The organization of the actin homologue MreB in bacteria

Abstract

A major research topic in cell shape research is the function of the protein MreB. Over the last 15 years several research groups have presented evidence, mostly based on fluorescence microscopy techniques, both in favour of and against a model that describes MreB as a helical structure, possibly spanning from pole to pole in rod-shaped bacteria, mostly *Escherichia coli* and *Bacillus subtilis*. Different properties of MreB have been studied such as length of filaments and speed of movement, as well as its response to nutrient and oxygen depletion. In this paper a selection of articles on this subject, published in highly regarded scientific journals, is reviewed and the different results and arguments are discussed. Based on the reviewed material I feel that filaments of a certain length, that extend a good part of a turn around the cell periphery and aligning relatively parallel to each other along the length of the cell are the likely candidates for MreB organization in healthy cells.

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Table of contents

Introduction

Bacteria are found in a wide variety of shapes and volumes, some are completely spherical while others form spirals or rod-like structures. Generally, most species maintain a uniform shape and size during their vegetative growth phase, and pass these morphological characteristics to their offspring. Hence, bacteria have to contain various mechanisms that control and modulate the cell into the correct shape. In rod-shaped bacteria, the bacterial actin homologue MreB has been identified as one of the key components to maintain the characteristic elongated shape (van den Ent, Amos, and Löwe 2001; Jones, Carballido-López, and Errington 2001). Depleting cells of MreB has been proven to increase the diameter of the bacteria and yield spherical cells that eventually die under normal growth conditions (Salje et al. 2011). Therefore, it is of great importance to understand how this protein organizes in the rod-shaped cells, since this offers important clues on how the bacteria maintain their morphology.

Initially, MreB was thought to form helical structures, spanning from one cell pole to the other, following a dynamic treadmill-type of movement (Jones,Carballido-López, and Errington 2001), similar to what had been observed with actin filaments. Polymerizing one side of the helix, while simultaneously depolymerizing the other side, will induce net movement of the filament , though maintaining a backbone-assembly that supports the cell structure (Mogilner and Oster 2003). Later studies, using confocal and total internal reflection (TIRF) microscopy, found evidence that challenges the presence of the extended helices in the rod-shaped cells. In these studies, MreB appeared to form patches and clusters which moved in a circumferential motion, perpendicular to the cell length due to their association to the cell wall synthesis machinery (Domínguez-Escobar et al. 2011; Garner et al. 2011; van Teeffelen et al. 2011). As a result, the actual distribution and function of MreB in rod-shaped cells is still debated at this moment. In general, the articles that were studied for this paper can be divided into three models that attempt to describe the organization and distribution of MreB. (1) The MreB proteins form helices that extend between both cell poles. (2) MreB proteins organize into filaments that wrap around the cell, that vary in length between a quarter turn and a few full turns. (3) MreB forms small patches or protein clusters that move rapidly around the cell.

The aim of this paper is to create an overview of significant experimental observations that have been made with regard to the organization and function of the actin homologue MreB in rod-shaped bacteria. After introducing some of the pertinent cell structures such as the peptidoglycan sacculus and actin, a more detailed description of the protein MreB is presented. Next, several important experimental observations and methods are presented, in separate sections, which influence the views of MreB localization. The last section of the paper attempts to summarizes all the findings.

The field of MreB research is enormous and reading all that has been published is impossible. A choice has been made about which papers to read and analyse. Obviously, the discussion of the MreB models is coloured by the choice of papers. Judging from the reference lists in each paper, it seems that the authors were aware of each other's work giving the selection of articles a level of cohesiveness. To what degree counter arguments to proposed theories are represented is very difficult to know, but most of the facets that are important for the MreB functionality and distribution discussion have both proponents and dissenters. Hopefully, this provides a diverse foundation of information to base this study on.

Description of some of the important cell shaping components

Peptidoglycan sacculus

In bacteria there is a peptidoglycan (PG) support structure which surrounds the cytosolic membrane in Gram-positive bacteria , while the PG lattice is fitted between the cytosolic and outer membrane of Gram-negative bacteria. This encasing network, called the sacculus, not only introduces mechanical strength to withstand osmotic changes and intracellular forces, but also has a great impact on the cell morphology. The glycan strands are synthesised by forming β-1,4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid (Höltje 1998, 181-203). These strands are then cross-linked by penicillin-binding proteins (PBPs) by interlinking short peptides to the glycan chains. Newly synthesised PG contain pentapeptides, and then are quickly shortened to tetra- or tripeptides by carboxypeptiases (Typas et al. 2012). Generally, Gram-positive and Gram-negative bacteria have different peptides, with varying amino acid compositions, which interconnect the various glycan strands. As the cell grows and divides, constant remodelling of the sacculus has to take place, or else the transpeptiation would only thicken the mesh without elongating the cell. This requires directed and highly specific cleavage of the PG layer to insert new PG monomers at the right position for proper shape maintenance. It has been estimated that ~40-50% of the total peptidoglycan structure has to be removed during the growth of each *E.coli* generation (Typas et al. 2012). This underlines that the cells require a proficient recycling system to ensure an efficient build-up of the sacculus.

