJ) using Coot 0.8.9.1 [69]. Using UCSF Chimera version 1.13.1 [72] each
6 subdomain of ScMreB5 was saved as separate PDB files. The subdomains, IA, IB,
7 IIA and IIB, of ScMreB5 were separately superposed on one of the protofilament of
8 the double protofilament of CcMreB. For superposition Match Maker option in
9 Chimera was used in default settings. Similarly, ScMreB5 subdomains were
10 superposed on other two monomers of the protofilament of CcMreB. This gave a
11 protofilament assembly for ScMreB5.

12 **QUANTIFICATION AND STATISTICAL ANALYSIS**

13 All the statistical analysis and the graphs plotting was performed using the software,
14 GraphPad Prism. Details of statistical tests used, errors bar shown, significance
15 values, the number of independent repeats and the number of cells (n) are
16 mentioned in results, figure legends and also described in the method details.

17

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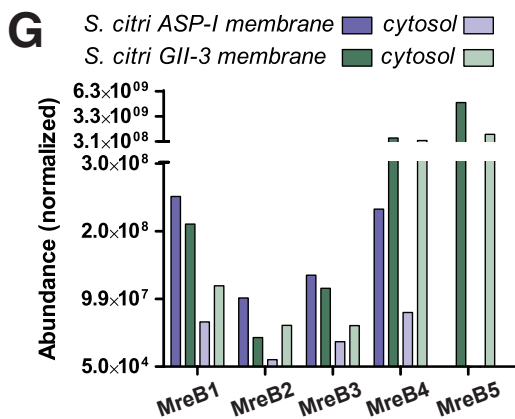
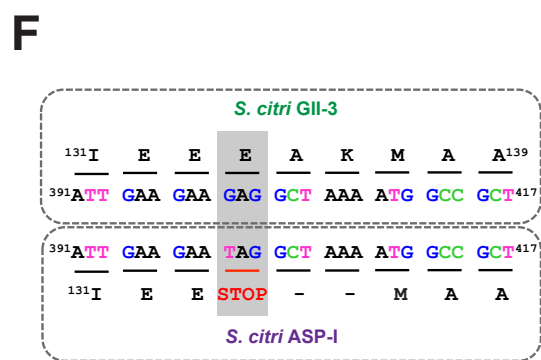
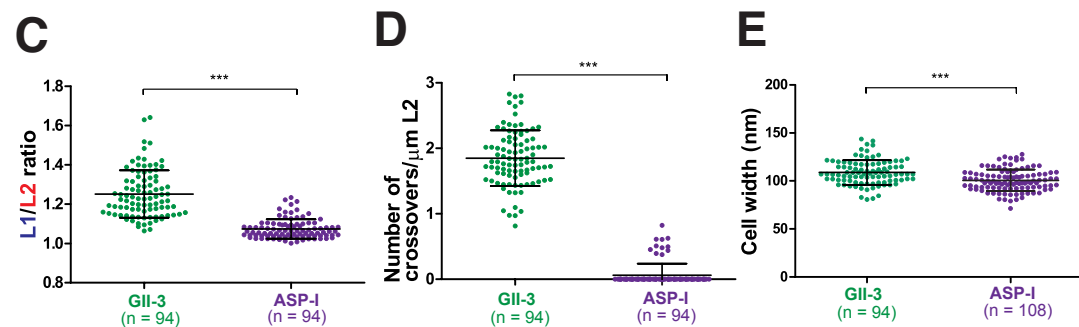
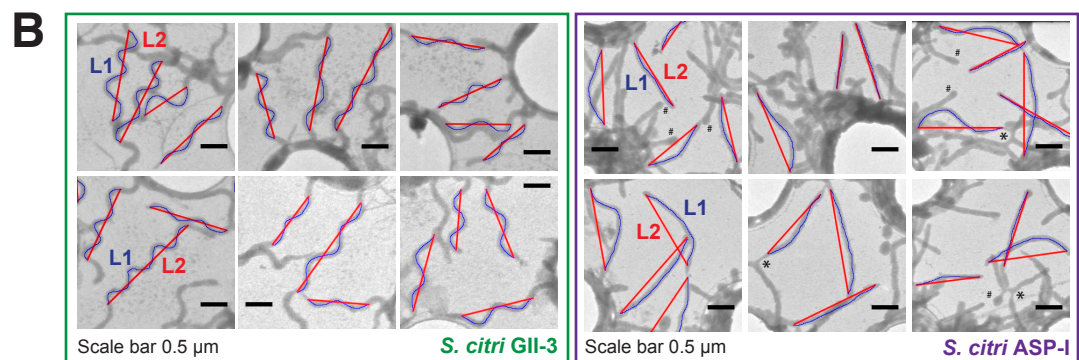
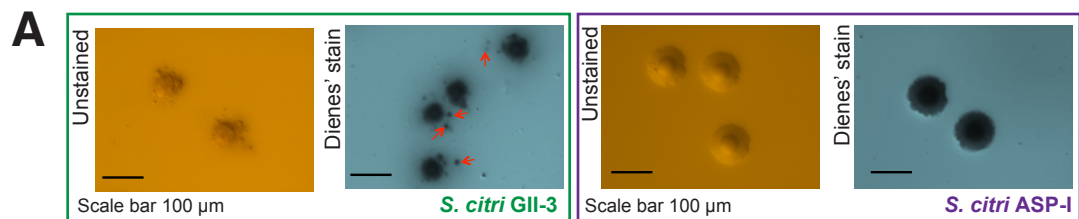
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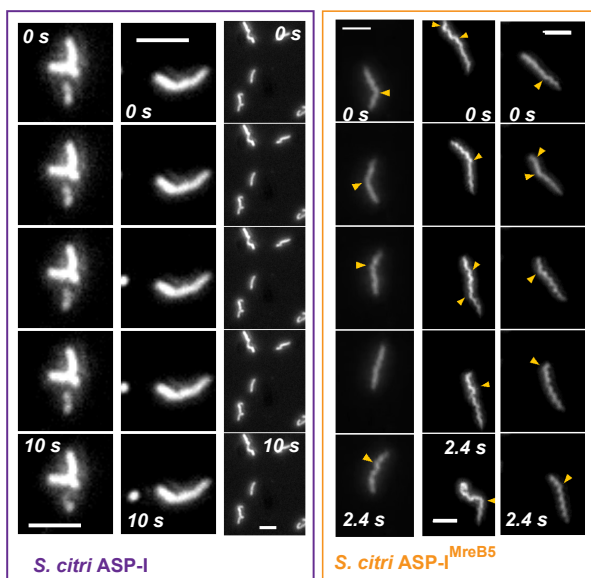
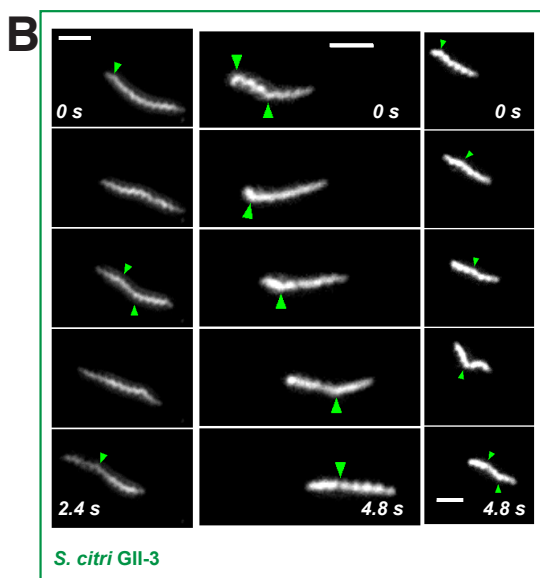
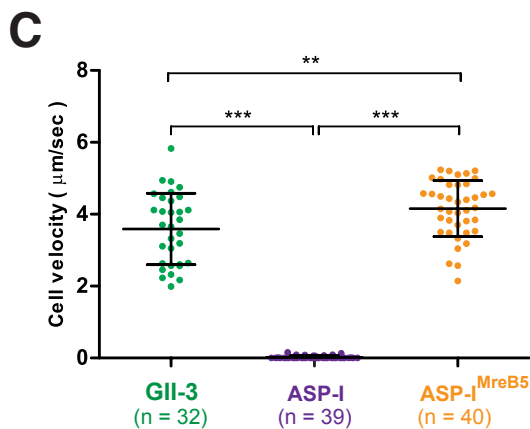
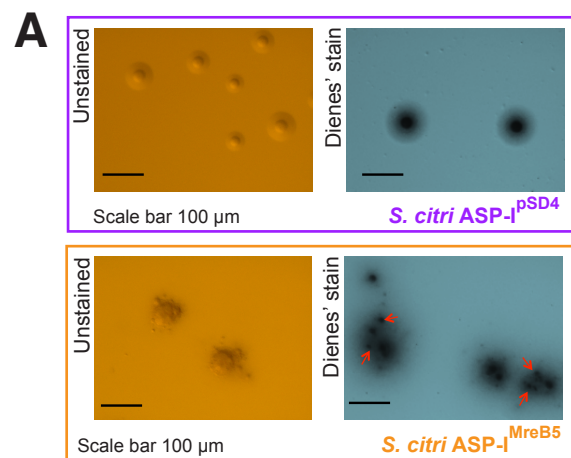
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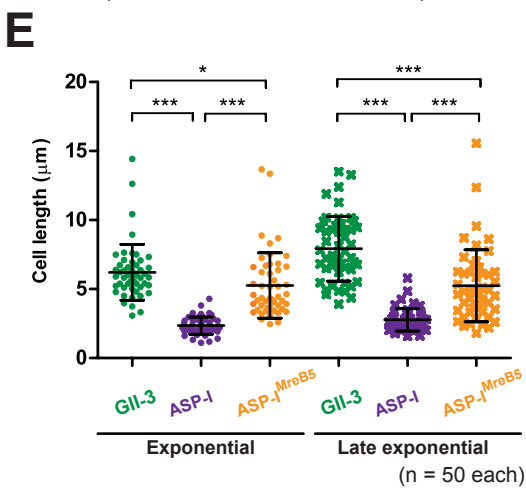
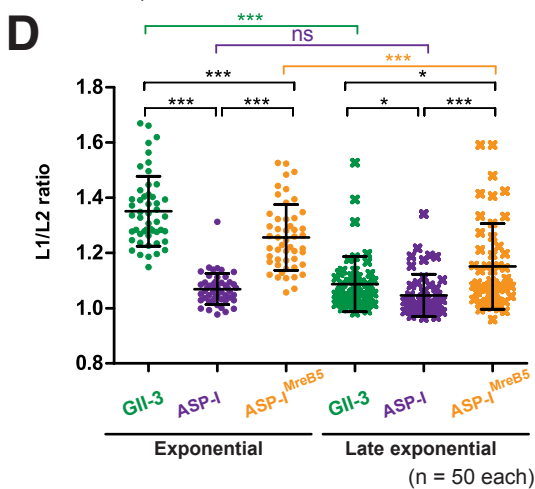


