Host-Polarized Cell Growth in Animal Symbionts

Graphical Abstract

Highlights
- Cell growth is host oriented in an animal symbiont
- Symbiont poles contain bacterial actin homolog MreB and are active growth sites
- The bacterial actin homolog is arranged into a medial ring-like structure
- The bacterial actin homolog is essential for symbiont growth and division

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In Brief
Pende et al. show that cell growth is host oriented in two marine nematode-attached bacteria. In contrast to what is observed in model rods, the actin homolog MreB of the symbionts is arranged parallel to the cell long axis throughout the cell cycle. This medial MreB ring is essential for symbiont growth and division.

Pende et al., 2018, Current Biology 28, 1039–1051
April 2, 2018 © 2018 The Author(s). Published by Elsevier Ltd.
https://doi.org/10.1016/j.cub.2018.02.028
Host-Polarized Cell Growth in Animal Symbionts

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https://doi.org/10.1016/j.cub.2018.02.028

SUMMARY

To determine the fundamentals of cell growth, we must extend cell biological studies to non-model organisms. Here, we investigated the growth modes of the only two rods known to widen instead of elongating, Candidatus Thiosymbion oneisti and Thiosymbion hypermnestrae. These bacteria are attached by one pole to the surface of their respective nematode hosts. By incubating live Ca. T. oneisti and T. hypermnestrae with a peptidoglycan metabolic probe, we observed that the insertion of new cell wall starts at the poles and proceeds inward, concomitantly with FtsZ-based membrane constriction. Remarkably, in Ca. T. hypermnestrae, the proximal, animal-attached pole grows before the distal, free pole, indicating that the peptidoglycan synthesis machinery is host oriented. Immunostaining of the symbionts with an antibody against the actin homolog MreB revealed that it was arranged medially—that is, parallel to the cell long axis—throughout the symbiont life cycle. Given that depolymerization of MreB abolished newly synthesized peptidoglycan insertion and impaired divisome assembly, we conclude that MreB function is required for symbiont widening and division. In conclusion, our data invoke a reassessment of the localization and function of the bacterial actin homolog.

INTRODUCTION

Investigations of micro-organisms with non-canonical growth modes are necessary to identify the conserved mechanisms underlying bacterial cell proliferation. Candidatus Thiosymbion oneisti and Candidatus T. hypermnestrae form virtually pure cultures on the surface of their respective nematode hosts, Laxus oneistus [1] and Robbea hypermnestra [2]. More precisely, each rod is attached by one pole (the proximal pole) to the host so that its long axis is perpendicular to the host surface. As a result, the bacterial coat looks like a palisade. Once extracted from their natural habitat (tropical, shallow water sediment), symbiotic nematodes can be maintained in seawater-filled dishes. However, Ca. Thiosymbion oneisti and T. hypermnestrae cannot be grown in culture without their hosts, and no genetic tools are available to study them. Despite their relatively poor experimental tractability, we showed the reproductive mode of these Gammaproteobacteria to be unique, as they are the only rods known to widen and to undergo FtsZ-based longitudinal fission [3, 4]. We hypothesized that longitudinal fission might require the evolution of a special cell growth mode, as building a whole new lateral side is mechanistically more challenging than building a new pole.

In many taxa, maintenance of the rod shape itself requires at least two well-studied modes of growth: incorporation of peptidoglycan (PG) along the sidewalls (lateral elongation) and the generation of nascent poles (septation). In these canonical cases, which are exemplified by the model organisms Escherichia coli and Bacillus subtilis, material at the poles remains inert, with no evidence of new PG incorporation or turnover [5–8]. In model rods, the protein systems mediating lateral elongation and septation—the elongasome and the divisome—are genetically different. However, they both position functionally similar PG modification and synthesis machineries: the elongasome inserts new PG along the length of the rod during growth, and the divisome completes the steps of constriction and new PG synthesis at the cell center during cell division [9]. Moreover, the elongasome and the divisome interact to prepare for cell division [10], and they both consist of scaffolding cytoskeletal-like proteins, inner membrane-spanning elements, and periplasmic enzymes, including PG syntheses and hydrolases [9, 11, 12]. This machinery works in concert to create the sacculus, a meshwork consisting of chains of two alternating sugar types, N-acetylgalactosamine and N-acetylmuramic acid, joined through beta-(1,4)-glycosidic bonds. The stiff glycan strands are cross-linked together via peptide chains, and in E. coli, they are oriented helically (near perpendicularly) relative to the cell's long axis [13, 14]. In dividing model rods, the guanosine triphosphate (GTP)-dependent polymerization of the tubulin-like FtsZ creates...
the Z-ring (or proto-ring) [15] at the cytoplasmic face of the membrane at the center of the cell [16–18]. Once assembled, the proto-ring organizes the localization and activity of over 10 essential divisome components [12], and recently, FtsZ GTPase activity was found to determine FtsZ dynamic treadmilling in E. coli and B. subtilis [19–21].

As for the spatiotemporal regulation of the elongosome, the membrane-associated actin-like protein MreB appears to be the major scaffold for coordinating PG precursor synthesis and polymerization [22–24]. Inactivation of the mreB gene in E. coli [25, 26] and B. subtilis results in a change in the cell shape from rod to round [27]. MreB interacts with the inner membrane proteins MreC, MreD, and RodZ [28–32], as well as lipid II synthesis enzymes MraY and MurG [33], and its interactions and movement during elongation depends on both the synthesis of essential PG components and the activity of PG syntheses [34–37]. Curiously, the intracellular pathogen Chlamydia—which lacks FtsZ—grows by placing new cell wall mostly at the septum using an MreB-dependent system [34].

However, alternative cell growth models do not invoke MreB polymers and hypothesize that local coordination of cell-wall-synthetizing enzymes in individual complexes is sufficient to maintain rod shape [35]. Moreover, ovococcoid bacteria that do not possess MreB homologs elongate slightly by PG synthesis that occurs in close proximity to the septation plane. This so-called preseptal growth is solely coordinated by FtsZ [36, 38, 39]. Even the model coccus Staphylococcus aureus grows by placing new cell wall mostly at the septum using an FtsZ-dependent system [40]. Finally, most polarly growing species, including actinobacteria and many alphaproteobacteria, do not require MreB to maintain their rod shape [41].

Based on their phylogenetic placement and shape, we hypothesized that the nematode symbionts would grow like model rods, that is predivisionally (by disperse growth) and then septally. However, ultrastructural and morphometric analyses [3, 4] indicated that widening starts at the poles of septating symbionts and proceeds toward midcell. To determine which cell wall growth mode would reconcile these observations with our predictions, we tracked the growth of Ca. Thiosymbion oneisti and T. hypermnestrae with a PG metabolic probe and analyzed their MreB and FtsZ localization patterns. We discovered that longitudinally dividing bacteria grow while dividing by septal, pole-to-midcell insertion of PG. Further, we showed that the actin homolog MreB is essential for both growth and division.

RESULTS

In Longitudinally Dividing Bacteria, New PG Insertion Starts at the Poles and Proceeds Centripetally

To determine the growth mode of longitudinally dividing bacteria, we incubated Ca. T. oneisti and T. hypermnestrae, still attached to their respective live nematode hosts, with a clickable bio-orthogonal D-amino acid dipeptide ethynyl-D-alanyl-D-alanine (EDA-DA) [34, 42]. Subsequently, symbiont cells were fixed and EDA-DA was clicked (i.e., conjugated to a fluorophore) and imaged by epifluorescence microscopy or three-dimensional structured illumination microscopy (3D SIM) (Figure 1). Among the tested incubation times, 30 min and 45 min were the shortest ones resulting in detectable incorporation of new PG incorporation in Ca. T. oneisti (Figures S1A–S1F and S2C) and T. hypermnestrae (Figures S1G–S1L). We will henceforth refer to these incubation times as short. In Ca. T. oneisti, the earliest EDA-DA signal (cyan in Figures 1A and 1B) appeared at both poles, albeit often with different intensities (Figure 1A, leftmost panel). In the asynchronously dividing rod Ca. T. hypermnestrae [4], new PG first appeared at the proximal and—only subsequently—at the distal pole (cyan in Figures 1E and 1F; note the lack of distal signal in Figure 1E, leftmost and middle panels). At later septation stages, EDA-DA signal appeared closer to the cell center in both symbionts (dashed white line in Figures 1B and 1F). Longer incubation times (90 min or above) resulted in a similar EDA-DA incorporation pattern, except that the septal signal was partly masked by the weak, diffuse signal likely resulting from incubating the cells for more than one generation (see representative Ca. T. oneisti and T. hypermnestrae cells in Figures 1C and 1D and 1G and 1H, respectively).