Actin

MreB is the bacterial homologue of the extensively studied actin protein. Actin forms filaments and is an indispensable element of the eukaryotic cytoskeleton, it is conserved in a broad variety of species, including yeast and humans. It appears to be the most abundant protein in numerous eukaryotic cells (van den Ent, Amos, and Löwe 2001). Due to the structural similarities between MreB and actin (van den Ent, Amos, and Löwe 2001), MreB was first suggested to adopt a similar organization pattern as actin under certain conditions. For instance, MreB was assumed to follow the treadmill-type of movement that had been proposed for actin. Actin is a 43 kDa globular protein, with an ATP-binding domain (Defeu Soufo and Graumann 2004), and its polymerization is a strictly ordered process, were growth occurs at the 'barbed' end of the filament. At this side the needed critical concentration of actin monomers is low, ~0.2 µM, which translates into a high affinity for new monomers. At the same time, actin monomers are released from the pointed end after ATP hydrolysis. On this side of the affinity for binding mew actin monomers is low, which means that the critical monomer concentration is higher (~0.7 µM) (Mogilner and Oster 2003). This combined process of assembly and disassembly, induces a net movement of the filament, better known as 'treadmilling', and requires a constant actin turnover.

Bacterial actin homologue MreB

MreB is a bacterial actin homologue and is found in most non-spherical cells. *E. coli* and *C. crescentus* only contain one form, while other bacteria, like *B. subtilis*, synthesise an additional MreB-like (Mbl) protein (Salje et al. 2011). In E. coli, MreB is built-up by 347 aa, and weighs 37.0 kDa. Besides being vital for inducing and maintaining a proper morphology in many rod-shaped cells, this protein is also capable of inducing negative curvature in liposomes. This observation is in agreement with the model that MreB is on the inside of the membrane near the lipids and bending the membrane (Salje et al.

2011). It has been shown that MreB, from both *E.coli* and *Thermotoga maritima*, interacts directly with the cell membrane. The proposed binding is suggested to occur via an insertion loop and/or a N-terminal amphipathic helix that gets embedded into the lipid surface. (Salje et al. 2011)

In general, the protein consists of two main domains, with the ATP-nucleotide binding pocket encased in-between (van den Ent, Amos, and Löwe 2001). When superimposing the crystal structures of actin and MreB, it is clear that there is a high resemblance between the two nucleotide binding sites since several of the active site amino acids are placed in similar positions. But there are small variations that could indicate that the two proteins have different nucleotide states (van den Ent, Amos, and Löwe 2001). In vitro experiments with MreB from *T. maritime* showed that the protein assembled into filaments and lateral sheets if ATP was present (van den Ent, Amos, and Löwe 2001; Popp et al. 2010). Interestingly, when the ATP binding site was blocked with A22, thereby inhibiting MreB polymerization, the cells were deformed and the distribution pattern of MreB was distorted (Gitai et al. 2005). But also several PG precursor synthesising proteins like MurA–MurG and MraY lost their normal MreB-like distribution pattern, when the cells were treated with A22. This implies that these proteins have direct or indirect cytosolic interactions with MreB (White and Gober 2012). There is also evidence that MreB collaborates with other proteins, like RodZ which helps enforcing a proper cell-shape (Bendezú et al. 2009), Crescentin which generates curvature in *Caulobacter cresentus* (Typas et al. 2012), PBPs which play an important role in cell wall synthesis, and even interacts with RNAses (van Teeffelen et al. 2011; Salje et al. 2011).

Aspects of MreBs localisation and organisation in the cell

Dimensions of MreB structures

There are clear variations between the observed localization patterns and lengths of the MreB polymers. One of the first articles published, written by Jones et al in 2001, suggested that the protein formed bands and filamentous structures in the *B. subtilis* cells. (See fig 1A, model 2) Also other groups support the view of shorter filamentous structures. Reimold et al. (2013) quantified that in fifty percent of all protein strands in a cell covered half a turn around the cell circumference, perpendicular to the length axis, whereas about a third of the proteins covered only a quarter turn, and one-sixth stretched three-quarters to a full turn (fig 1D). This implies that the majority of the filaments are long enough to couple MreB-associated or other membrane proteins on the opposite sides of the cell circumference. While more recent papers, using conventional and high resolution fluorescent imaging of YFP-MreB (fig 1 B and E, model 1), observed pole- to-pole spanning helices covering the entire length of E. coli, with multiple turns (Grotjohann et al. 2011).

