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Controlling septum thickness by a large protein ring

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Abstract

Gram-positive bacteria divide by forming a thick cross wall. How the thickness of this septal wall is controlled is unknown. In this type of bacteria, the key cell division protein FtsZ is anchored to the cell membrane by two proteins, FtsA and SepF. We have isolated SepF homologues from different bacterial species and found that they all polymerize into large protein rings with diameters varying from 19 to 41 nm. Importantly, these values correlated well with the thickness of their septa. To test whether ring diameter determines septal thickness, we tried to construct different SepF chimeras with the purpose to manipulate the diameter of the SepF protein ring. This was indeed possible and confirmed that the conserved core domain of SepF determines ring diameter. Importantly, when SepF chimeras with a smaller diameter were expressed in the bacterial host *Bacillus subtilis*, the thickness of its septa also became smaller. These results strongly support a model in which septal thickness is controlled by curved molecular clamps formed by SepF polymers attached to the leading edge of nascent septa. This also implies that the intrinsic shape of a protein polymer can function as a mould to shape the cell wall.

Significance Statement

Many bacteria form a thick cell wall and divide by forming a cross wall. How they control the thickness of their cell wall and cross wall is unknown. In this study we show that in these bacteria the cell division protein SepF forms very large protein rings with diameters that correspond to the diameter of their cross walls. Importantly, when we reduced the diameter of SepF rings in the bacterial host *Bacillus subtilis* the cross wall also became thinner. These results provide strong evidence that a large protein ring can function as a mould to control the thickness of the cell wall that divides these bacterial cells.

Introduction

The hallmark of Gram-positive bacteria is their thick cell wall composed of multiple layers of peptidoglycan. They divide by synthesizing a crosswall in between the newly formed daughter cells, and the thickness of the nascent division septum approaches that of the lateral cell wall. How Gram-positive bacteria regulate the thickness of their division septum is not known.

Bacterial cell division is accomplished by a complex multi-protein machinery called the divisome. Assembly of the divisome begins with polymerization of the tubulin homologue FtsZ at midcell into a ring-like configuration, the so called Z-ring (1). This structure forms a scaffold for the late cell division proteins that are responsible for synthesis of the dividing septal wall (2). Several cell division proteins support the formation of the Z-ring, and a key step is the anchoring of FtsZ polymers to the cell membrane. This is achieved by the conserved peripheral membrane proteins FtsA and SepF. Both proteins directly interact with FtsZ and use an amphipathic α -helix to bind the cell membrane (3, 4). FtsA can be found in both Gram-positive and Gram-negative bacteria, whereas SepF is widely conserved in Gram-positive and Cyanobacteria, but has no known homologue in Gram-negatives (5, 6). Other Z-ring proteins are the conserved protein ZapA, which interlinks FtsZ polymers (7), and the bitopic transmembrane proteins EzrA (Gram-positives) and ZipA (Gram-negatives) (8, 9). Once the Z-ring is assembled, the late cell division proteins arrive. These conserved transmembrane proteins form a complex comprising the peptidoglycan glycosyltransferase FtsW (10), the transpeptidase Pbp2B (FtsI in Gram-negatives) (11, 12), and the heterotrimeric complex composed of FtsL, DivIC and DivIB (FtsL, FtsB and FtsQ in Gram-negatives, respectively) (13, 14). It is assumed that the latter three proteins

regulate the assembly of late cell division proteins, although it is not yet clear how the late proteins are recruited to the Z-ring in Gram-positive bacteria.

Some bacteria, such as the Gram-positive model organism *Bacillus subtilis*, contain both FtsA and SepF, and this organism needs only one of them for Z-ring formation. However, the absence of SepF results in highly deformed septa, which is not the case when FtsA is absent (6). This indicates that SepF must have an additional function related to septum formation. A curious property of purified *B. subtilis* SepF is that it forms large ring structures with an inner diameter of 40 nm (15). Based on the SepF crystal structure, these rings must encompass at least 80 to 100 SepF molecules (15). *In vitro*, these protein rings are able to bundle FtsZ polymers into very long microtubule-like structures with SepF rings stacked perpendicularly to the FtsZ polymers (16). However, such microtubular structures have never been observed in bacteria, and later studies showed that the membrane-binding amphipathic α -helix of SepF is likely located inside the ring, which seems to rule out ring formation *in vivo* (15).

Interestingly, the inner diameter of SepF rings is about the same size as the thickness of the septal wall (43 nm). We wondered whether this relationship is relevant, and if so, whether SepF rings might actually control the thickness of the septum. To examine this, we purified SepF from different Gram-positive bacteria and found that all these proteins bind to lipid membranes and form large protein rings, albeit with different diameters. Importantly, also in these organisms there was a correlation between SepF ring diameter and septum thickness. To confirm that the SepF ring diameter determines septal width, we expressed SepF chimeras with smaller diameters in *B. subtilis*. Indeed, this reduced the thickness of septa. These results provide strong evidence that Gram-positive bacteria regulate the thickness of their septal wall by the strong curvature of SepF polymers at the

106 leading edge of nascent septa. This also implies that the intrinsic form of a protein
107 polymer can function as a mold that can shape a cell wall.