To gain a better resolution of the PG insertion pattern, we performed 3D SIM of Ca. T. oneisti and T. hypermnestrae cells subjected to long EDA-DA incubations (Figures 1D and 1H). In the case of Ca. T. oneisti, we detected polar arcs of newly inserted PG (Figure 1D, left panel). After septation onset, weak EDA-DA signal spanned the strong polar arcs of EDA-DA signal (Figure 1D, middle panel), and in the last septation stage, an EDA-DA ring of homogeneous signal strength appeared (Figure 1D, right panel). In the case of the Ca. T. hypermnestrae, we detected a strong proximal arc of EDA-DA incorporation at the septation onset (Figure 1H, left panel). At later septation stages, a distal EDA-DA incorporation arc and faint medial signal spanning the two polar arcs became visible (Figure 1H, middle panel). Finally, at the terminal stage of septation, the ring of newly synthesized PG was almost continuous (Figure 1H, right panel).

We conclude that longitudinally dividing rod-shaped Gammaproteobacteria grow “medially,” with “medial” referring to the plane passing through the angular points (zeniths) of the symbiont poles and, therefore, parallel to the cell long axis. Medial growth implies that in nematode symbionts membrane regions of both high and low curvature are sites of active growth. Although septation and cell widening appeared to be concomitant, that is, we could not detect the disperse growth typical of model Gammaproteobacteria, we cannot exclude some predivisional widening. Finally, the temporal pattern of EDA-DA incorporation (Figures S1 and S2) suggests a symbiont generation time between 90 and 180 min (under experimental conditions).

FtsZ Colocalizes with Septal Insertion of New PG

The mature divisome provides the biochemical and mechanical activities required for septal PG synthesis, septation, and separation [43]. Moreover, GTPase-based FtsZ treadmilling dynamics direct the processive movement of the septal cell wall synthesis machinery in E. coli, thereby ensuring uniform septal cell wall synthesis and correct polar morphology, without limiting septal synthesis rate [20]. To determine whether FtsZ may play and mediate new PG insertion at the septum of longitudinally dividing bacteria, we immunostained symbionts subjected to long pulses of EDA-DA with anti-FtsZ antibody (Figures 2 and S3; Movie S1). In both Ca. T. oneisti and T. hypermnestrae, FtsZ localized with septal sites of PG incorporation at early (Figures 2A and 2C) and later stages of septation.
In Ca. T. hypermnestrae cells displaying an indentation of the proximal membrane (early septation stage), we detected only one proximal arc of EDA-DA incorporation (Figure 2C, upper panel) and one proximal FtsZ arc (Figure 2C, middle panel). However, at later septation stages, a distal arc of EDA-DA (Figure 2D, upper panel) and a distal arc of FtsZ (Figure 2C, middle panel) appeared. Of note, the EDA-DA and FtsZ signals were almost completely overlapping. We conclude that FtsZ localization pattern is consistent with this tubulin homolog placing and mediating septal PG insertion in longitudinally dividing rods.
Moreover, we plotted the total MreB fluorescence of non-constricted and constricted cells against the normalized cell width (Figures 3B and 3D for Ca. T. oneisti and T. hypermnestrae, respectively). MreB accumulates in the central part of the short axis of constricted Ca. T. oneisti and T. hypermnestrae (Figures 3B and 3C, respectively). Of note, at late septation stages (Figure S4), distal accumulation of Ca. T. hyermnestrae MreB mirrored distal PG insertion (Figures 1F and S4E) and distal FtsZ accumulation (Figure 2 and as shown in [4]).

In order to confirm that MreB polymers are aligned parallel to the long axis and to gain a better resolution of their localization, we subjected immunostained Ca. T. oneisti (Figures 4A–4E) and T. hypermnestrae to 3D SIM analysis (Figures 4F–4J; Movies S2 and S3). In non-constricted Ca. T. oneisti and T. hypermnestrae cells, MreB is medial, and a discontinuous ring running from pole to pole is clearly recognizable in the latter (Figures 4A and 4B for Ca. T. oneisti and Figure 4F for Ca. T. hypermnestrae). However—as the septum forms and closes—MreB is progressively excluded from the septum and localizes medially in each of the two prospective daughter cells (Figures 4C–4E for Ca. T. oneisti and Figures 4G–4J for Ca. T. hypermnestrae). Despite being medial (Figures 3 and 4), MreB barely overlaps with septal EDA-DA (Figure S5), suggesting that FtsZ, rather than MreB, mediates the building of the septum.

In conclusion, we showed that MreB accumulates medially throughout the cell cycle in longitudinally dividing bacteria and it is not excluded from membrane regions of high curvature as observed in E. coli and B. subtilis.

**MreB Accumulates Medially Prior to FtsZ**

In E. coli, MreB midcell rings colocalized with and were never observed independently of FtsZ proto-rings [10, 49]. To analyze MreB localization with respect to FtsZ, we immunostained Ca. T. oneisti with anti-FtsZ and -MreB antibodies (Figure 5). Comparison of FtsZ (Figure 5B) and MreB (Figure 5A) demographics, and of total FtsZ and total MreB fluorescence plotted against normalized cell width (Figures 5C and 5D), as well as images of representative cells (Figures 5E–5H), indicated that MreB is medially localized prior to FtsZ. Colocalization of MreB and FtsZ fluorescence signals was quantified by using the Pearson’s coefficient, and we observed a considerable overlap between the two fluorescence signals in all cells (average Pearson’s coefficient of 0.45 on a scale of 0.0 to 1.0; Figure S6).

We conclude that—in contrast to what was reported for E. coli—in Ca. T. oneisti and T. hypermnestrae, MreB is localized at the prospective septation plane prior to divisome assembly. Therefore, based on its localization pattern, MreB may mediate FtsZ proto-ring assembly in longitudinally dividing bacteria.

**MreB Depolymerization Blocks Insertion of Newly Synthesized PG**

Localization pattern analysis of newly inserted PG, FtsZ, and MreB suggested that FtsZ mediates septum synthesis (Figures 2 and 5) and that MreB mediates preseptal widening and divisome assembly (Figures 3, 4, and 5). Given that direct interaction of FtsZ and MreB supports septum synthesis and cell division in E. coli [49], we applied the MreB polymerization inhibitor S-3,4-dichlorobenzyl-isothiourea (A22) to the symbionts to understand the role of MreB polymers in Ca. T. oneisti and T. hypermnestrae division.
Importantly, A22 is not toxic to Chromadorea nematodes at the used concentration and incubation times (Table S1). Further, the amino acids that make up the ATP-binding pocket to which A22 is predicted to bind, as well as the amino acids that—if mutated—confer A22 resistance are conserved between E. coli, Ca. T. oneisti, and Ca. T. hypermnestrae [50] (Figure S7). As expected from the symbiont MreB protein primary structures, A22 incubation affected medial MreB polymerization in both non-constricted and constricted symbionts (Figures 6A, 6B, and 6E for Ca. T. oneisti and Figures 6C–6E for T. hypermnestrae). Surprisingly, insertion of newly synthesized PG in Ca. T. oneisti and T. hypermnestrae was not only reduced but abolished (Figure 6E). Moreover, we observed a decrease of total medial FtsZ fluorescence (Figures 6F and 6G for Ca. T. oneisti and Figures 6I–6K for T. hypermnestrae), and FtsZ ring-like structures were barely recognizable in non-constricted and constricted Ca. T. oneisti (Figure 6H) and not at all in Ca. T. hypermnestrae (Figure 6L). Consistently, the average number of constricted cells decreased significantly (p = 0.001 for Ca. T. oneisti and p = 0.003 for Ca. T. hypermnestrae; Figures 6M and 6N, respectively; Table S4).