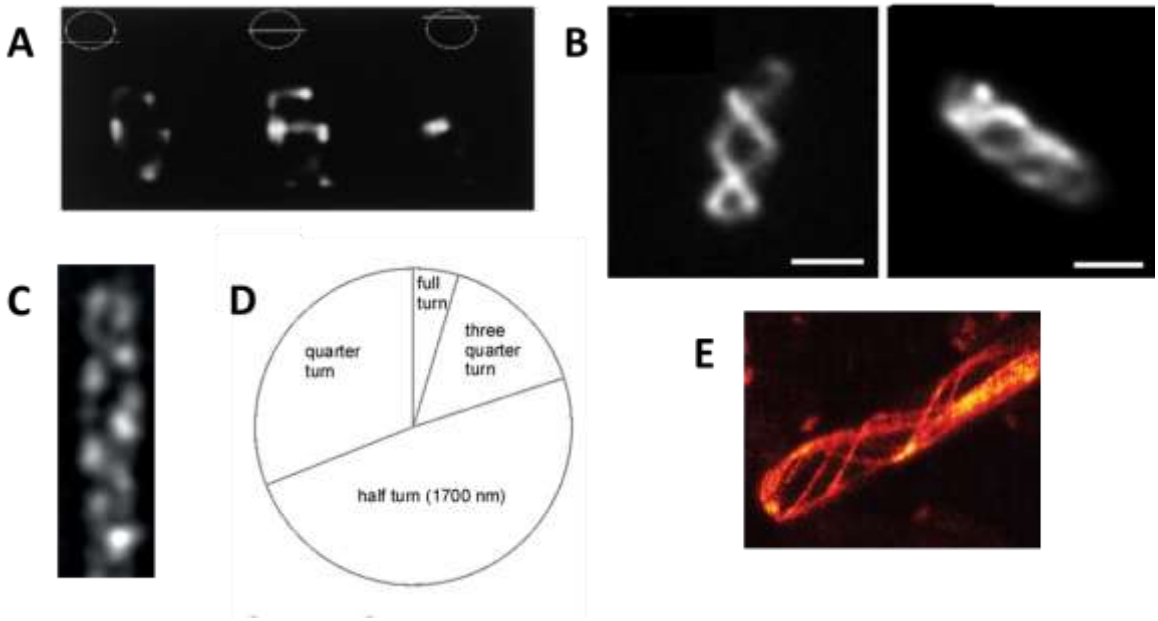

Figure 1. A) Z-stack images of c-Myc-MreB in *B. subtilis* which were detected with monoclonal antibody against c-Myc (Jones, Carballido-López, and Errington 2001). B) Fluorescent images of YFP-MreB in *E. coli* (Vats and Rothfield 2007). C) GFP-MreB localization in *B. subtilis* (Domínguez-Escobar et al. 2011). D) Relative portion of filaments within a particular size range(Reimold et al 2013). E) a RESOLFT high-resolution image of rsEGFP-MreB in *E.coli* (Grotjohann et al. 2011).

In contrast, articles written by (Garner et al. 2011; Domínguez-Escobar et al. 2011), both published 8th of July 2011, suggest a more patch-like form of MreB along the cell wall (fig 1C, model 3), and is in clear contradiction to the proposed helical structures. Swulius et al. (2011) reported that no long helical filaments were observed near or embedded in the inner membrane of six different rod-shaped bacterial species. But it must be mentioned that filament-like structures were observed by Garner et al, and therefore do not completely rule out the existence of larger MreB assemblies.

Dynamics of MreB structure

In general, the structure of MreB could either be permanent over the cell lifetime, without significant renewal . While the other option is that the polymer strands are constantly rebuilt, perhaps by

polymerizing on one end and de-polymerizing on the other end. Shorter filaments or patches are in theory more mobile because fewer connections need to be broken and or re-established upon movement. That way the cell may respond faster to changes in its environment. The shape control could be exerted in a dynamic way, moving around the cell surface, as opposed to a scaffold which simply delivers static support. But it is important to realize that shorter fragments will need to move faster to be able to induce similar shape control and forces on the membrane when compared to long polymer stretches. Extended structures have the advantage of providing more long-range forces and therefore do not need move as fast to ensure a proper cell, but are usually more rigid. Computer simulations performed by van Teeffelen et al. (2011) (figure 2) show that rotating MreB was crucial for actively maintaining a smoother rod-shape. Without rotation, the rod showed bulges on all sides and resulted in misshaped cells.

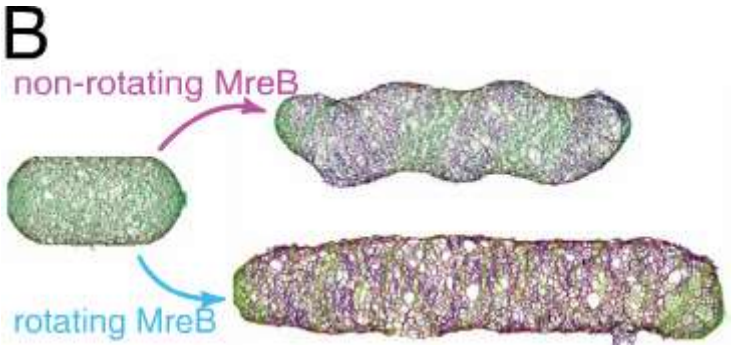

Figure 2. Computer simulation modelling the predicted cell wall shape after a threefold increase in cell length inserting strands from rotating and non-rotating MreB (van Teeffelen et al. 2011).

Conceptually, MreB was viewed at first as having a similar function to actin, as the two proteins have significant structural similarities(van den Ent, Amos, and Löwe 2001). Therefore it was speculated that MreB also moved using a treadmilling mechanism. Due to the speed and directional movement of MreB in the cells, it was argued that *if* MreB´s motion was caused by whole-filament movement, the timespan in which single MreB-YFP fusions were visual, would only depend on photobleaching and therefore not be timescale limited. In contrast, treadmilling MreB monomers would also be affected by photobleaching, but also be influenced by the depolymerisation at the polymer end. Since the average time required for photobleaching was longer than the average measured time before a single YFP-MreB disappeared out of view, it was show that there must be protein turnover in the filaments, thereby showing that MreB adopts treadmilling mechanism (Kim et al. 2006).

Conversely, fluorescence recovery after photobleaching (FRAP, see fig 3) and inverse FRAP experiments have argued against treadmilling. As a certain turnover of MreB is expected at the ends of the protein filaments, the fluorescent signal should recover in time after bleaching a YFP-MreB cluster. However, the fluorescent signal did not recover. Also the negative control, using iFRAP, did not expose a significant drop in fluorescent signal. Confirming that there was no measurable protein turnover in the protein cluster. Hence, MreB does not apply a treadmilling mechanism (Domínguez-Escobar et al. 2011). Other discovered evidence against treadmilling, was that MreB clusters were seen to undergo fusion and fission events (Domínguez-Escobar et al. 2011). So far it remains unclear whether MreB moves due to a treadmilling mechanism or that other factors induce the motion.

Figure 3. TRIF-FRAP partially bleaching YFP-MreB cluster, no bleach recovery while the motility was unaffected (Domínguez-Escobar et al. 2011).