Results

SepF rings and tubules

Purified *B. subtilis* SepF forms large protein rings when observed with transmission electron microscopy (TEM) using negative staining with uranyl acetate (15). To confirm these findings with an independent method, we examined whether the protein can form rings under more physiological conditions using atomic force microscopy (AFM). It appeared that under these conditions purified *B. subtilis* SepF also forms rings with diameters of 41 nm (Fig. 1). Rings were 2 to 4 nm high (Fig. 1C, D), which corresponds to a single or double molecule stacking, since the crystal packing has shown that SepF polymers can be either 1.5 or 2.6 nm wide, depending on the orientation of SepF dimers (15).

Previously, SepF was purified as a maltose-binding protein (MBP) fusion followed by proteolytic cleavage and removal of MBP using ion-exchange chromatography (15, 16). We noticed that after cleavage, SepF precipitates, possibly due to the presence of calcium in the digestion buffer. After resuspension in calcium-free buffer, SepF formed stacks and tubules with diameters corresponding to that of SepF rings when observed by TEM (Fig. 1E). After ion-exchange chromatography only rings are found (Fig. 1F). These findings, together with the AFM data, show that the circular polymerization of *B. subtilis* SepF is a robust feature that may also occur *in vivo*.

SepF from other species

To determine whether ring formation is a conserved feature of SepF, we purified the protein from different organisms. *Bacillus cereus* is an important food-spoiling bacterium (17, 18) and the causative agent of rainforest anthrax (19–21) and was

chosen as a close relative of *B. subtilis*. We further selected *Clostridium perfringens*, *Mycobacterium tuberculosis*, and *Streptococcus pneumoniae*, all of which are important human pathogens. *S. pneumoniae* differs from the rest since it forms cocci instead of rods, and *M. tuberculosis* is one of the bacterial species that lack an FtsA homologue. An amino acid sequence alignment of the different SepF homologues is shown in Fig. 2A. The central domain of the protein, which contains the FtsZ binding site (15), is particularly conserved. All proteins were successfully purified as MBP fusions. *C. perfringens*, *M. tuberculosis*, and *S. pneumoniae* SepF precipitated after proteolytic cleavage from MBP and were collected by centrifugation, whereas *B. cereus* SepF remained largely soluble and was isolated by ion-exchange chromatography.

Membrane binding

A key property of *B. subtilis* SepF is its capacity to bind to the cell membrane, which is achieved by an N-terminal amphipathic α -helix (amino acids 1-13) (15). This N-terminal amphipathic helix is reasonably conserved in the different SepF variants (Fig. 2A, see Fig. S1 for helical wheel depictions), although the helices differ in their hydrophobicity and hydrophobic moment (Fig. S2). To confirm that the different SepF molecules are able to bind to lipid membranes, the purified proteins were mixed with liposomes. This caused a strong aggregation of liposomes, and deformed them into small vesicles, when observed with high-resolution structured illumination microscopy (SIM). One example, *S. pneumoniae* SepF, is presented in Fig. 2B, C (see Fig. S3 for all variants). Such liposome deformation is a typical characteristic of membrane-interacting amphipathic α -helices (22), and a *B. subtilis* SepF variant without the N-terminal α -helix does not possess this property (15). These results indicate that the membrane binding

activity of SepF is conserved.

Ring formation

Another characteristic of *B. subtilis* SepF is its tendency to polymerize into a curved structure. If this feature is important for the activity of SepF, it should not be restricted to the *B. subtilis* protein. To check this, the purified SepF homologues were spotted on TEM grids and negatively stained with uranyl acetate. Indeed, as shown in Fig. 2D, all SepF variants formed large rings of a remarkably constant size. However, the average ring diameters varied between species, from ~20 up to ~40 nm (Fig. 2F).

Correlation with septum thickness

The different ring diameters provided a first proof of the hypothesis that SepF ring diameter determines septum thickness. To confirm this we performed TEM of the different bacterial species and measured the thickness of nascent cell division septa (Fig. 2E, G). Because of its high pathogenicity we used published data for *M. tuberculosis* (23). As shown in Fig. 2I, there is a clear correlation between inner ring diameters and the thickness of septa in the different species. Only *B. cereus* did not follow this trend, with SepF rings that are clearly smaller than the width of its septa (Fig. 2F-H).

Core domain defines ring diameter

Proteolytic trimming has shown that the conserved C-terminal domain of *B. subtilis* SepF, spanning amino acids 57 to 151, is sufficient for the formation of rings (15). Therefore, we assumed that this core domain determines ring diameter. To test this, we replaced the conserved core domain of *B. subtilis* SepF with those of the

SepF proteins from the other species, maintaining the first 67 and last 13 amino acids of *B. subtilis* SepF. These chimeras were then expressed in *E. coli*, purified, and visualized by TEM using negative staining. All SepF chimeras were able to form rings, and importantly, the ring diameters of these chimeras corresponded very well to the diameters of the original SepF variants (Fig. 3A-D).