We conclude that the bacterial actin homolog is required for cell growth and division in longitudinally dividing Gammaproteobacteria.

**DISCUSSION**

We investigated the growth mode of two longitudinally dividing symbionts to know whether (1) it may be polarized toward the host attachment site and whether (2) transversal arrangement and polar exclusion of MreB polymers are essential for rod shape maintenance. We found that Ca. T. hypermnestrae orients its cell growth synthesis machinery toward the host attachment site. Indeed, in this symbiont, new PG insertion starts at the proximal, nematode-attached pole by default. Strikingly, at the molecular level, the novelty of symbiont cell growth is three-fold: (1) these Gammaproteobacteria start to grow at PG regions traditionally thought to be inert in model rods; (2) cell wall growth is mainly (if not only) septal as observed in model ovococci and cocci, but—differently from these—it is MreB-mediated; and (3) MreB appears to localize medially prior to divisome assembly and is required for septal growth.

Beside the nematode symbionts, the actinobacterium Streptomicies coelicolor and the Alphaproteobacteria Agrobacterium tumefaciens, and Brucella suis [51] also have growing poles. However, Ca. T. hypermnestrae is unique in that PG insertion initiates at the host-attached pole and is accompanied by asymmetric, FtsZ-mediated membrane invagination [4]. Although asymmetric cell growth has been observed in a variety of gram-negative bacteria, including Proteus mirabilis and Caulobacter crescentus [51], Ca. T. hypermnestrae is the first to show animal host polarization of the PG synthesis machinery. The septal EDA-DA incorporation observed in dividing symbionts is similar to that reported for model cocci [52] and suggests that cell widening occurs during septation. Whereas we could not observe predivisional, disperse growth in the nematode symbionts, in *E. coli* and *B. subtilis*, disperse growth of the lateral walls is detectable even upon short fluorescent D-amino acids pulses [42].

Recent work indicates that, in *E. coli*, MreB preferably localizes to and directs PG synthesis laterally (i.e., at regions of low curvature) and not at the poles [32, 44, 45, 53]. This can be explained by the fact that the poles are enriched in anionic phospholipids (phosphatidylglycerol and cardiolipin), which preferentially interact with monomeric MreB in vitro [45, 54]. Therefore, it has been hypothesized that exclusion of MreB polymers at the poles is necessary to enable cells to elongate bidirectionally only in their cylindrical part [45]. Although the lipid composition of the symbionts’ membranes is still under investigation, symbiont MreB localizes in areas of both low and high
curvature (i.e., not only in the cylindrical part but also at the poles). The presence of MreB throughout the cell long axis in both Ca. T. oneisti and T. hypermnestrae would enable them to branch and double their poles. However, differently to what was observed in E. coli cells that artificially express MreB at their poles [45], symbiont cells maintain their rod shape and polarity despite polar bifurcation. One possible explanation could be that—in the symbionts—polar bifurcation is mediated by medial PG insertion and this, in turn, is coupled to FtsZ-based longitudinal division.

When E. coli is treated with the MreB-depolymerizing drug A22, MreB localization becomes diffuse and ubiquitous, and the cells become round after at least two generations [25, 26, 55, 56]. Moreover, the orientation of MreB polymers relative to the E. coli long axis correlates with cell diameter, which led to propose that MreB polymers are responsible for the diameter of the cell wall [50]. On the other hand, mreB mutants capable to accommodate to different cell widths suggested that MreB might function as a width-maintaining rather than a width-determining factor [37, 57]. In Ca. T. oneisti and T. hypermnestrae, A22 altered both MreB and FtsZ localization patterns (Figure 6), but the nematode symbionts did not become round (Table S2). Instead, in line with PG incorporation abolishment, A22 treatment resulted in a decrease in the number of constricted Ca. T. oneisti and T. hypermnestrae cells (Figures 6M and 6N, respectively). Thus, the presence of medial, functional MreB and its direct interaction with FtsZ may be required for symbiont division. As the symbiont MreB amino acid required for FtsZ interaction is conserved [49] (Figure S7), loss of MreB-FtsZ interaction could directly affect FtsZ proto-ring formation. The septation defects observed upon MreB depolymerization are in line with what was reported for pathogenic Chlamydia. In these obligate intracellular pathogens that lack FtsZ, inhibition of MreB polymerization blocks PG synthesis and affects replication [34]. As for E. coli, mreB gene loss of function also affects the division rate. However, this only seems to be an indirect effect of the A22-induced spherical shape, which requires the construction of a bigger septum [10]. Indeed, division rate is not affected in mreB deletion mutants in which PBP3, PBP1B, and FtsZ are overproduced [25, 58]. What the symbionts, pathogenic Chlamydia and E. coli have in common is that—despite the

Figure 4. 3D SIM Analysis of MreB Localization Pattern in Ca. T. oneisti and T. hypermnestrae

Five representative Ca. T. oneisti cells (A–E) and five representative Ca. T. hypermnestrae cells (F–J) sorted from the thinnest (top) to the thickest (bottom) immunostained with anti-MreB antibody. Dotted white lines indicate cell outlines. A frontal view (left) and a corresponding 90° shifted side view (right) are shown for each cell. The scale bar represents 1 µm. See also Figures S3 and S5 and Movies S2 and S3.
Figure 5. MreB and FtsZ Localization Pattern in Ca. T. oneisti

(A and B) Demographics of Ca. T. oneisti cells (n = 2,038) immunostained with (A) anti-MreB and (B) anti-FtsZ antibody. Each cell is represented as a pixel-wide bar whose width corresponds to the cell short axis. Symbiont cells were sorted according to increasing width from left to right. Dotted white line indicates the center of the short axis.

(C) Average FtsZ and MreB fluorescence (a.u.) plotted against normalized cell width (%) of Ca. T. oneisti cells divided into two morphological classes, nc (dashed line; n = 1,594) and c (full line; n = 444). MreB is shown in red and FtsZ in black.

(D) Average FtsZ and MreB fluorescence (a.u.) plotted against normalized cell width (%) of Ca. T. oneisti cells divided into six width classes (class 1 is 0.53–0.759 μm, n = 462; class 2 is 0.759–0.8376 μm, n = 462; class 3 is 0.8376–0.9246 μm, n = 462; class 4 is 0.9246–1.117 μm, n = 462; class 5 is 1.117–1.295 μm, n = 462; class 6 is 1.295–2.25 μm, n = 230). MreB is shown in red and FtsZ in black.

(E–H) Representative non-constricted (E), early septating (F), slightly constricted (G), and deeply constricted (H) Ca. T. oneisti cells immunostained with anti-MreB and anti-FtsZ antibodies (red and green in the overlay images, respectively). White dotted lines represent the cell outlines deduced from the corresponding phase contrast images. The scale bar represents 1 μm. See also Figures S3 and S6.
Figure 6. Effect of MreB-Depolymerizing Drug A22 on Ca. T. oneisti and T. hypermnestrae
(A–D) 3D SIM images of a non-constricted (A) and constricted (B) Ca. T. oneisti cell and a non-constricted (C) and constricted (D) Ca. T. hypermnestrae cell upon A22 treatment. Cells were treated with 20 μg/mL A22 for 120 min prior to addition of 10 mM EDA-DA (cyan; barely detectable). After an additional 90-min-long incubation, cells were fixed, clicked, and immunostained with anti-MreB antibody (red). Dotted white line indicates cell outline. A frontal view (left) and a corresponding 90° shifted side view (right) is shown of each cell. The scale bar represents 1 μm.

(E) Averaged EDA-DA fluorescence (a.u.) plotted against normalized cell length (%) of A22-treated (dotted line) and untreated (full line) Ca. T. oneisti (black) and Ca. T. hypermnestrae (gray) cells. Number of cells is indicated next to each line.

(F and G) Averaged FtsZ fluorescence (a.u.) plotted against normalized cell length (%) of A22-treated and untreated (full line) Ca. T. oneisti subdivided into two morphological classes, nc (F) and c (G). n, number of analyzed cells.
A22-induced mislocalization of MreB—misplacement of their septation planes was never observed.