MreB speed

The measured speed of MreB patches and filaments in *B. subtilis* was fairly consistent, ranging between 20 – 50 nm/s with maximum speeds of about 90 nm/s. (Garner et al. 2011; van Teeffelen et al. 2011; Domínguez-Escobar et al 2011; Reimold et al 2013) Interestingly, MreBs speed in *E. coli* was slightly lower, moving at 7 nm/s. (van Teeffelen et al. 2011) Comparing the shape of the two bacteria we observe that *E. coli* has a larger diameter 1.4 µm (van Teeffelen et al. 2011) as opposed to the diameter of *B. subtilis*, 1 µm (Domínguez-Escobar et al. 2011). Knowing that MreB movement is necessary for cell shape maintenance, makes it logic from a physics point of view, that the shape controlling molecules need to encircle the cell faster, i. e. move at higher speed to exert the required inward directed force to maintain a smaller diameter. But this needs to be confirmed, and other additional factors may also be at play, because the difference in MreB speed between *B. subtilis* and *E. coli* is too large for this to be the sole explanation.

Furthermore, the MreB filament speed decreases in time. When Reimold et al (2013) imaged the same cell ~5 min later, the speed nearly halved from ~90 nm/s to 52 nm/s. This is possibly an effect of cell stress, e. g. oxygen depletion or mechanical pressure from the imaging slides, and strongly suggests that MreB behaviour is linked to the cell wellbeing. If significant speed changes can occur within a few minutes, it is not unthinkable that the structural organization or MreB also can be altered in the same timescale, hence, possibly explaining the observed discrepancies in MreB localization and organization.

Angle of filament with long axis of cell

Viewing MreB as a spring wound around a rod one observes that a tightly wound spring has loops at an angle of almost 90^{o} with the long axis of the cell, whereas a loosely wound spring makes an angle that is significantly smaller. The angle at which filaments or helices cross the central axis is a characteristic of the winding and measuring it may clarify its structure. For a pole-to-pole helix one expects to observe strands crossing at the same angle, i.e. in parallel, when viewing the top slice of the cell, and certainly not crossing each other. In case MreB is organized in shorter filaments or (partial) rings one would expect a larger spread in angles, and neighbouring strands need not be (perfectly) parallel.

Several have measured or calculated the angle that MreB makes with the long-axis of the cell. Again, there is an overall agreement in the measured angle distributions, it was centred at 90^{o} with a full

width at half maximum (FWHM) of about 30°. This spread clearly indicates that there was a significant variation in the angle of the filaments within a cell (Garner et al. 2011; van Teeffelen et al. 2011; Domínguez-Escobar et al. 2011; Reimold et al. 2013). Angles of 50° were also readily observed, clearly underlining a wide spread (Reimold et al. 2013). If long helices were present in the cells, one would expect a narrower distribution, with a fixed angle that was either smaller than or larger than 90°, hence that the strands were oriented in the same direction. In other words, these results support theory 2, where MreB forms filaments that orient more randomly in the cell, without the strict organization of a perfect helix spanning from cell pole-to-pole.

While performing the angle measurements, nascent MreB bands were sometimes oriented parallel to each other with small angle variations, which is an assumed prerequisite for a helix structure. The average distance between the MreB tracks was found to be 0.44 µm (Defeu Soufo and Graumann 2004). Other times, neighbouring bands had opposite angles relative to 90° degrees, thereby suggesting shorter filaments were not part of an overall helical structure (fig. 4A). When tracking MreB foci, the clusters sometimes paused or reversed direction (fig 4B), while neighbouring clusters kept moving in a unidirectional motion (Garner et al. 2011; Domínguez-Escobar et al. 2011).

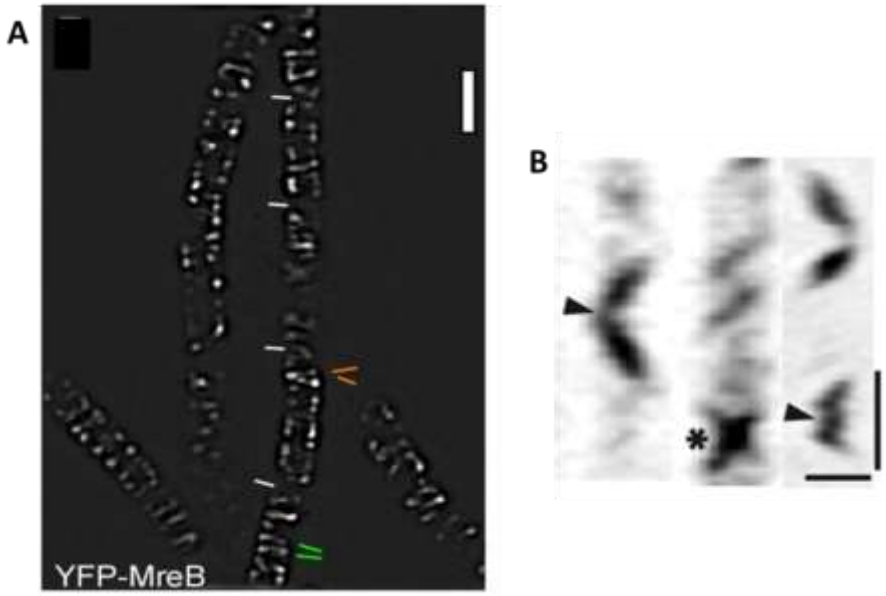

Figure 4. A) YFP-MreB N-SIM images. Green stripes indicates MreB bands with different angles and the orange strips indicate bands that have opposite angles relative to 90°. (Reimold et al. 2013) B) Kyamograph images of MreB, arrowheads represent a reversal in movement direction, asterisk represent a crossover of MreB patches. Supplemental image (Domínguez-Escobar et al. 2011)

Most agree that the MreB filaments do not cross each other, which could be explained by MreBs adherence to the cell membrane as one of the alpha helices is embedded in the cell membrane (Salje et al. 2011; Reimold et al. 2013). Although, it was claimed that MreB foci crossed-over each other, when mapping their trajectories in a kymograph (Domínguez-Escobar et al. 2011). (see fig 4B) However it must be said that this would seem unlikely, since MreB is known to embed an α-helix in the cell membrane, it would appear more likely that the clusters moved next to each other in close proximity.