Functional chimeras

If it is true that the diameter of SepF rings determines the thickness of septa, then expressing a SepF variant with a smaller diameter in *B. subtilis* should result in thinner septa. It is unlikely that SepF from other species function in *B. subtilis*, since the N- and C-termini of SepF, which are much less conserved, are crucial for its activity (15, 16). Therefore, the chimeras provided a unique opportunity to test this. To determine whether these chimeras are active in *B. subtilis*, they were expressed in a $\Delta sepF$ strain. *B. subtilis* is one of the few species that can grow without SepF, although this leads to strongly deformed septa (6). Since these deformed septa are lined by the cytoplasmic membrane, this phenotype can be easily observed using high-resolution SIM microscopy by fluorescently labelling the cell membrane (Fig. 4A). Only the *S. pneumoniae* chimera failed to rescue the $\Delta sepF$ phenotype, while the *B. cereus*, *C. perfringens*, and *M. tuberculosis* chimeras were all able to restore normal septum formation in the $\Delta sepF$ background (Fig. 4A). To check that the membrane deformations observed by SIM are indeed indicative of deformed cell wall septa, we confirmed these findings by TEM (Fig. 4B).

The inner ring diameter of the *C. perfringens* SepF chimera (24 nm) is considerably smaller than *B. subtilis* SepF (42 nm). When this chimera was expressed in the $\Delta sepF$ strain, the septum thickness decreased substantially from

43 to 28 nm (Fig. 4C, D). A similar decrease in septum thickness was observed, when the *M. tuberculosis* SepF chimera (35 nm diameter) was expressed in the $\Delta sepF$ background (Fig. 4C-E). These results strongly suggests that the SepF ring diameter control septum thickness.

Finally, the diameter of *B. cereus* SepF is approximately 10 nm smaller than *B. subtilis* SepF (Fig. 2F), and so is the diameter of the *B. cereus* SepF chimera (Fig. 3B). However, *B. cereus* has an average septum thickness comparable to that of *B. subtilis* (Fig. 2G). If the core domain of SepF determines septum thickness, it is likely that expression of the *B. cereus* chimera in *B. subtilis* $\Delta sepF$ results in septa with a thickness comparable to that of wild type *B. subtilis* cells. This was indeed the case (Fig. 4C-E).

Discussion

In this study we have shown that the membrane binding and ring forming activities are conserved in SepF homologues, that the correlation between ring diameter and septum thickness can be found in different Gram-positive bacteria, and that the septum thickness of *B. subtilis* cells can be reduced by expressing SepF mutants with a smaller diameter. Together these findings strongly suggest that the diameter of curved SepF polymers define septum thickness. The positioning of SepF polymers at the division site cannot be directly observed using high-resolution cryo-electron microscopy due to the electron density of the bacterial cytoplasm and cell wall. However, our findings provide support for a SepF clamp model (Fig. 5). Since the membrane-binding amphipathic α -helix of SepF is located inside the rings, it is likely that SepF polymers do not form rings, but instead form arcs, wrapping the leading edge of nascent septa, on top of which FtsZ polymers bind and align. Since these arcs control the freedom of movement of FtsZ polymers, including the peptidoglycan synthetic apparatus that is linked to them, the diameter of SepF rings will determine the thickness of the septal wall. Many SepF arcs must line the leading edge of the nascent septum to maintain an even thickness. This could be facilitated by the propensity of SepF rings to form stacks (Fig. 1F).

Previous structural work has indicated that SepF rings are built of tightly packed dimers. These dimers are formed by two parallel oriented monomers (15), as a consequence all SepF dimers have the same orientation in a ring. However, FtsZ polymers treadmill in both directions in the Z-ring (26), and it is likely that they bind SepF rings in both polymer orientations. SepF binds to the C-terminal domain of FtsZ (27), which is connected to the main protein domain by a long

flexible linker (28). This flexibility might enable FtsZ polymers with opposite orientations to interact with the same SepF ring.

B. cereus SepF behaved slightly differently and showed an average inner ring diameter of 30 nm, which is smaller than its average septum thickness of 43 nm. Possibly, *B. cereus* SepF polymers are more flexible in a cellular environment, enabling a wider diameter, or there might be other factors involved in controlling septum diameter in this organism in addition to the physical confinement of SepF polymers. In fact, *B. subtilis* can still make septa, albeit irregular in shape, when SepF is absent, suggesting that other factors provide some control of FtsZ polymers. FtsA, which also forms polymers, might be such factor.

Gram-negative bacteria contain a cell wall that consists of a single-layer of peptidoglycan. The lipoproteins LpoA and LpoB, which reach through the peptidoglycan layer to contact and regulate the cell membrane-anchored peptidoglycan-synthetic enzymes, are thought to regulate peptidoglycan thickness in these species (24). Possibly, this is why Gram-negative bacteria do not need a SepF-like protein for septum thickness control. It should be mentioned that *E. coli* FtsA has been shown to form ring structures *in vitro* as well (25). However, the inner diameter of these rings is ~15 nm, which is considerably smaller than the width of septa.

Regulation of the shape of septal walls by SepF through its intrinsic curvature is reminiscent of the activity of MreB, which controls the rod shape of many bacteria (29). MreB forms polymers of defined curvature that orientate and move transversely to the long axis of cells, along the direction of greatest membrane curvature (30, 31), thereby guiding the cell wall synthetic machinery in a way that enforces and maintains a rod shape (32, 33). However, unlike SepF, the intrinsic curvature of MreB filaments, which has been estimated to have a

276 diameter of roughly 200 nm (30), does not correlate with the diameter of the cell,
 277 which is much larger (30). Thus, the control of septum thickness by a molecular
 278 clamp, such as a SepF arc, is a novel concept of how protein polymers can control
 279 the shape of growing cell walls.