A22 can have an MreB-independent toxic effect on bacterial growth [59]. This toxicity is consistent with the finding that, given that it binds to MreB as a low-affinity ADP imitator, A22 may affect other nucleotide-binding processes [60]. In this study, we employed A22 at 20 μM based on a previous work showing that this concentration depolymerized MreB filaments but affected neither the growth rate of *E. coli* nor its DNA replication or segregation [55]. The amino acids that make up the ATP-binding pocket to which A22 is predicted to bind, as well as the amino acids that—if mutated—confer A22 resistance, are conserved between *E. coli*, *Ca. T. oneisti*, and *Ca. T. hypermnestrae* (Figure S7). Therefore, it is safe to assume that the affinity of A22 for MreB is similar among these three *Gammaproteobacteria*. However, to confirm that the A22-induced septation defects observed in the symbionts are not due to off-target toxicity, additional inhibitor studies employing less toxic A22 analogs, such as MP266, should be performed.

The fact that MreB is medially throughout the life of the symbionts suggests that, differently to what was observed previously [49], medial FtsZ polymers are not necessary to recruit MreB to the septation plane. Considering the FtsZ localization and septation defects observed in A22-treated symbionts, the opposite scenario seems likelier (i.e., MreB-mediated FtsZ localization).

The conservation of protein classes for PG synthesis machinery across gram-negative and positive bacteria, and between the elongasome and divisome, suggests a general strategy for shaping the bacterial cell, as well as a common evolutionary history [61]. However, if penicillin-binding proteins (PBPs) catalyze PG growth and maturation throughout the bacterial kingdom, different PBPs have different functions. The *E. coli* class A (bi-functional) PBPs PBP1A, PBP1B, and PBP1C have glycosyltransferase and transpeptidase activities. The class B PBPs are monofunctional transpeptidases that crosslink PG subunits [62]. Two of these, PBP2 and PBP3, are present in *E. coli* and have different cellular functions [63]: inactivation of PBP2 inhibits cell elongation and results in spherical shape, whereas inactivation of PBP3 blocks new cell pole synthesis (septation) and results in filamentation. However, PBP2 also localizes transiently at midcell, thereby overlapping and interacting with PBP3 during the onset of the divisome [10, 64]. It was therefore proposed that the transient positioning of the elongasome and divisome at midcell in a preparative division phase mediates preseptal PG synthesis. In our symbionts, following short incubations with EDA-DA, the first detectable PG insertion was at the leading edges of the constricting cell envelope. This could be due to the fact that, in *E. coli*, completely new PG is synthesized for septal growth, whereas dispersed insertion of already available lipid II elongates its cylindrical part [7]. If this holds true for the symbionts, septal EDA-DA incorporation would be favored over preseptal incorporation and, as a consequence, if this is not as extensive as in *E. coli*, would not be detectable by using EDA-DA. Nevertheless, (1) polar incorporation in non-constricted symbionts (Figures 1A and 1E) and (2) the bone-like and pear-like shape of *Ca. T. oneisti* and *T. hypermnestrae* cells that have just started septation, respectively, suggest that MreB might mediate PG incorporation at the poles shortly before divisome maturation. In analogy with *E. coli*, PBP1A/PBP2 might synthesize new cell wall prior to PBP3 at the septation plane [10]. Although the available genome drafts indicate the presence of PBP1A, PBP2, and PBP3/FtsI, as well as RodA, RodZ, and the MraY and Mur enzymes for lipid II synthesis, their localization patterns await to be described.

Intriguingly, MreB exclusion from the divisome in concomitance with furrow ingression is in line with published models where the absence of MreB polymers is a prerequisite for septation [10, 65–67]. As the divisome matures and a PG-synthesizing enzyme (possibly a PBP3 ortholog) accumulates medially, the elongasome would be more and more excluded until it cannot participate in septal PG synthesis (in case it ever did).

Our data suggest that MreB mediates and places divisome assembly, but what drives centripetal new PG insertion at the septum? It has been recently discovered that *B. subtilis* FtsZ treadmills circumferentially around the division ring and drives the motions of the PG-synthesizing enzymes. The FtsZ treadmilling rate controlled both the rate of PG synthesis and cell division [21]. Similarly, GTPase-activity-coupled FtsZ treadmilling guides the progressive insertion of new cell wall by building increasingly smaller concentric rings of PG to divide the cell in *E. coli* [20]. Given that FtsZ signal overlapped with new PG insertion (in particular at the leading edges of membrane constriction), we hypothesize that FtsZ, possibly together with FtsA, organizes septal cell wall in the symbionts. In the absence of available genetic tools, in *vitro* reconstitution of cell wall synthesis will hopefully shed light on how the FtsZ-mediated processes of septation and membrane ingression are coupled.

To sum up, we propose that longitudinally dividing rods grow as follows (Figure 7): in new-born cells, MreB is localized medially and, before FtsZ polymerizes into a ring-like structure, new PG is inserted at the poles (both poles in the case of *Ca. T. oneisti* and at the proximal pole only in the case of *Ca. T. hypermnestrae*).
Notably, functional MreB is required for medial FtsZ proto-ring formation in the symbionts. As septation proceeds, FtsZ organizes progressive and centripetal PG insertion at the septum. Given the limited width of the symbionts, septal growth alone could be sufficient for cell widening. However, we cannot exclude the existence of some MreB-mediated preseptal growth. As septation approaches completion, MreB is more and more excluded from the constricting membrane but already medially localized in each of the two prospective daughter cells.

If it is clear that the geometric cues that have been proposed to guide MreB localization in *E. coli* or *B. subtilis* are reversed in the symbionts, one may argue that the symbionts’ growth mode has not evolved by reorienting the cytokinetic and PG synthesizing machineries 90 degrees to position the septation plane parallel to the cell long axis. Instead, the symbionts represent standard Gammaproteobacteria with an extremely short long axis and a considerably increased cell width. In this valid, evolutionary sound scenario, the symbiont cell poles would be functionally equivalent to the *E. coli* lateral sides (and vice versa) and the medial position of symbiont would correspond to the midcell position of *E. coli*. Because, in this scenario, the symbionts would consist almost exclusively of virtually inert poles, MreB would have the same circumferential orientation as in *E. coli* but only have a thin (medial) band to localize within, distant from the inert sides of the cell. If we consider the symbionts as merely “squeezed” *E. coli* cells, the cytokinetic (FtsZ-based) machinery and the cell growth machinery would mesh to a point where—due to the extremely low amount of “active” membrane regions—it would be hard to observe predivisional widening and/or septal FtsZ prior to septal MreB. However, if *Ca. T. oneisti* may be regarded as a “squeezed” *E. coli*, this cannot be the case for *Ca. T. hypermnestrae*, which shows a strong polarization of both cytokinetic and growth machineries. The latter point implies that *Ca. T. hypermnestrae* has three functionally different membrane regions instead of the canonical two: the lateral side, the free pole, and the nematode-attached pole previously shown to be fimbriae-rich [4]. Future research will hopefully clarify how reversed geometric cues are integrated with host-attachment cues to guide symbiont MreB and FtsZ localization.

MreB-mediated septal growth has only been observed in animal-associated rods. However, given that we do not know how the free-living counterparts of the symbionts—if existing—reproduce, it is unclear whether host-polarized cell growth is an adaptation to the symbiotic lifestyle. Longitudinal fission and concomitant septal growth might have evolved to guarantee host attachment to both daughter cells while maximizing symbiont cell number. To grasp the functional significance of host-polarized cell growth, we need to better understand symbionts’ physiology. Nevertheless, our work suggests that environmental cues can induce an inversion of the cellular dimensions so that even fundamental biological processes can be reoriented without the need to substitute the underlying molecular key players.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, four tables, and three movies and can be found with this article online at https://doi.org/10.1016/j.cub.2018.02.028.