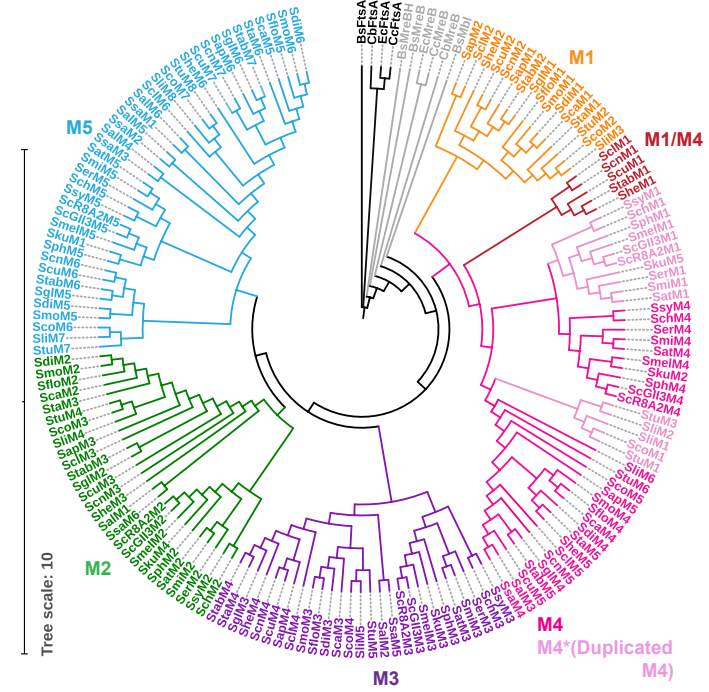


Scale bar 3 μm

Scale bar 3 μm

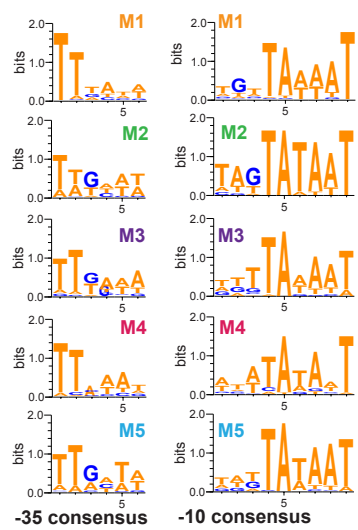
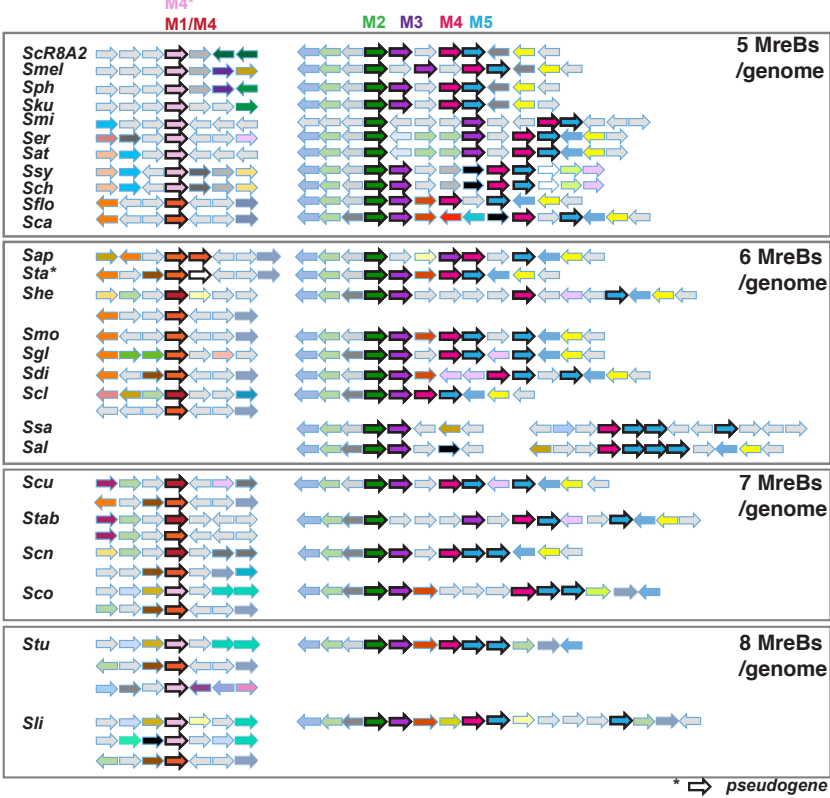
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