Material and Methods

Strain construction

All strains used in this study are listed in Table S1, plasmids in Table S2, and primers in Table S3. Plasmids for purification of SepF variants were constructed by PCR amplification of *sepF* from the respective organisms, followed by restriction cloning into the pMalC2 plasmid (34), using the *Xba*I and *Sma*I or *Eco*RI restriction sites. pMalC2-based plasmids for purification of chimera proteins and pAPNC-213-kan-based plasmids (35) for integration of *sepF* variants into the *aprE* locus in the *B. subtilis* genome were constructed by Gibson assembly (36). pMalC2-derived purification plasmids were transformed into calcium-competent *E. coli* BL21 (DE3). pAPNC-213-kan-derived integration plasmids were transformed into *B. subtilis* 168 using a standard starvation protocol (37). Deletions of the *sepF* and *ftsA* genes were introduced by transforming the resulting *B. subtilis* strains with chromosomal DNA isolated from YK204 (*sepF::spc*) (38), BFA2863 (*sepF::ery*) (6), and TB94 (*ftsA::cat*), respectively. TB94 was created by transformation of *B. subtilis* 168 with chromosomal DNA of strain MD133 *ftsA::cat aprE::kan Pspac-ftsA* (38) followed by selection on chloramphenicol resistance but kanamycin sensitivity.

Protein purification

E. coli BL21 (DE3) strains carrying pMalC2 plasmids for purification of SepF variants were grown overnight in LB containing 100 µg/ml ampicillin at 37 °C under continuous shaking. Cultures were diluted 1:100 and allowed to grow until an OD₆₀₀ of 0.4 in the presence of ampicillin. Expression of maltose-binding protein (MBP)-tagged SepF proteins was induced by addition of 0.5 mM IPTG. Cells were harvested by centrifugation after 4 h of induction and subsequently washed in

phosphate-buffered saline supplemented with 1 mM phenylmethylsulfonyl fluoride to prevent protein degradation. Cell pellets were flash-frozen in liquid nitrogen and stored at -80 °C until further use. Cell pellets were resuspended in buffer AF (50 mM Tris-HCL, pH 7.4, 200 mM KCl, 5 mM EDTA, 0.5 mM DTT) supplemented with one Complete mini protease inhibitor tablet (Roche) and disrupted by French Press. Cell debris was removed by ultracentrifugation at 31,000 x g, and the resulting supernatant was filtered through 0.2 µm filter membranes prior to loading onto a Tricorn 10/20 column (GE Healthcare) packed with 2 ml amylose resin (New England Biolabs) equilibrated with buffer AF. After loading, the column was washed with 5 column volumes buffer AF, followed by buffer BF (50 mM Tris-HCl, pH 7.4) until the baseline was stable. MBP-tagged SepF variants were eluted with buffer BF containing 10 mM maltose. Appropriate fractions were pooled and digested with factor Xa protease (New England Biolabs) in the presence of 2 mM CaCl₂ at 4 °C overnight. *B. cereus* SepF was soluble while the others precipitated after cleavage (*B. subtilis*, *S. pneumoniae*, *C. perfringens*, *M. tuberculosis*, all chimera proteins) due to presence of calcium. Insoluble proteins were separated from soluble MBP and factor Xa by centrifugation at 10,000 x g. Pellets containing pure SepF were dissolved in buffer BF without CaCl₂, flash-frozen in liquid nitrogen and stored at -80 °C until further use. SepF variants that were soluble after factor Xa cleavage were separated from MBP and Factor Xa protease by ion exchange chromatography. To this end, digested samples were loaded onto a 1 ml HiTrap Q column (GE Healthcare) equilibrated with buffer BF. The column was washed with buffer BF until the baseline was stable, followed by washing with 7.5% buffer CF (50 mM Tris-HCl, pH 7.4, 1 M KCl) resulting in elution of MBP, and washing with 17.5% buffer CF resulting in elution of factor Xa. Pure SepF was eluted with 50% buffer CF, flash-frozen in liquid nitrogen, and stored at -80 °C.

332

333 **Atomic force microscopy**

334 Purified *B. subtilis* SepF was diluted 1:10 in buffer BF and added onto a freshly
 335 cleaved highly oriented pyrolytic graphite (HOPG) surface (Agar Scientific) and
 336 allowed to settle for 20 min at room temperature prior to imaging in buffer solution
 337 (39). Images were taken with an atomic force microscope from Nanotec
 338 Electronica (Tres Cantos) operated in jumping mode at room temperature.
 339 Olympus OMCL-RC800PSA rectangular, silicon-nitride cantilevers with a nominal
 340 tip radius of 15 nm and a nominal spring constant of 0.05 N/m were used. Images
 341 were processed and analyzed with WSxM software (40).

342

343 **Fluorescence light microscopy of liposomes**

344 Liposomes were prepared from *E. coli* polar lipid extract (Avanti Polar Lipids) as
 345 described previously (3). Liposomes were extruded through 0.2 μ m filters.
 346 Samples were stained with 1 μ g/ml Nile red, spotted on 1.2% agarose films,
 347 covered with poly-dopamine-coated cover slips (41), and immediately imaged with
 348 a Nikon Eclipse Ti equipped with a CFI Plan Apochromat DM 100x oil objective, an
 349 Intensilight HG 130 W lamp, a C11440-22CU Hamamatsu ORCA camera, and NIS
 350 elements software. Images were analyzed using ImageJ (National Institutes of
 351 Health).