**ACKNOWLEDGMENTS**

This work was supported by the Austrian Science Fund (FWF) grant P22470 (N.L. and S.B.), a uni:docs fellowship and a PhD completion grant 2016 from...
the University of Vienna (N.P.), grant 201506760048 from the China Scholarship Council (J.W.), FWF project P28593 (P.M.W.), grants R01GM51986 and R33GM122556 from the NIH (Y.V.B.), and grant R01GM113172 from the NIH (M.S.V. and Y.V.B.). We are extremely grateful to Lijuan Zhang and Kareem Elsayad (Vienna Biocenter Core Facility for Advanced Microscopy) for technical support with 3D SIM, to Norbert Vischer for image analysis programming, and to Gabriela Paredes (Dep. Ecogenomics and Systems Biology, University of Vienna) and Veronika Plichta for statistical analysis. The wild-type C. elegans, NGM, FuDR, and M9 buffer in this study were provided by Belinda Koenders-van Sintanneland (Faculty of Science, University of Amsterdam), whom we thank for her help with A22 toxicity tests on nematodes. We are extremely grateful to Sven van Teefelen, K.C. Huang, Martin Loose, and two anonymous reviewers for their very constructive comments. This work is a contribution from the Carrie Bow Cay Laboratory, Caribbean Coral Reef Ecosystem Program, National Museum of Natural History, Washington, DC.

AUTHOR CONTRIBUTIONS

N.P. did most of the experiments and display items and commented on the manuscript; J.W. did experiments; P.M.W. did experiments and display items and commented on the manuscript; J.V. did preliminary experiments; E.K. contributed to experimental design, did preliminary experiments, and commented on the manuscript; S.K.-M.R.R. did statistical analysis and commented on the manuscript; M.S.V. synthesized and provided peptidoglycan metabolic probes; Y.V.B. contributed to experimental design and commented on the manuscript; T.d.B. contributed to experimental design and results analysis and commented on the manuscript; and S.B. conceived the work, provided most of the funding, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 16, 2017
Revised: December 13, 2017
Accepted: February 15, 2018
Published: March 22, 2018

REFERENCES

1. Polz, M.F., Distel, D.L., Zarda, B., Amann, R., Felbeck, H., Ott, J.A., and Cavanaugh, C.M. (1994). Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode. Appl. Environ. Microbiol. 60, 4461–4467.

2. Ott, J.A., Gruber-Vodicka, H.R., Leisch, N., and Zimmermann, J. (2014). Phylogenetic confirmation of the genus Robbea (Nematoda: Desmodoridae, Stilbonematinae) with the description of three new species. Syst. Biodivers. 12, 434–455.

3. Leisch, N., Verheul, J., Heindl, N.R., Gruber-Vodicka, H.R., Pende, N., den Blauw, T., and Bulgheresi, S. (2012). Growth in width and FtsZ ring longitudinal positioning in a gammaproteobacterial symbiont. Curr. Biol. 22, R831–R832.

4. Leisch, N., Pende, N., Weber, P.M., Gruber-Vodicka, H.R., Verheul, J., Vischer, N.O.E., Abby, S.S., Geer, B., den Blauw, T., and Bulgheresi, S. (2016). Asynchronous division by non-ring FtsZ in the gammaproteobacterial symbiont of Robbea hyenomestra. Nat. Microbiol. 2, 16182.

5. Mobley, H.L., Koch, A.L., Doyle, R.J., and Streips, U.N. (1984). Insertion and fate of the cell wall in Bacillus subtilis. J. Bacteriol. 158, 169–179.

6. Schlaeppi, J.M., Schaefer, O., and Karamata, D. (1985). Cell wall and DNA cosegregation in Bacillus subtilis studied by electron microscope autoradiography. J. Bacteriol. 164, 130–135.

7. de Pedro, M.A., Quintela, J.C., Hölting, J.V., and Schwarz, H. (1997). Murein segregation in Escherichia coli. J. Bacteriol. 179, 2823–2834.

8. Janakiraman, A., and Goldberg, M.B. (2004). Evidence for polar positional information independent of cell division and nucleoid occlusion. Proc. Natl. Acad. Sci. USA 101, 835–840.

9. Tynas, A., Banzhaf, M., Gross, C.A., and Vollmer, W. (2011). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat. Rev. Microbiol. 10, 123–136.

10. van der Ploeg, R., Verheul, J., Vischer, N.O.E., Alexeeva, S., Hoogendoorn, E., Postma, M., Banzhaf, M., Vollmer, W., and den Blauw, T. (2013). Colocalization and interaction between elongasome and divisome during a preparative cell division phase in Escherichia coli. Mol. Microbiol. 87, 1074–1087.

11. Margolin, W. (2009). Sculpting the bacterial cell. Curr. Biol. 19, R812–R822.

12. Egan, A.J.F., and Vollmer, W. (2013). The physiology of bacterial cell division. Ann. N Y Acad. Sci. 1277, 8–28.

13. Deng, Y., Sun, M., and Shaeowitz, J.W. (2011). Direct measurement of cell wall stress stiffening and turgor pressure in live bacterial cells. Phys. Rev. Lett. 107, 158101.

14. Wang, S., Furchtgott, L., Huang, K.C., and Shaeowitz, J.W. (2012). Helical insertion of peptidoglycan produces chiral ordering of the bacterial cell wall. Proc. Natl. Acad. Sci. USA 109, E595–E604.

15. Rico, A.I., Krupka, M., and Vicente, M. (2013). In the beginning, Escherichia coli assembled the proto-ring: an initial phase of division. J. Bacteriol. Chem. 288, 20830–20836.

16. Bi, E.F., and Lukkenhaus, J. (1991). FtsZ ring structure associated with division in Escherichia coli. Nature 354, 161–164.

17. Löwe, J., and Amos, L.A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. Nature 391, 203–206.

18. Mukherjee, A., and Lukkenhaus, J. (1998). Dynamic assembly of FtsZ regulated by GTP hydrolysis. EMBO J. 17, 462–469.

19. Loose, M., and Mitchison, T.J. (2014). The bacterial cell division proteins FtsA and FtsZ self-organize into dynamic cytoskeletal patterns. Nat. Cell Biol. 16, 38–46.

20. Yang, X., Lyu, Z., Miguel, A., McQuillen, R., Huang, K.C., and Xiao, J. (2017). GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. Science 355, 744–747.

21. Blisson-Filho, A.W., Hsu, Y.P., Squyres, G.R., Kuro, E., Wu, F., Jukes, C., Sun, Y., Dekker, C., Holden, S., Vanhuenenwehe, M.S., et al. (2017). Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science 355, 739–743.

22. Esue, O., Cordero, M., Witzig, D., and Tseng, Y. (2009). The assembly of MreB, a prokaryotic homolog of actin. J. Biol. Chem. 284, 2628–2635.

23. Salje, J., van den Ent, F., de Boer, P., and Löwe, J. (2011). Direct membrane binding by bacterial actin MreB. Mol. Cell 43, 478–487.

24. Ozyaman, E., Kolman, J.M., and Kornei, A. (2013). Bacterial actins and their diversity. Biochemistry 52, 6928–6939.

25. Wachi, M., Doi, M., Tanaki, S., Park, W., Nakajima-Iijima, S., and Matsuhashi, M. (1987). Mutant isolation and molecular cloning of mre genes, which determine cell shape, sensitivity to mectillin, and amount of penicillin-binding proteins in Escherichia coli. J. Bacteriol. 169, 4935–4940.

26. Doi, M., Wachi, M., Ishino, F., Tomioka, S., Ito, M., Sakagami, Y., Suzuki, A., and Matsuhashi, M. (1988). Determinations of the DNA sequence of the mreB gene and of the gene products of the mre region that function in formation of the rod shape of Escherichia coli cells. J. Bacteriol. 170, 4619–4624.

27. Jones, L.J.F., Carbellal-Díez, R., and Errington, J. (2001). Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. Cell 104, 913–922.

28. Kruse, T., Bork-Jensen, J., and Gerdes, K. (2005). The morphogenetic MreBCD proteins of Escherichia coli form an essential membrane-bound complex. Mol. Microbiol. 55, 78–89.

29. van den Ent, F., Leaver, M., Bendezú, F., Errington, J., de Boer, P., and Löwe, J. (2008). Dimeric structure of the cell shape protein MreC and its functional implications. Mol. Microbiol. 62, 1631–1642.
30. Shiomi, D., Sakai, M., and Niki, H. (2008). Determination of bacterial rod shape by a novel cytoskeletal membrane protein. EMBO J. 27, 3081–3091.

31. Aliyaha, S.A., Alexander, R., Costa, T., Henriques, A.O., Emonet, T., and Jacobs-Wagner, C. (2009). RodZ, a component of the bacterial core morphogenic apparatus. Proc. Natl. Acad. Sci. USA 106, 1239–1244.