Mobility versus Motility

One of the issues arising in the discussion of how MreB works is what causes it to move. Is there a driving force that sweeps MreB along, like a raft on a river (mobility) or is MreB able to move on its own, like a motor boat (motility). It has been shown that MreB does not move alone: RodA, RodZ, MreC, MreD and several other parts of the cell wall synthesis machinery (PGEM) move in concert and interact with MreB (Garner et al. 2011). MreB and the PGEM move with comparable velocities, following roughly the same circumferential pathway (Typas et al. 2012). Depleting RodA, RodZ, and PBP slowed down MreB gradually over time (Garner et al. 2011).

Furthermore, treating the cells with specific antibiotics, stopped the movement of the MreB and Mbl clusters. The addition of vancomycin and phosphomycin, were used to inhibit PG precursor incorporation into the sacculus and PG precursor synthesis, respectively. The drug addition was reversible, since the clusters became mobile again after drug removal. (Domínguez-Escobar et al 2011). Reducing the ATPase activity with a D158A mutation, lead to a significant drop in number of MreB filaments and slowed the protein dynamics. The mutant filaments barely moved. (Reimold et al. 2013) All this suggests that the PG machinery and MreB together provide the required energy to move along the cell wall and that MreB is co-piloting, if not piloting, the morphology control.

Helices an artefact of the fluorescent protein fusion?

Generally, most choose between a N- or C-terminal fusion when adding a fluorescent protein. Another option, is to use a sandwich construction, were the fluorescent protein is inserted into an external loop of the studied protein, in this case MreB. However, it is important to keep in mind that the extra addition can influence the folding and/or the functionality of the protein of interest. It has been argued that fusing a fluorescent tag to the N-terminal caused the MreB molecules to polymerize into helices that spanned the cell length, whereas making a sandwich fusion with a fluorescent protein did not induce this type of polymerization. In other words, it was claimed that the cell-long helices were an artefact of the N-terminal fusion and not an inherent property of the MreB (Swulius and Jensen 2012).

The authors imaged MreB under varying conditions, using electron cryo-tomography and fluorescent microscopy. (see fig 5A-E) Overexpressing and hyper-overexpressing YFP-MreB, yielded cells were filaments were detected (fig. 5B, C). But, when the MreB proteins lacked a fluorescent tag, no filaments were visible or could be detected in the cells, both at endogenous and overexpression levels (fig 5A, D). Also, the MreB-RFP^{sw} images did not render any visible long filaments (fig 5E). However, it must be mentioned that MreB-RFP^{sw} was expressed at endogenous levels, while the N-terminal fusion was overexpressed. It is worth noting that MreB has been shown to form filaments in vitro, and this is most likely an intrinsic property of the protein (van den Ent, Amos, and Löwe 2001). Furthermore, there is evidence that MreB attaches itself to the cell membrane by inserting an N-terminal amphipathic helix and/or an insertion loop. This observation could explain why some N- and C-terminal GFP fusions in literature were not fully functional, since the fusion could distort the normal membrane interactions (Salje et al. 2011).