352

353 **Structured illumination (SIM) microscopy**

354 Liposomes for SIM were prepared from *E. coli* polar lipid extract (Avanti Polar
 355 Lipids) as described previously (3). Liposomes were extruded through 0.8 μ m
 356 filters to obtain large enough vesicles. 0.25 mg/ml of the respective SepF variants
 357 was mixed with 2 mg/ml liposomes in SepF binding buffer. Samples were stained

with 0.5 µg/ml mitotracker green, spotted on 1.2% agarose films, covered with poly-dopamine-coated cover slips (41), and immediately imaged with a Nikon Eclipse Ti N-SIM E microscope setup equipped with a CFI SR Apochromat TIRF 100x oil objective (NA1.49), a LU-N3-SIM laser unit, an Orca-Flash 4.0 sCMOS camera (Hamamatsu Photonics K.K.), and NIS elements Ar software. SIM microscopy of bacteria was performed by staining cells with 0.5 µg/ml mitotracker green for 1 min, spotted on a thin film of 1.2% agarose (21).

Transmission electron microscopy of proteins

Protein samples were spotted on glow-discharged 200 mesh formvar/carbon-coated copper grids (Agar Scientific) and incubated for 1 min at room temperature. Excess liquid was removed with paper tissue and samples were negatively stained by adding 100 µl 2% uranyl acetate drop by drop. Excess staining solution was removed with paper tissue and samples were allowed to air dry. Samples were examined with a JEOL1010 at 60 kV.

Growth conditions for microscopy

B. cereus, *S. pneumoniae*, and *C. perfringens* were grown on tryptic soy agar plates containing 5 % sheep blood (BioMerieux). After three days of aerobic (*B. cereus* and *S. pneumoniae*) or anaerobic incubation (*C. perfringens*) at 37 °C, colonies were transferred to fresh plates and incubated for another 48 h prior to suspension in phosphate-buffered saline and preparation for electron microscopy. *B. subtilis* strains were grown in Luria-Bertani (LB) broth at 37 °C under steady agitation. The medium was supplemented with 0.1 mM IPTG to induce expression of SepF variants, where appropriate. It is important not to use more IPTG since SepF overproduction causes membrane deformations that obscure septa (42).

Overnight cultures were grown with appropriate antibiotic concentrations (100 µg/ml spectinomycin, 10 µg/ml chloramphenicol, 5 µg/ml kanamycin, 1 µg/ml erythromycin), where necessary. Cells were grown until exponential phase (OD_{600} = 0.4) prior to microscopy.

Transmission electron microscopy of bacteria

Electron microscopy of bacteria was performed according to van Wezel *et al.* (43) (*B. cereus*, *S. pneumoniae*, *C. perfringens*) or to a novel method that uses immobilization of bacterial cells in one plane on an agarose layer prior to fixation and embedding (44) (*B. subtilis*). Samples were examined with a JEOL 1010 at an electron voltage of 60 kV.

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Conflict of Interest

The authors declare that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

MW planned and performed experiments and wrote the paper. INCG constructed strains and performed experiments. YG constructed strains. JW and MGM performed experiments. GJLW and WHR provided tools and planned experiments. LWH designed the study and wrote the paper.

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Figure legends

Fig. 1 SepF forms rings and tubules. **(A)** Plane AFM images of three different SepF rings imaged in buffer solution. **(B)** 3D projections of the same images. **(C)** Height measurements of the individual SepF rings shown in A and B. **(D)** Height measurements of SepF rings derived from AFM data. **(E)** TEM image of SepF stack and tubules after cleavage from MBP. **(F)** TEM image of SepF rings after ion-exchange chromatography. Scale bars 100 nm.

Fig. 2 Membrane binding, ring formation and septum thickness. **(A)** Sequence alignment (Clustal Omega) of SepF homologues used in this study. Dark and light grey marks identical and similar amino acids, respectively. Arrows mark the beginning and end of the highly conserved core domain. **(B)** Fluorescence light microscopy image showing the aggregation of small liposomes (~200 nm) by purified *S. pneumoniae* SepF. Liposomes were stained with Nile red. **(C)** SIM image showing deformation of large liposomes (800 nm) by purified *S. pneumoniae* SepF. Liposomes were stained with mitotracker green. 0.25 µg/ml SepF was mixed with 2 mg/ml liposomes. Scale bars 1 µm. See Fig. S3 for the results with other SepF variants. **(D)** TEM pictures of purified SepF from the different species. Scale bar 50 nm. **(E)** Quantification of the inner SepF ring diameter. Ring diameter was consistently measured as the inner diameter of the SepF ring at its widest point. Black bars show mean with standard error of the mean. A minimum of 45 rings was measured. **(F)** TEM images of nascent cell division septa of the respective species. **(G)** Quantification of the thickness of the nascent septa of the respective organisms. Black bars show mean with standard error of the mean. A minimum of 50 septa was measured. **(H)** Comparison of inner ring diameters with nascent

septum thickness. Average +/- standard deviations is indicated. *Data for *M. tuberculosis* were taken from the literature (23). **(I)** Correlation between inner SepF ring diameter and septum thickness ($R^2=0.74$).