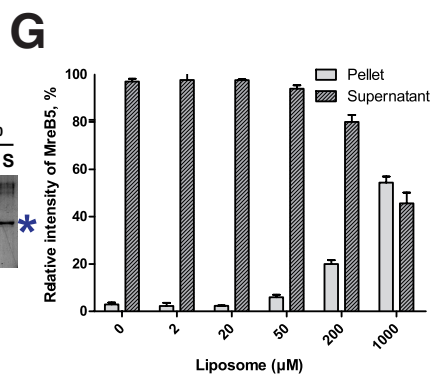
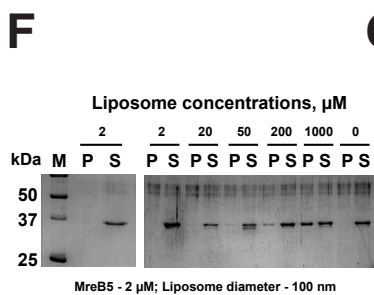
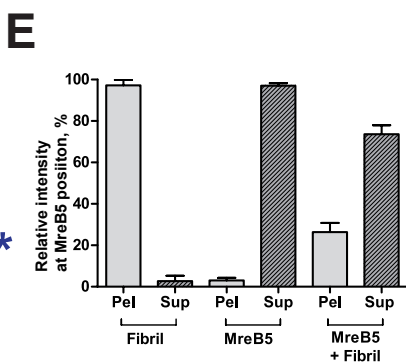
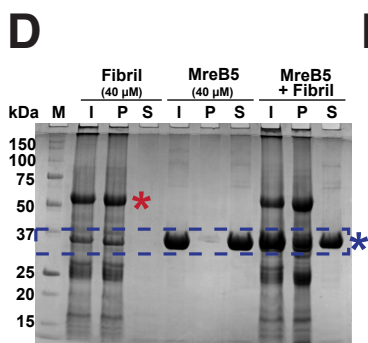
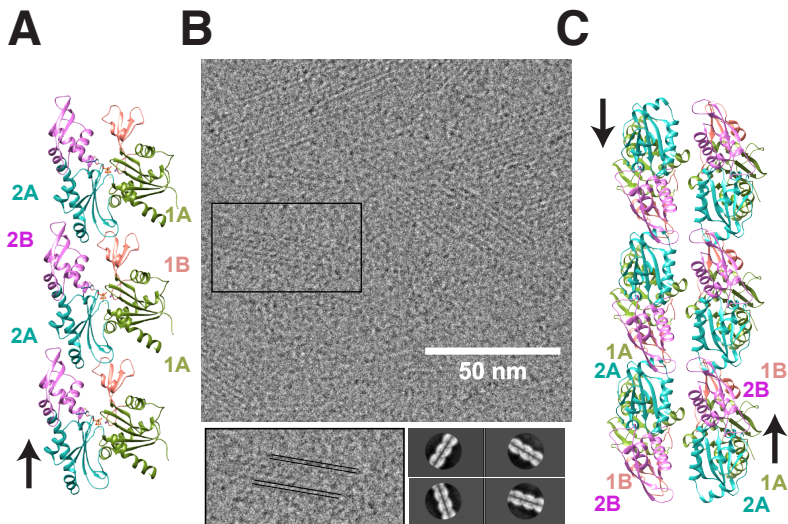
A**B**