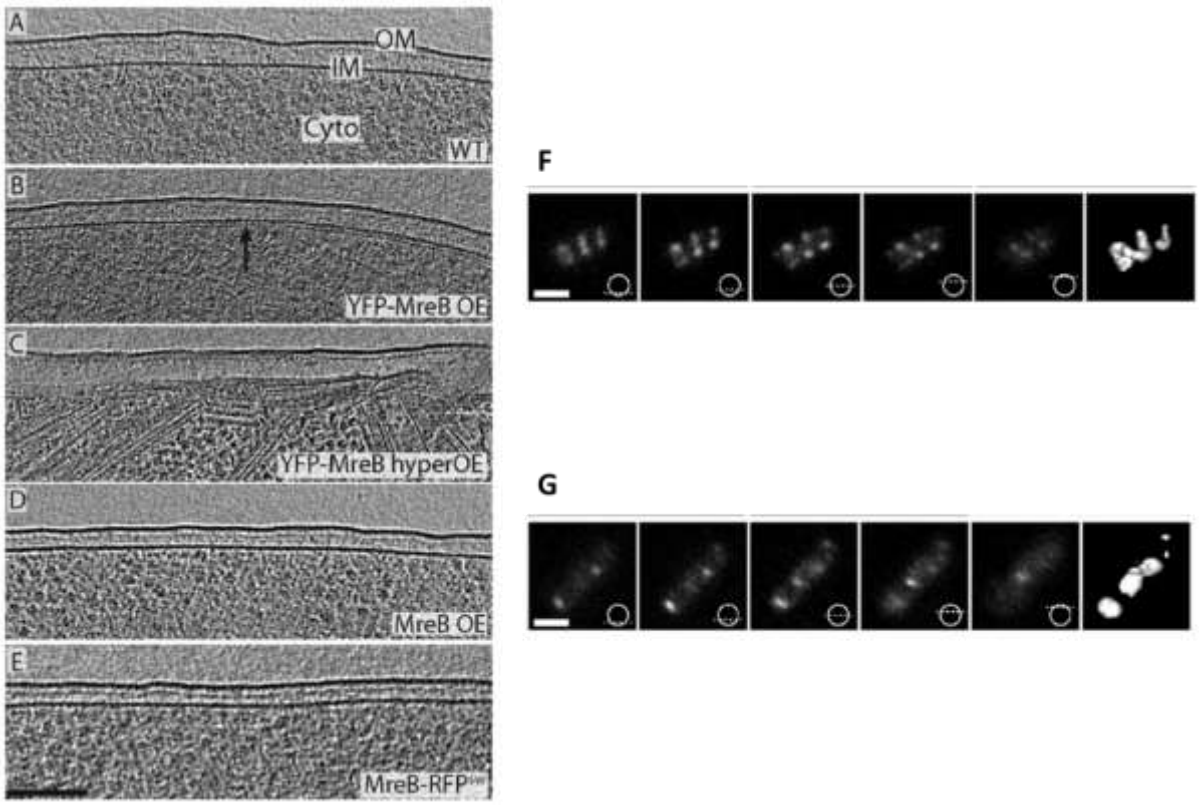

Figure 5. A) WT MreB, B) YFP-MreB overexpressed, C) YFP-MreB hyper-overexpressed, D) MreB over expressed, E) MreB-RFP^{sw} endogenous expression, F) 3D- reconstruction of YFP-MreB, G) 3D- reconstruction of MreB-RFP^{sw}.(Swulius and Jensen 2012)

Immunofluorescence

An alternative approach to fused fluorescent protein labelling is immunofluorescence. In immunofluorescence there are two main approaches, either a specific antibody can be developed for the desired protein, or a antigen sequence can be fused to into it which will be recognized by a more general antibody. In most cases the latter method is choses, since it causes less effort and often gives better labelling. Although there fusions also may cause perturbations in the protein of interest, they are often assumed to be less severe since the insert-size is significantly smaller that a fluorescent protein. Also, the fluorescent markers used in immunofluorescence are small, like AlexaFluor594-immunostain, and usually have a completely different structure then the classical fluorescent proteins, making it less likely that these fluorophores also would induce large-scale polymerization. In addition, the immunofluorophore are added to the cell after the MreB structures have been formed, making it less likely that it can change the existing structure. Interestingly, Jones et al. (2001) used both common methods of immunofluorescence, with c-Myc as a fusion tag for antibody recognition, and polyclonal anti-MreB antiserum which was specific for MreB. Interestingly, both the c-Myc fusion and the z-stack images using the specific antiserum for MreB did indicate that the cells contained MreB filaments, see fig. 1A and 6, respectively.

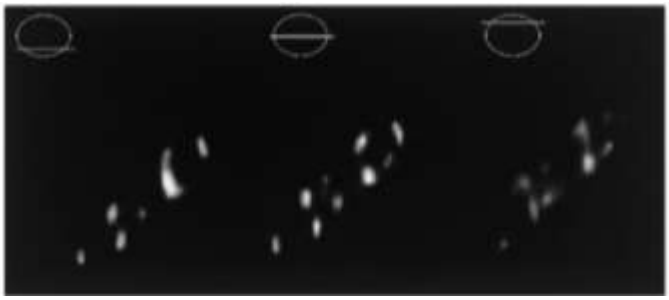

Figure 6. Deconvoluted z-stack of wild-type cells stained with polyclonal anti-MreB antiserum (Jones, Carballido-López, and Errington 2001).

Microscopy and measurement techniques

Comparing images

Comparing the images of MreB-RFP^{sw} in two different publications written by Swulius and Jensen (2012) and Reimold et al. (2013), they appear to observe different localization patterns of MreB. The images from Swulius et al., used to make a 3D reconstruction of the MreB (see fig 5F, G), were clustered and did not seem to form filaments like what was observed in the images by Reimold et al. (2013). This shows that even though the protein construct is the same, the images show variations that can lead to different interpretations. Whether this is caused by different levels of expression, microscopy techniques, cell health, preparation technique, cell to cell variations, or other factors is hard to determine. Inhomogeneity between cell samples can lead to different interpretations of the protein localization of MreB.

Depth of focus

In the review article by White & Gober (2012) it is pointed out that different microscopy techniques probe different volumes in the cell. In total internal reflection fluorescence (TIRF) microscopy, laser light is reflected at an angle at the glass plate – cell interface. Although most of the intensity is reflected, some penetrates about 100 nm into the cell where it can excite the fluorescence tags. Hence, this technique allows only for probing the bottom or top 100 nm of a cell. Using this technique, MreB appeared to organized into patches rather than filaments. It has been argued that because of the limited depth of focus, it is not possible to fully exclude the presence of helices or filaments, even though they have not been observed (White and Gober 2012).

Other microscopy techniques allow imaging through the entire cell, by moving the focus plane, taking an image at each plane and building up a stack (z-stack) in this way. This enables a 3-D reconstruction of the cell, and can visualize larger protein structures. (see fig 5F, G) In this image an YFP-MreB helical structure is clearly visualized.