Fig. 3 The conserved core domain of SepF determines ring diameter. **(A)** TEM pictures of purified SepF chimera proteins. **(B)** Quantification of ring diameter. Black bars represent mean with standard error of the mean. A minimum of 50 rings was measured. **(C)** Comparison of the inner ring diameters of the original and chimera proteins. Average +/- standard deviation is given. **(D)** Correlation between original and chimera ring diameter ($R^2=0.71$).

Fig. 4 SepF ring diameter determines septum thickness. **(A)** SIM images of *B. subtilis* $\Delta sepF$ cells expressing different SepF chimeras. The chimeras were expressed from the IPTG-inducible *Pspac* promotor. Cells were grown in the presence of 100 μ M IPTG until mid-log phase prior to membrane staining and microscopy. Lack of SepF results in severely deformed septa, which shows as highly fluorescent membrane patches due to membrane invaginations and double membranes. Scale bar 2 μ m. **(B)** Representative TEM images of septa of $\Delta sepF$ strains expressing the different SepF chimeras. Scale bar 1 μ m. **(C)** Quantification of the septum thickness of the different strains. Black bars represent mean with standard error of the mean. p values for *C. perfringens* and *M. tuberculosis* chimeras compared to *B. subtilis* are <0.0001 . **(D)** Comparison and **(E)** correlation of the inner ring diameter of SepF chimeras and nascent septum thickness of *B. subtilis* $\Delta sepF$ expressing the chimeras ($R^2=0.57$).

Fig. 5 Clamp model illustrating how SepF polymers can control septal thickness.

589 SepF forms large rings with an average inner ring diameter (d) that corresponds
 590 to the thickness of septa (t). Crossections of a nascent septum (grey) is depicted.
 591 FtsZ polymers (Z) bind to the outside of SepF rings, in a perpendicular fasion. The
 592 transmembrane peptidoglycan synthesis machinery (P) is associated with FtsZ
 593 polymers. Of note, it is not yet know how these transmembrane proteins are linked
 594 to FtsZ polymers in Gram-positive bacteria, and this association might be indirect.

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Fig. 4.

A

B

D

	<i>B. subtilis</i>	<i>B. cereus</i>	<i>C. perfringens</i>	<i>M. tuberculosis</i>
inner ring diameter chimeras (nm)	41 ± 3	31 ± 3	24 ± 3	23 ± 3
nascent septum thickness chimeras (nm)	43 ± 8	44 ± 9	28 ± 7	35 ± 10

C

E

Fig. 4 SepF ring diameter determines septum thickness. **(A)** SIM images of *B. subtilis* $\Delta sepF$ cells expressing different SepF chimeras. The chimeras were expressed from the IPTG-inducible *Pspac* promoter. Cells were grown in the presence of 100 μ M IPTG until mid-log phase prior to membrane staining and microscopy. Lack of SepF results in severely deformed septa, which shows as highly fluorescent membrane patches due to membrane invaginations and double membranes. Scale bar 2 μ m. **(B)** Representative TEM images of septa of $\Delta sepF$ strains expressing the different SepF chimeras. Scale bar 1 μ m. **(C)** Quantification of the septum thickness of the different strains. Black bars represent mean with standard error of the mean. p values for *C. perfringens* and *M. tuberculosis* chimeras compared to *B. subtilis* are <0.0001. **(D)** Comparison and **(E)** correlation of the inner ring diameter of SepF chimeras and nascent septum thickness of *B. subtilis* $\Delta sepF$ expressing the chimeras ($R^2=0.57$).

Fig. 5.

Fig. 5 Clamp model illustrating how SepF polymers can control septal thickness. SepF forms large rings with an average inner ring diameter (d) that corresponds to the thickness of septa (t). Crosssections of a nascent septum (grey) is depicted. FtsZ polymers (Z) bind to the outside of SepF rings, in a perpendicular fasion. The transmembrane peptidoglycan synthesis machinery (P) is associated with FtsZ polymers. Of note, it is not yet know how these transmembrane proteins are linked to FtsZ polymers in Gram-positive bacteria, and this association might be indirect.

Supplementary Information

Controlling septum thickness by a large protein ring

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- **Table S1** Strains used in this study
- **Table S2** Plasmids used in this study
- **Table S3** Primers used in this study
- **Fig. S1** Comparison of the amphipathic helices of SepF variants
- **Fig. S2** Hydrophobic moment plot of the putative N-terminal α -helices of SepF variants
- **Fig. S3** Liposome aggregation and deformation by SepF variants
- **References**

Table S1 Strains used in this study.