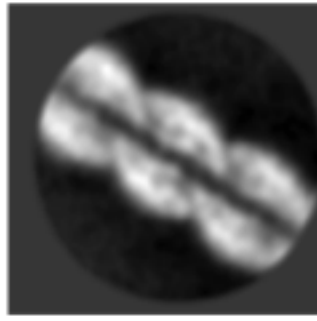
Sequence identity %, *S. citri* MreBs

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M1		53	40	86	53
M2	50		42	53	63
M3	33	38		39	44
M4	74	52	37		53
M5	50	60	37	53	

D**C**

- Color key for neighbors**
- ABC transporter ATP-binding protein
 - Acetaldehyde dehydrogenase
 - Arginyl-tRNA synthetase
 - Chromosome partitioning protein ParA
 - Class III heat-shock ATP-dependent Lon A protease (all proteases)
 - 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (all kinases)
 - DNA directed RNA polymerase subunit beta
 - DNA 3-methyladenine glycosidase
 - DNA polymerase III subunit epsilon
 - ECF transporter S component
 - Ferredoxin
 - GTP binding protein EngA
 - GTP-binding protein YsxC
 - GTP-dependent nucleic acid-binding protein
 - HAD family hydrolase
 - Heavy metal transporting ATPase
 - Holliday junction DNA helicase RuvA
 - Hypothetical protein
 - Inorganic pyrophosphatase
 - MerR family transcription regulator
 - Outer surface protein
 - Phosphonate ABC transporter, phosphonate-binding protein
 - Phosphonate transport system ATP-binding protein
 - Primase-like protein
 - Putative adhesion P123
 - Putative deoxyribonuclease (all nucleases)
 - Putative dimethyladenosine transferase (all transferases)
 - Pyruvate formate lyase activating enzyme
 - Putative NADH-flavin reductase
 - Recombination factor protein RarA
 - Ribose/Galactose ABC transporter substrate-binding protein
 - Ribonuclease M5
 - 50S ribosomal protein L7/L12
 - 30S ribosomal protein S21
 - Site-specific DNA-binding protein ParB
 - Spiroplasma plectovirus-related protein
 - tRNA modification GTPase TrmE
 - Transmembrane protein
 - Transmembrane protein pearmease (Pseudo gene) (all permeases)