Stressed cells

The health status and the external environment can affect the main priorities of a cell. Reducing the amount of oxygen to exponentially growing cells for three hours, lead to loss of filamentous structures and the formation of YFP-MreB patches. (Fig 7A, 0h) Interestingly after re-exposing the cells to oxygen by shaking, the patch-like structures disappeared and longer filaments became visible again. At first, the filaments often aligned parallel to the long axis of the cell (fig 7C), but within a few hours the protein strands regained the normal perpendicular conformation. Perpendicular filaments

were never observed when the cells were grown under optimal conditions. It is unclear whether the patches were reshaped into filaments or whether the patches were broken down and built-up into new filaments (Reimold et al. 2013). When exponentially growing cells were shifted to an isotonic medium without nutrients, the helical pattern of MreB was obliterated within minutes. (Defeu Soufo and Graumann 2004) Stationary-phase cells also displayed clusters randomly distributed in the cells (Defeu Soufo and Graumann 2004).

Based on these observations, there is a correlation between cell health and MreB's organization, cells with lower stress levels have longer filaments, while stressed cells are less likely to form helices and have more compact structures of MreB.

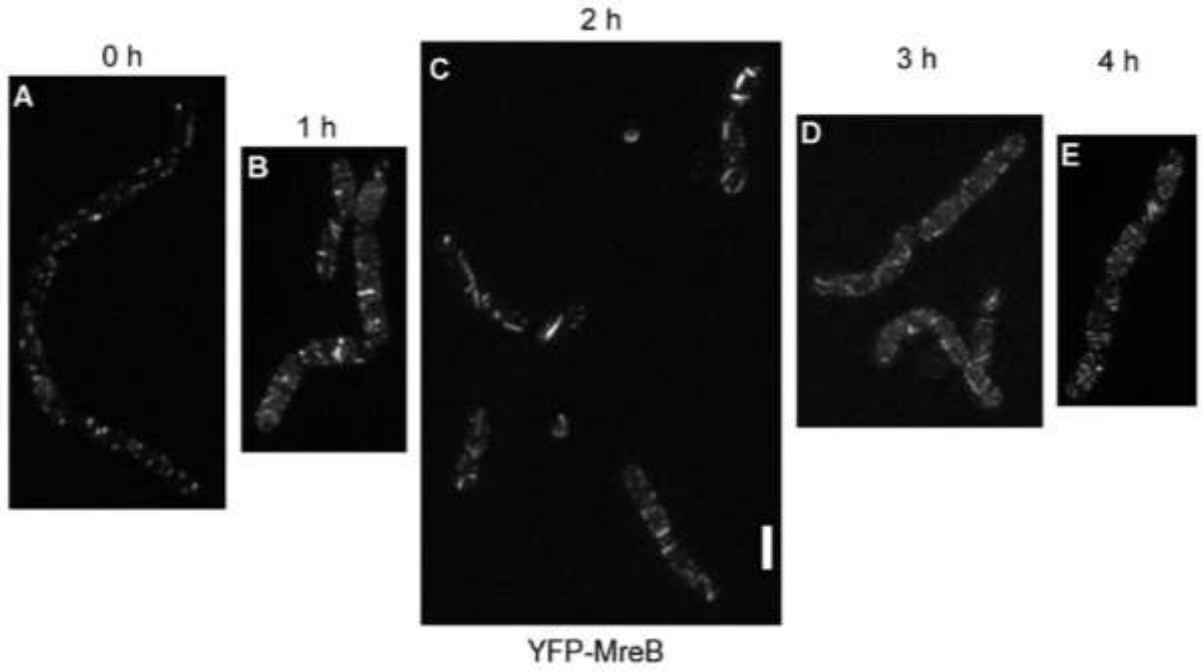

Figure 7. STED images of YFP-MreB in cells that had A) not been shaken for three hours. B) 1h, C) 2h, D) 3h, E) 4h after cell culture shaking was resumed (Reimold et al. 2013).

Discussion

MreB's strong structural homology to actin, made it natural to hypothesize that MreB possibly could have a similar cell organization and localization as actin. Fluorescence microscopy experiments done by Jones et al (2001) suggested that MreB formed helical structures in the cells. Later images made by Vats et al. (2007) clearly observed double MreB helices that spanned from pole-to-pole in the cell, forming a figure eight-like construction. (see fig 1) Also, Garner et al. 2011 found that perturbing MreBs function had adverse effect on the normal cell shape, i.e. allowing rod-shaped cells to deform into more spherical shapes, which ultimately lead to cell death in some cases. To recap, initial research on MreB suggested that the protein formed large shape-controlling scaffolds in the form of helices, and was vital for proper cell morphology, underlining that distortions of this protein's function could have a fatal effects.

Some more recent experimental findings strongly support shorter MreB structures, filaments and patches/clusters, and oppose the formation of large helical structures (Garner et al. 2011; Domínguez-Escobar 2011; van Teeffelen 2011; Reimhold 2013). In these papers the dynamic behaviour of MreB was central, measuring the speed of movement, the trajectory angle with respect to the longitudinal axis, and path length, to determine the distribution functions of these parameters. Typically the speeds ranged between 20 – 50 nm/s, with van Teeffelen reporting a much lower value, ~ 7 nm/s. However, the latter was studied in *E. coli* whereas the others observed the behaviour in *B. subtilis*. It is likely that the speed of movement of MreB is species dependent. At this moment it is still under debate whether MreBs movement is caused by polymerization/ depolymerisation, or that the entire filament moves simultaneously. Treadmilling could have explained the observed MreB movement but it had been excluded through the FRAP experiments by Domínguez-Escobar 2011. However, Kim et al. (2006) found clear evidence that MreB clearly followed a treadmilling mechanism in *C. crescentus*. It is important to note that since MreB has been shown to insert an alpha helix into the membrane surface, a considerable amount of drag would have to be overcome if large stretches of protein were moved at once. Therefore, MreBs high speed of movement supports a shorter filament view of MreB because shorter strands are more likely to move along the cell wall with a significant speed.