strains	genotype	reference
<i>B. subtilis</i> 168	<i>trpC2</i>	(1)
<i>B. subtilis</i> TB92	<i>trpC2 sepF::spc</i> (YK204 in 168CA)	(2)
<i>B. subtilis</i> TB94	<i>trpC2 ftsA::cat</i> (MD133 in 168CA)	(2)
<i>B. subtilis</i> LH28	<i>trpC2 ezrA::cat</i>	(3)
<i>B. subtilis</i> BFA2863	<i>trpC2 sepF::ery</i>	(4)
<i>B. subtilis</i> GYQ457	<i>trpC2 aprE::kan Pspac-sepF(subtilis-cereus)</i>	this study
<i>B. subtilis</i> GYQ475	<i>trpC2 aprE::kan Pspac-sepF(subtilis-pneumoniae)</i>	this study
<i>B. subtilis</i> GYQ544	<i>trpC2 aprE::kan Pspac-sepF(subtilis-perfringens)</i>	this study
<i>B. subtilis</i> GYQ474	<i>trpC2 aprE::kan Pspac-sepF(subtilis-tuberculosis)</i>	this study
<i>B. subtilis</i> MW18	<i>trpC2 aprE::kan Pspac-sepF(WT) sepF::spc</i>	this study
<i>B. subtilis</i> GYQ458	<i>trpC2 aprE::kan Pspac-sepF(subtilis-cereus)</i>	this study
<i>B. subtilis</i> MW89	<i>trpC2 aprE::kan Pspac-sepF(subtilis-pneumoniae)</i>	this study
<i>B. subtilis</i> MW92	<i>trpC2 aprE::kan Pspac-sepF(subtilis-perfringens)</i>	this study
<i>B. subtilis</i> MW86	<i>trpC2 aprE::kan Pspac-sepF(subtilis-tuberculosis)</i>	this study
<i>E. coli</i> BL21(DE03) pNC1	<i>pMalC2-sepF(coelicolor3)</i>	this study
<i>E. coli</i> BL21(DE03) pNC2	<i>pMalC2-sepF(cereus)</i>	this study
<i>E. coli</i> BL21(DE03) pNC4	<i>pMalC2-sepF(tuberculosis)</i>	this study
<i>E. coli</i> BL21(DE03) pNC5	<i>pMalC2sepF(coelicolor2)</i>	this study
<i>E. coli</i> BL21(DE03) pNC6	<i>pMalC2-sepF(coelicolor1)</i>	this study
<i>E. coli</i> BL21(DE03) pNC7	<i>pMalC2-sepF(perfringens)</i>	this study
<i>E. coli</i> BL21(DE03) pNC9	<i>pMalC2-sepF(pneumoniae)</i>	this study
<i>E. coli</i> BL21(DE03) pNC12	<i>pMalC2-sepF(subtilis)</i>	(5)
<i>E. coli</i> BL21(DE03) pYQ62	<i>pMalC2-sepF(subtilis-cereus)</i>	this study
<i>E. coli</i> BL21(DE03) pYQ94	<i>pMalC2-sepF(subtilis-tuberculosis)</i>	this study
<i>E. coli</i> BL21(DE03) pYQ95	<i>pMalC2-sepF(subtilis-perfringens)</i>	this study
<i>E. coli</i> BL21(DE03) pYQ96	<i>pMalC2-sepF(subtilis-pneumoniae)</i>	this study
<i>B. cereus</i> ATCC14579		(6)
<i>S. pneumoniae</i> ATCC6305		(7, 8)
<i>C. perfringens</i> ATCC13124		(9)

Table S2 Plasmids used in this study.

plasmid	genotype	reference
pMW8	<i>pAPNC-213-kan-Pspac-sepF(WT)</i>	this study
pYQ90	<i>pAPNC-213-kan-Pspac-sepF(subtilis-cereus)</i>	this study
pYQ97	<i>pAPNC-213-kan-Pspac-sepF(subtilis-tuberculosis)</i>	this study
pYQ98	<i>pAPNC-213-kan-Pspac-sepF(subtilis-perfringens)</i>	this study
pYQ99	<i>pAPNC-213-kan-Pspac-sepF(subtilis-pneumoniae)</i>	this study
pNC1	<i>pMalC2-sepF(coelicolor3)</i>	this study
pNC2	<i>pMalC2-sepF(cereus)</i>	this study
pNC4	<i>pMalC2-sepF(tuberculosis)</i>	this study
pNC5	<i>pMalC2sepF(coelicolor2)</i>	this study
pNC6	<i>pMalC2-sepF(coelicolor1)</i>	this study
pNC7	<i>pMalC2-sepF(perfringens)</i>	this study
pNC9	<i>pMalC2-sepF(pneumoniae)</i>	this study
pNC12	<i>pMalC2-sepF(subtilis)</i>	(5)
pYQ62	<i>pMalC2-sepF(subtilis-cereus)</i>	this study
pYQ94	<i>pMalC2-sepF(subtilis-tuberculosis)</i>	this study
pYQ95	<i>pMalC2-sepF(subtilis-perfringens)</i>	this study
pYQ96	<i>pMalC2-sepF(subtilis-pneumoniae)</i>	this study

Table S3 Primers used in this study.