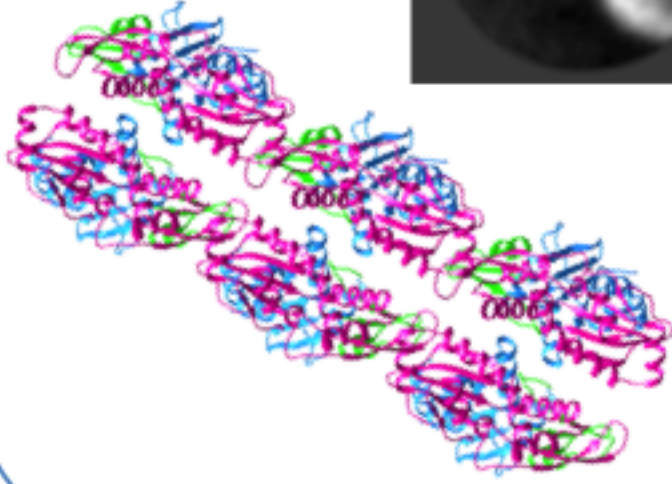




MreB5

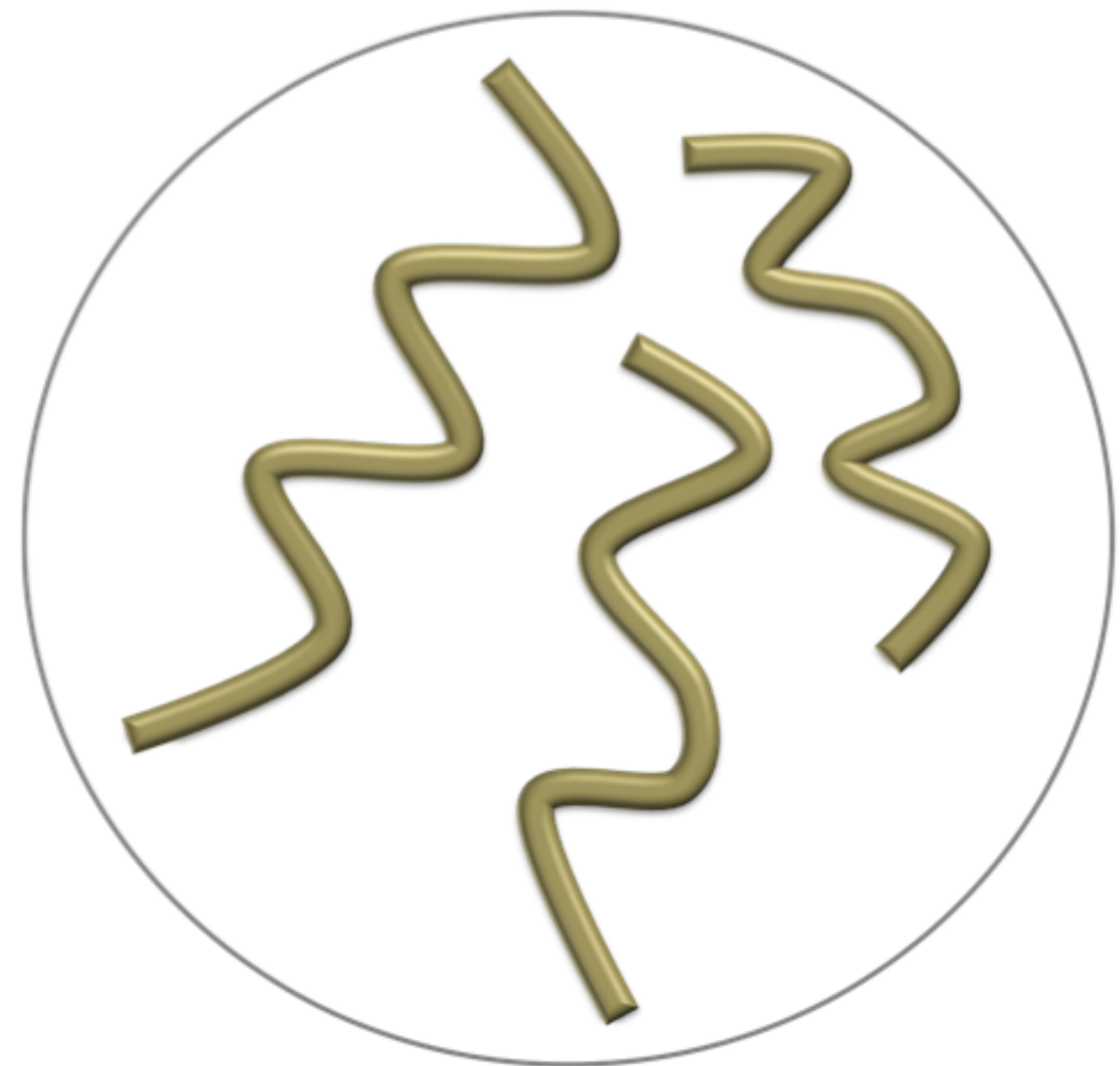
Antiparallel double
protofilaments

Binds liposomes
Binds fibril



Non-helical and Non-motile

+ MreB5



Helical and Motile