32. Bendezú, F.O., and de Boer, P.A. (2008). Conditional lethality, division defects, membrane involution, and endocytosis in mre and mrd shape mutants of Escherichia coli. J. Bacteriol. 190, 1792–1811.

33. Mohammadi, T., Karczmarek, A., Crouvoisier, M., Bouchss, A., Mengin-Lecreulx, D., and den Blaauwen, T. (2007). The essential peptidoglycan glycosyltransferase MurG forms a complex with proteins involved in lateral envelope growth as well as with proteins involved in cell division in Escherichia coli. Mol. Microbiol. 65, 1106–1121.

34. Liechti, G., Kuru, E., Packiam, M., Hsu, Y.P., Tekkam, S., Hall, E., Rittichier, J.T., VanNieuwenhze, M., Brun, Y.V., and Maurelli, A.T. (2016). Pathogenic Chlamydia lack a classical sacculus but synthesize a narrow, mid-cell peptidoglycan ring, regulated by MreB, for cell division. PLoS Pathog. 12, e1005590.

35. Nguyen, L.T., Gumbart, J.C., Beeby, M., and Jensen, G.J. (2015). Coarse-grained simulations of bacterial cell wall growth reveal that local coordination alone can be sufficient to maintain rod shape. Proc. Natl. Acad. Sci. USA 112, E3689–E3698.

36. Pinho, M.G., Kjos, M., and Veening, J.-W. (2013). How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. Nat. Rev. Microbiol. 11, 601–614.

37. Harris, L.K., Dye, N.A., and Theriot, J.A. (2014). A Caulobacter MreB mutant with irregular cell shape exhibits compensatory widening to maintain a preferred surface area to volume ratio. Mol. Microbiol. 94, 988–1005.

38. Tsui, H.T., Boersma, M.J., Vella, S.A., Koga, O., Kuro, E., Peceny, J.K., Carlson, E.E., VanNieuwenhze, M.S., Brun, Y.V., Shaw, S.L., and Winkler, M.E. (2014). Ppb2p localizes separately from Ppb2b and other peptidoglycan synthesis proteins during later stages of cell division of Streptococcus pneumoniae D39. Mol. Microbiol. 94, 21–40.

39. van Teeffelen, S., Wang, S., Furchgott, L., Huang, K.C., Wingreen, N.S., Shaeowitz, J.W., and Gots, Z. (2011). The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. Proc. Natl. Acad. Sci. USA 108, 15822–15827.

40. Monteiro, J.M., Fernandes, P.B., Vaz, F., Pereira, A.R., Tavares, A.C., Ferreira, M.T., Pereira, P.M., Veiga, H., Kuru, E., VanNieuwenhze, M.S., et al. (2015). Cell shape dynamics during the staphylococcal cell cycle. Nat. Commun. 6, 8055.

41. Cameron, T.A., Zupan, J.R., and Zambraysky, P.C. (2015). The essential features and modes of bacterial polar growth. Trends Microbiol. 23, 347–353.

42. Liechti, G.W., Kuru, E., Hall, E., Kalinda, A., Brun, Y.V., VanNieuwenhze, M., and Maurelli, A.T. (2014). A new metabolic cell-wall labelling method reveals peptidoglycan in Chlamydia trachomatis. Nature 506, 507–510.

43. de Boer, P.A.J. (2010). Advances in understanding E. coli cell fission. Curr. Opin. Microbiol. 13, 730–737.

44. Billings, G., Ouzounov, N., UrSELL, T., Desmarais, S.M., Shaeowitz, J., Gots, Z., and Huang, K.C. (2014). De novo morphogenesis in L-forms via geo-synthesis machinery and MreB filament in B. subtilis. Science 333, 222–225.

45. Gamner, E.C., Bernard, R., Wang, W., Zhuang, X., Rudner, D.Z., and Mitchison, T. (2011). Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in B. subtilis. Science 333, 222–225.

46. Fenton, A.K., and Gerdes, K. (2013). Direct interaction of FtsZ and MreB is required for septum synthesis and cell division in Escherichia coli. EMBO J. 32, 1953–1965.

47. Ouzounov, N., Nguyen, J.P., Bratton, B.P., Jacobowitz, D., Gots, Z., and Shaeowitz, J.W. (2016). MreB orientation correlates with cell diameter in Escherichia coli. Biophys. J. 111, 1035–1043.

48. Yao, Q., Jewett, A.L., Chang, Y.W., Oikonomou, C.M., Beeby, M., Iancu, C.V., Briegel, A., Ghosal, D., and Jensen, G.J. (2017). Short FtsZ filaments can drive asymmetric cell envelope constriction at the onset of bacterial cytokinesis. EMBO J. 36, 1577–1589.

49. Kuro, E., Hughes, H.V., Brown, P.J., Hall, E., Tekkam, S., Cava, F., de Pedro, M.A., Brun, Y.V., and VanNieuwenhze, M.S. (2012). In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent D-amino acids. Angew. Chem. Int. Ed. Engl. 51, 12519–12523.

50. Strahl, H., Bürmann, F., and Hamoen, L.W. (2014). The actin homologue MreB organizes the bacterial cell membrane. Nat. Commun. 5, 3442.

51. Karczmarek, A., Martínez-Arteaga, R., Alexeeva, S., Hansen, F.G., Vicente, M., Nanninga, N., and den Blaauwen, T. (2007). DNA and origin region segregation are not affected by the transition from rod to sphere after inhibition of Escherichia coli MreB by A22. Mol. Microbiol. 65, 51–63.

52. van der Ploug, E., Goulard, S.T., and den Blaauwen, T. (2015). Validation of FRET assay for the screening of growth inhibitors of Escherichia coli reveals elongasome assembly dynamics. Int. J. Mol. Sci. 16, 17637–17654.

53. Shi, H., Colavin, A., Bigos, M., Tropini, C., Mondes, R.D., and Huang, K.C. (2017). Deep phenotypic mapping of bacterial cytoskeletal mutants reveals physiological robustness to cell size. Curr. Biol. 27, 3419–3429.e4.

54. Wachi, M., and Matsushashi, M. (1989). Negative control of cell division by mre, a gene that functions in determining the rod shape of Escherichia coli cells. J. Bacteriol. 171, 3123–3127.

55. Takacs, C.N., Poggio, S., Charbon, G., Puchault, M., Vollmer, W., and Jacobs-Wagner, C. (2010). MreB drives de novo rod morphogenesis in Caulobacter crescentus via remodeling of the cell wall. J. Bacteriol. 192, 1671–1684.

56. Bean, G.J., Fickinger, S.T., Westler, W.M., McCloud, M.E., Sept, D., Weibel, D.B., and Amann, K.J. (2009). A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB. Biochemistry 48, 4852–4857.

57. Szwedziak, P., and Löwe, J. (2013). Do the divisome and elongasome share a common evolutionary past? Curr. Opin. Microbiol. 16, 745–751.

58. Saugveau, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol. Rev. 32, 234–258.

59. Spratt, B.G. (1975). Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12. Proc. Natl. Acad. Sci. USA 72, 2999–3003.

60. Den Blaauwen, T., Aarsman, M.E.G., Vischer, N.O.E., and Nanninga, N. (2003). Penicillin-binding protein PBP2 of Escherichia coli localizes preferentially in the lateral wall and at mid-cell in comparison with the old cell pole. Mol. Microbiol. 47, 539–547.

61. Vats, P., and Rothfield, L. (2007). Duplication and segregation of the actin (MreB) cytoskeleton during the prokaryotic cell cycle. Proc. Natl. Acad. Sci. USA 104, 17795–17800.

62. Jiang, H., Si, F., Mangolin, W., and Sun, S.X. (2011). Mechanical control of bacterial cell shape. Biophys. J. 101, 327–335.
67. Swulius, M.T., and Jensen, G.J. (2012). The helical MreB cytoskeleton in *Escherichia coli* MC1000/pLE7 is an artifact of the N-terminal yellow fluorescent protein tag. J. Bacteriol. 194, 6382–6386.