primer	description	sequence	used for
MW135	pAPNC-213-kan-GA-for	GGCGTTAGCCCAAGCGC	pMW8
MW139	pAPNC-213-kan-GA-rev	ACACCCCCTGTTTCATTTCCCTAGCAGGTCAA TTGTGAGCGC	pMW8
MW140	<i>sepF</i> -WT-GA-for	GGAAATGAAACAGGGGGTGTACAGCAATGAG TATGAAAAATAAACTGAAAACTTTTTCTCAAT GGAAGATGAAG	pMW8
MW141	<i>sepF</i> -WT-GA-rev	GCGCTTGGGCTAACGCCTTACCACCTCTGATG TTCGTCTTCAGATATG	pMW8
eg122	<i>sepF-cereus</i> -for	GACGAATTCATGAGTTGGTCAAAAG	pNC2
eg123	<i>sepF-cereus</i> -rev	GACTCTAGATTACCACCTCTTTAT	pNC2
inc15	<i>sepF-pneumoniae</i> -for	GTAGGAGCCCGGGATGTCTTTAAAGATAG	pNC9
inc16	<i>sepF-pneumoniae</i> -rev	GCTAGACTCTAGATTATCGTACTCTATTTTC	pNC9
inc9	<i>sepF-perfringens</i> -for	GATTAGCCCGGGATGTGTATGTCAAAAG	pNC7
inc10	<i>sepF-perfringens</i> -rev	GACTATCTAGATTATTTTGAAGCCCAGTTG	pNC7
inc7	<i>sepF-tuberculosis</i> -for	GGTTACGGAATTCGTGAATAGTCACTGTAG	pNC4
inc8	<i>sepF-tuberculosis</i> -rev	GGCGACCGTCTAGACTATTGGTAGGCGTAG	pNC4
inc17	<i>sepF-coelicolor</i> 1-for	GTGAGAGGAGGAATTCATGGGATCGGTAC	pNC6
inc18	<i>sepF-coelicolor</i> 1-rev	TGTGCGGCTCTAGATCAGCTCTGGTTGAAG	pNC6
inc19	<i>sepF-coelicolor</i> 2-for	GAGGACTCCCGGGATGGCCGGCGCGATG	pNC5
inc20	<i>sepF-coelicolor</i> 2-rev	ACCGGTAGTCTAGATCAGCTCTGGTTGAAG	pNC5
eg139	<i>sepF-coelicolor</i> 3-for	GACGAATTCGTGAAATCGGGGGAGC	pNC1
eg140	<i>sepF-coelicolor</i> 3-rev	GACTCTAGATCACACTCCCGGCAC	pNC1
YQ276	<i>sepF-cereus</i> -for	AATCCTCTAAAGTGGTGTGTTAGAACACGC ACATATTCGGA	pYQ62, 90
YQ277	<i>sepF-cereus</i> -rev	CGCACCAACAATATCTACATTTTC	pYQ62, 90
YQ278	pMalC2-for	ATGTAGATATTGTTGGTGCGATTTCTGAGCTC ATATCTGAAGAC	pYQ62, 90
YQ279	pMalC2-rev	CAACACCACTTTAGAGGATTTCTG	pYQ62, 90
YQ349	pMalC2-rev	TTTCTGAACACTTTGCAAGCTCAC	pYQ94-99
YQ350	<i>sepF-tuberculosis</i> -for	GAGCTCATATCTGAAGACGAACATC	pYQ94, 97
YQ351	pMalC2-for	GCTTGCAAAGTGTTTCAGAACTCTCGAAGATC ACCACGCT	pYQ94-99
YQ352	<i>sepF-tuberculosis</i> -rev	TCGTCTTCAGATATGAGCTCGCGCTCCTCGGG GGACACATC	pYQ94, 97
YQ353	<i>sepF-perfringens</i> -	GCTTGCAAAGTGTTTCAGAAAACAGCTAAATTA	pYQ95, 98

	for	ATGATAACTAAACCA	
YQ354	<i>sepF-perfringens</i> - rev	TCGTCTTCAGATATGAGCTCCTTTAATTCATTG CTTACTTCAAC	pYQ95, 98
YQ355	<i>sepF-pneumoniae</i> - for	GCTTGCAAAGTGTTTCAGAAAAAGGTCATTATA GATGTTTCGTTATC	pYQ96, 99
YQ356	<i>sepF-pneumoniae</i> - rev	TCGTCTTCAGATATGAGCTCGATATCTTCAAC ATTACAATAACATTC	pYQ96, 99

Fig. S1 Comparison of the amphipathic helices of SepF variants. Helical wheel projections (RZ lab) show the first 13 amino acids of the different proteins. Hydrophilic residues are represented as circles, hydrophobic residues as diamonds, negatively charged as triangles, and positively charged as pentagons. Hydrophobicity is color coded with green indicating the highest and yellow the lowest (zero) hydrophobicity. Hydrophilic residues are depicted in red with the amount of red decreasing to orange proportionally to the hydrophilicity. Potentially charged residues are depicted in light blue.

Fig. S2 Hydrophobic moment plot of the putative N-terminal α -helices of SepF variants. High H values indicate strong hydrophobicity. The hydrophobic moment (μH) is a measure for the amphipathicity of an α -helix (high values indicating strong amphipathicity). The plot of H and μH indicates the probability of an α -helix to reside in the globular or surface-exposed domains of a protein and can be used to predict transmembrane or amphipathic membrane-binding helices. Black dots indicate known globular (*Bacillus thermoproteolyticus* thermolysin, aa 67-88), transmembrane (*B. subtilis* EzrA, aa 1-21) and amphipathic (*B. subtilis* FtsA, aa 424-440) helices for comparison. N-terminal α -helices of SepF homologues are depicted in red.

Fig. S3 Liposome aggregation and deformation by SepF variants. **(A)** Fluorescence light microscopy images of small liposomes (200 nm) stained with Nile red. **(B)** SIM images of large liposomes (800 nm) stained with mitotracker green. 0.25 μg/ml of the different SepF proteins (species names indicated) were mixed with 2 mg/ml liposomes prepared from *E. coli* polar lipid extract. Scale bars 1 μm.

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