In addition to speed, the angle which the protein cluster/filament made with respect to the long axis of the cell was also determined. Either by directly measuring the angle of the filament, or by calculating the average angle from the tracking analysis of a cluster in time. Most measurements show an average angle of 90^{o} with a spread of 15^{o} on either side, suggesting either a very tightly wound helix or more ring-like structures. Beside a spread in angles, neighbouring filaments demonstrated both parallel and opposing angles. These findings argue against the formation of cell spanning helices, as this would require a fairly constant angle between all protein bands, and that the bands would have a bias towards one side of 90^{o}. However, it does not exclude the presences of larger filament structures, which encompass the cell with structures that range between 0.5 to >1 turns.

Dominguez-Escobar et al. (2011) propose very short filaments or patches as organizational model for MreB. They observed fission and fusion of fluorescent spots and assume that it is unlikely for larger structures to behave this way, concluding that MreB organizes as patches.

Evidence presented by Swulius et al. (2012), suggested that the MreB helix, in fact, was an artefact caused by the N-terminal fusion of YFP to MreB. The claim was based on a combination of cryo-tomography and fluorescent microscopy images, using a N-terminal MreB fusion and a sandwich construction were the RFP was inserted into a loop of MreB. When overexpressing and hyper overexpressing YFP-MreB, MreB filaments appeared in the cells. But when wild type MreB and MreB-RFP^{sw} were expressed with its endogenous promotor, no filaments were found in the images. Hence, Swulius et al. (2012) concluded that helices were an artefact caused by the N-terminal fusion of the fluorescent protein to MreB. But it must be argued that not seeing large helices or filaments with a sandwich fusion does not automatically imply that they are not formed in the cell. It is possible that the untagged MreB still formed filaments, but that they were simply harder to detect. It is known that MreB inserts an α-helix in the membrane, and is oriented closely to the lipid bilayer. It is clear that fusing a fluorescent tag to MreB, could affect its normal membrane binding, thereby distorting its normal behaviour. (Salje et al. 2011) As was remarked by Swulius et al. (2012), the density of the lipids of the membrane were comparable to the protein. Which would imply that if MreB was embedded deeper into the membrane, it would be harder to detect in cryo-electron images. Perhaps the N-terminal YFP fusion, pulls the MreB slightly out of the membrane, due to its hydrophilic surface, making it easier to detect.

Swulius et al. (2012) claims were also supported by 3D-reconstructions of fluorescent images. (Fig. 5). Based on the available images and supplemental movies that were presented, an additional observation should be added, that there was an obvious correlation between the number of filaments present in the cell and the expression level of YFP-MreB. The paper clearly stated that the MreB-RFP^{sw} construct was expressed using MreB's wild-type promotor. Hence, the expression level of MreB-RFP^{sw} proteins was lower than that of YFP-MreB. With the expression-level dependence of YFP-MreB in mind, one has to be careful when comparing overexpressed YFP-MreB cells to cells expressing MreB-RFP^{sw} at endogenous levels. It would have been of greater value to compare YPF-MreB and MreB-RFP^{sw} cells with equal expression levels. At this point it can only be speculated whether the promotor, hence protein expression level, would have an indirect influence on the final localization pattern of MreB, but it is worth testing, as this could be key point information in elucidating the actual localization pattern of MreB under normal conditions.

Immunofluorescence techniques often use smaller fluorophores and either implement inserts that are shorter than classic fluorescent proteins or simply use a specific antiserum for the protein of interest. Therefore it is less likely that immunofluorescence would induce the exact same structural artefacts as the fluorescent protein fusion, in certain cases. This is because the protein of interest, can organize itself in to its normal structure before the antibody is added, letting MreB adjust into its normal localization pattern. Of course the labelling efficiency has to be high to get informative images. Nevertheless both Jones et al. (2001) and Vats et al. (2007) observed helices, or at least partial helical structures with different types of antigen fusions. Jones et al (2001) even used polyclonal antiserum that was specific for MreB, meaning that no tag was inserted into MreB and still observed filamentous MreB structures. These observations clearly support the formation of helices, if not pole-to-pole spanning structures, at least the presence of partial helices and larger filamentous structures of MreB in rod-shaped cells.

Cell-health and growth state appear to have a clear impact on the observed MreB organization. The fact that O_2 deprived cells had a different organization and orientation of the MreB filaments

(Reimold et al. 2013), underlines that the fitness of the cell can have a significant impact on how the protein seems to be organized. Although there are many factors that could attribute to the wide spread of localization phenotypes, it is certain that cell health and external stress factors have an influence on MreBs localization. Of course, one tries to image cells under the best possible circumstances, but it is nearly impossible to ensure that the cells do not experience some form of stress. It is logical that a cell would prioritise life-saving pathways over cell membrane-modulating machineries, at least for short-term survival, thereby sending its resources to needed processes and shutting down parts that require energy which are non-essential over a short time frame. Since MreBs movement speed nearly halved within five minutes, it is clear that significant changes in MreB behaviour are taking place in a very short timeframe. This could be one of the main reasons that there is such a wide spread in MreB localization patterns, which include helices spanning from cell pole-to pole, filaments and patches.

Based on the papers that have been studied for this study, I think that a slight overweight in evidence for model 2, implying that MreB proteins organize into filaments that vary in length, ranging between a quarter turn and a few full turns, thereby wrapping the cell and moulding it into the correct rod-like shape. But it must be underlined that the evidence still is not completely convincing, and future work is needed to figure out the exact organization pattern.

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