68. Koppelman, C.-M., Aarsman, M.E.G., Postmus, J., Pas, E., Muljsers, A.O., Scheffers, D.-J., Nanninga, N., and den Blaauwen, T. (2004). R174 of *Escherichia coli* FtsZ is involved in membrane interaction and protofilament bundling, and is essential for cell division. Mol. Microbiol. 51, 645–657.

69. Stiernagle, T. (2006). Maintenance of *C. elegans*. WormBook, 1–11.

70. Mitchell, D.H., Stiles, J.W., Santelli, J., and Sanadi, D.R. (1979). Synchronous growth and aging of Caenorhabditis elegans in the presence of fluorodeoxyuridine. J. Gerontol. 34, 28–36.

71. Petersen, J.M., Kemper, A., Gruber-Vodicka, H., Cardini, U., van der Geest, M., Kleiner, M., Bulgheresi, S., Mußmann, M., Herbold, C., Seah, B.K.B., et al. (2016). Chemosynthetic symbionts of marine invertebrate animals are capable of nitrogen fixation. Nat. Microbiol. 2, 16195.

72. Meisner, J., Montero Llopis, P., Sham, L.-T., Garner, E., Bernhardt, T.G., and Rudner, D.Z. (2013). FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in *Bacillus subtilis*. Mol. Microbiol. 89, 1069–1083.

73. Vischer, N.O.E., Verheul, J., Postma, M., van den Berg van Saparoea, B., Galli, E., Natale, P., Gerdes, K., Luirink, J., Vollmer, W., Vicente, M., and den Blaauwen, T. (2015). Cell age dependent concentration of *Escherichia coli* divisome proteins analyzed with ImageJ and ObjectJ. Front. Microbiol. 6, 596.

74. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-MreB | den Blauwen, University of Amsterdam [55]; | N/A |
| Rabbit polyclonal anti-FtsZ | den Blauwen, University of Amsterdam [68]; | N/A |
| Rabbit polyclonal anti-Ca. T. oneisti FtsZ | This paper; Genosphere Biotech | N/A |
| **Bacterial and Virus Strains** |        |            |
| *Escherichia coli* BI21(DE3) plysS | Invitrogen | Cat#69451 |
| *Escherichia coli* DH5α | Widely distributed | N/A |
| *Escherichia coli* OP50 | Caenohabditis Genomics Center [69]; | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Ampicillin | Sigma-Aldrich | Cat#A9518 |
| Chloramphenicol | Sigma-Aldrich | Cat#C0378 |
| A22 | Sigma-Aldrich | Cat#5ML0471 |
| EDA-DA | VanNieuwenhze, Indiana University Bloomington [34]; | N/A |
| IPTG | Duchefa | Cat#I1401 |
| H-S-MreB | This study | N/A |
| **Critical Commercial Assays** |        |            |
| AKTA FPLC Serial #1688191 | GE Healthcare | Cat#29018224 |
| HiTrap TALON crude | GE Healthcare | Cat#28953767 |
| Click-iT EdU Alexa Fluor 488 Imaging Kit | Invitrogen | Cat# C10337 |
| **Deposited Data** |        |            |
| TnnrreB | This paper | GenBank accession number MF350658 |
| ThnrreB | This paper | GenBank accession number MF317948 |
| **Experimental Models: Organisms/Strains** |        |            |
| Caenohabditis elegans N2 | Laboratory of Stanley Brul | [http://www.wormbase.org/species/c_elegans/strain/N2#02–10](http://www.wormbase.org/species/c_elegans/strain/N2#02–10) |
| Candidatus Thiosymbion oneisti | Environmental sample collected by the authors for this paper | N/A |
| Candidatus Thiosymbion hypermnestrae | Environmental sample collected by the authors for this paper | N/A |
| **Oligonucleotides** |        |            |
| pML118-his-sumo-losMreB-fw = 5’-TCACAGAGAACAGATTGTCGGGATGTTTCTCCGAGCGACATTCG-3’ | This paper | N/A |
| pML118-his-sumo-losMreB-rev = 5’-GTCCTGCAGTCCACGGGGCTCTCGCATAGCCACAGGTC-3’ | This paper | N/A |
| pML118-vector-his-sumo-fw = 5’-GCCCGGGTGACTGCAGGAAG-3’ | This paper | N/A |
| pML118-vector-his-sumo-rev = 5’-CCCACCAATC-TGTTTCTCTGGA-3’ | This paper | N/A |
| LosRhsMreB_F = 5’-ATGGTTTCTCCGACGCGCATTCGAG-3’ | This paper | N/A |
| LosMreB_R = 5’-CTACTCGCTCATAGCCACAGGTC-3’ | This paper | N/A |
| RhsMreB_R = 5’-TTACTCGACGCGTGAAACAAATC-3’ | This paper | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Silvia Bulgheresi (silvia.bulgheresi@univie.ac.at).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Both Ca. T. oneisti and T. hypermnestrae are not cultivable and can only be accessed by collecting their marine nematode hosts, L. oneistus and R. hypermnestra, respectively. The latter were collected on multiple field trips (2014-2016) in approximately 1 m depth from a sand bar off Carrie Bow Cay, Belize (16°48’11.01”N, 88°4’54.42”W). Specimens of L. oneistus and R. hypermnestra were extracted from the sand by stirring it in seawater and pouring the supernatant through a 63 μm mesh sieve. The retained material was transferred into a Petri dish and single nematodes were handpicked using pipettes under a dissecting microscope. Symbiotic nematodes were transported from Carrie Bow Cay to the University of Vienna deep-frozen.

METHOD DETAILS

A22 toxicity tests on Caenorhabditis elegans

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EDA-DA and A22 incubation of live symbionts

To track symbiont cell wall growth, batches of approx. 50 live symbiotic nematodes were each incubated in a 1.5 mL tube containing 500 μl of 10 mM ethynyl-D-alanyl-D-alanine (EDA-DA, a D-amino acid carrying a clickable ethynyl group) in filter sterilized natural seawater (FSW) for 5 to 480 min. For each A22 treatment, batches of approx. 40 live symbiotic nematodes were incubated in 1.5 mL tubes containing 500 μl of 20 μg/ml A22 (Sigma-Aldrich) in FSW for 2 h prior addition of EDA-DA and an additional 90 min-long incubation (A22 was not removed upon addition of EDA-DA). Given that A22 was dissolved in DMSO so that a 1:10,000 dilution of this compound was finally present in the incubation medium, DMSO was added in the same amount

REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant DNA     | This paper | N/A |
| Plasmid: pTB146-His-sumo-MreB, see methods details | This paper | N/A |

Software and Algorithms

| Software and Algorithms | SOURCE |
|-------------------------|--------|
| ImageJ                  | NIH    |
| Fiji                    | ImageJ |
| ObjectJ                 | University of Amsterdam |
| SoftWoRx                | GE Healthcare Life Sciences |
| NIS-Elements AR 4.20.01 | Nikon  |
| ProgRes Capture Pro 2.8.8 | Jenoptik |
| Photoshop CS6           | Adobe Systems |
| Illustrator CS 6        | Adobe Systems |
| CodonCode Aligner 3.7.1 software | CodonCode Corporation |
| SPSS 19.0               | SPSS   |

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Silvia Bulgheresi (silvia.bulgheresi@univie.ac.at).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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Protein (H-S-MreB) was expressed in E. coli strain BL21(DE3) pLysS. Primers used to obtain the fragments for Gibson assembly were pML118-his-sumo-losMreB-fw (5’-ATGGTTCTCCGACGCATTGGAG-3’) and the reverse primers LosMreB_R (5’-CTACTCGCTCATAGCCAAACGGTC-3’) or RhsMreB_R (5’-TTACTCGACCACGGTGAAACAATC-3’). PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 75 s, followed by a final elongation step at 72°C for 10 min. We randomly picked and fully sequenced four clones containing the Ca. T. oneisti mreB gene fragment and four clones containing the Ca. T. oneisti mreB gene fragment in both directions. Sequences were aligned and compared with CodonCode Aligner 3.7.1 software (CodonCode Corporation, Dedham, MA, USA). A predicted 348 aa-long MreB protein (ToMreB) is encoded by a 1,047 nt-long ORF in Ca. T. hypermnestrae (accession number MF317948).

Cloning of symbiont mreB genes
We used the genome drafts of Ca. T. oneisti [71] and Ca. T. hypermnestrae (available under request at http://rast.nmpdr.org/rast.cgi) to design specific primers against Ca. T. hypermnestrae and Ca. T. oneisti mreB genes, respectively. Methanol fixed deep-frozen worms were rehydrated in phosphate-buffered saline (PBS) and bacteria were detached by sonication. Subsequently, 2 µl of bacterial suspension were used as template in each 50 µl PCR reaction. A 1.047 nucleotide (nt)-long and a 1.059 nt-long mreB gene fragment were amplified from Ca. T. oneisti and Ca. T. hypermnestrae, respectively, using the forward primer LosRhsMreB_F (5’-ATGTTTCTCCGACGCAATTGGAG-3’) and the reverse primers LosMreB_R (5’-CTACTCGCTCATAGCCAAACGGTC-3’) or RhsMreB_R (5’-TTACTCGACCACGGTGAAACAATC-3’). PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 75 s, followed by a final elongation step at 72°C for 10 min. We randomly picked and fully sequenced four clones containing the Ca. T. hypermnestrae gene fragment and four clones containing the Ca. T. oneisti mreB gene fragment in both directions. Sequences were aligned and compared with CodonCode Aligner 3.7.1 software (CodonCode Corporation, Dedham, MA, USA). A predicted 348 aa-long MreB protein (ToMreB) is encoded by a 1,047 nt-long ORF in Ca. T. oneisti (GenBank accession number MF350658) and a 352 aa-long MreB protein (ThMreB) is encoded by a 1,059 nt-long ORF (GenBank accession number MF317948) in Ca. T. hypermnestrae.

Cloning, expression and purification of MreB from Ca. T. oneisti
The mreB gene of Ca. T. oneisti was cloned into pTB146 vector [72] by Gibson assembly and a His-tagged MreB recombinant protein (H-S-MreB) was expressed in E. coli cells. The recombinant protein was induced by adding 0.5 mM IPTG. After 3 h induction, the culture was pelleted at 8,000 rpm and resuspended in 8M urea phosphate buffer (pH = 8). Cells were passed through a French press and cell lysates separated into pellet and supernatant fractions by ultracentrifuge 210,000 rpm 45 min. The purification was performed by ÄKTA FPLC (GE Healthcare). The supernatant was applied to HiTrap TALON® crude (GE Healthcare) 1 mL column, and the column was washed by phosphate buffer (pH = 8) with 30 µM urea and 10 mM imidazole followed by elution of His-S-MreB by phosphate buffer (pH = 8) supplemented with 8 M urea and 200 mM imidazole.

Immunostaining
Deep-frozen methanol fixed nematodes were rehydrated and washed in PBS containing 0.1% Tween 20 (PBT), followed by permeabilization of the bacterial PG by incubation for 10 min with 0.1% (wt/vol) lysozyme at room temperature. Blocking was carried out for 1 h in PBT containing 2% (wt/vol) bovine serum albumin (blocking solution) at room temperature. Ca. T. oneisti and T. hypermnestrae were incubated with a 1:1 dilution of immunoaffinity purified rabbit polyclonal anti-E. coli MreB antibody (see previous section), as well as with a 1:500 dilution of rabbit polyclonal anti-Ca. T. oneisti FtsZ peptide antibody (Genosphere Biotech) in the case of
Ca. T. oneisti, or with a 1:200 dilution of rabbit polyclonal anti-E. coli FtsZ antibody [4, 73] in the case of Ca. T. hypermnestrae. All primary antibodies were incubated overnight at 4°C in blocking solution. Upon incubation with primary antibody (or without in the case of the negative control) samples were washed three times in PBT and incubated with a 1:500 dilution of secondary Alexa555-conjugated anti-rabbit antibody (Jackson ImmunoResearch, USA) in blocking solution for 1 h at room temperature. Unbound secondary antibody was removed by three washing steps in PBT and worms were sonicated for 40 s to dissociate Ca. T. oneisti and T. hypermnestrae cells from their hosts prior mounting. 1 μl of the bacterial solution was mixed with 0.5 μl of Vectashield mounting medium (Vector Labs).

**Click-chemistry**

Deep frozen methanol fixed nematodes were rehydrated and washed in PBS containing 0.1% Tween 20 (PBT). Blocking was carried out for 30 min in PBT containing 2% (wt/vol) bovine serum albumin (blocking solution) at room temperature. An Alexa488 fluorophore was covalently bound to EDA-DA via copper catalyzed click-chemistry by following the user manual protocol for the Click-iT reaction cocktail (Click-iT EdU Imaging Kit, Invitrogen). The nematodes were incubated with the Click-iT reaction cocktail for 30 min at RT in the dark. Unbound dye was removed by a 10-min wash in PBT and one wash in PBS. Worms were sonicated for 40 s to dissociate Ca. T. oneisti and T. hypermnestrae prior mounting. For immunostaining of clicked bacterial cells, worms were washed for 10 min in PBT and subsequently incubated with blocking solution for 30 min at room temperature in the dark. From here on, immunostaining was performed as described above, except that PG permeabilization was omitted and all steps were carried out in the dark to prevent bleaching of clicked cells.

**Morphometric and fluorescence measurements**

Symbiont cells were dissociated from fixed untreated and A22-treated *L. oneistus* nematodes, and from untreated and A22-treated *R. hypermnestrae* nematodes by sonication. Cell suspensions were applied to an 1% agarose covered microscopy slide [68] and imaged using a Nikon Eclipse 50i microscope equipped with either DS-Qi1mc camera, an Orca Flash 4.0 (Hamamatsu, Japan) or a MFCool camera (Jenoptik, Germany). Epifluorescence images were acquired using the NIS-Elements AR 4.20.01 software (Nikon) or the ProgRes Capture Pro 2.8.8 software (Jenoptik) and processed using the public domain program ImageJ [74] in combination with plugin ObjectJ and a modified version of Coli-Inspector [10, 73]. Cell outlines were traced and cell length, width and fluorescence patterns were measured automatically. Automatic cell recognition was double-checked manually. For the average fluorescence plots and for the demographs, cells were automatically grouped into morphological classes based on phase-contrast images, each cell was resampled to the same length and the fluorescence intensities added up and averaged. For assessing the effects of A22, symbiont cells were grouped into constricted and non-constricted based on visual inspection. Data analysis was performed using Excel 2016 (Microsoft Corporation, USA), plots were created with Excel 2016 and figures were compiled using Photoshop CS6 and Illustrator CS 6 (Adobe Systems Inc. USA).

**Three-Dimensional Structured Illumination Microscopy (3D SIM) imaging and analysis**

Cell suspensions were applied on high precision coverslips (No. 1.5H, Sigma-Aldrich) coated with 0.01% (wt/vol) of Poly-L-Lysin. After letting the cell dry onto the surface of the coverslip, antifade mounting medium (Vectashield) was applied and the coverslip was sealed to a slide. 3D SIM was performed on a Delta Vision OMX v4 microscope equipped with an Olympus 60X/1.42 Oil Plan Apo N objective and 2 sCMOS cameras. The samples were excited with lasers at 488 nm and at 568 nm, the emission was detected through emission filters 477/32 nm and 571.5/19 nm (Center/Bandpass), respectively. The image reconstruction and registration was performed as described above, except that PG permeabilization was omitted and all steps were carried out in the dark to prevent bleaching of clicked cells.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis of number of constricted cells**

For assessing the effects of A22 on septation, symbiont cells dissociated from 10 untreated and 10 A22-treated *L. oneistus* nematodes, and from five untreated and five A22-treated *R. hypermnestrae* nematodes were grouped into constricted and non-constricted based on visual inspection (*Table S3*). We analyzed whether the number of constricted cells for untreated and A22-treated Ca. T. oneisti and T. hypermnestrae were normally distributed by performing two tests of normality (Kolmogrov-Smirnov and Shapiro-Wilk; *Table S4*). An unpaired t-Test revealed significant difference (p < 0.05) between untreated and A22-treated cells for both Ca. T. oneisti and T. hypermnestrae (*Table S4*; a significance level of 95% (α = 0.05) was set for all the tests). Boxplots were created by SPSS 19.0 (SPSS, IL USA).