eLife

Reviewed Preprint
v1 • September 4, 2026
Not revised

For correspondence:
bnan@tamu.edu

Competing interests: No competing interests declared

Reviewing editor: Kumaran Ramamurthi, National Cancer Institute, United States

Reprogrammed peptidoglycan elongation reveals plasticity in bacterial growth modes

Carolina Basurto De Santiago, Isabella S Lin, Beiyan Nan
Department of Biology, Texas A&M University, College Station, United States

eLife Assessment

This paper demonstrates that relocating the MreB-Rod complex to the poles of E. coli can reprogram cell-wall synthesis, causing cells to elongate through peptidoglycan incorporation at the poles rather than along the cell length. This is a **valuable** finding for understanding how bacterial growth can be spatially reprogrammed and provides insight into how distinct modes of bacterial growth might arise. However, the evidence is currently **incomplete**, particularly regarding the nature of the polar structures, their relationship to cell shape and growth, and the requirement for a pre-existing pole.

https://doi.org/10.7554/eLife.112612.1.sa4

Abstract

Most bacteria are enclosed by a peptidoglycan (PG) cell wall that must be expanded for growth. In rod-shaped species, PG elongation is spatially organized in a species-specific manner, occurring either at the cell poles or along the lateral wall. MreB filaments organize the Rod machinery and are typically required for dispersed, nonpolar PG elongation but are dispensable for polar growth. Whether elongation modes are inherently fixed or can be reprogrammed remains unclear. *Escherichia coli* and *Myxococcus xanthus* both elongate PG in a dispersed, nonpolar fashion. Here, we show that heterologous expression of *M. xanthus* MreB in *E. coli* relocalizes native MreB to the cell poles, thereby redirecting the Rod machinery and PG elongation to polar sites. Moreover, direct targeting of the Rod synthase PBP2 to the poles is sufficient to drive polar PG elongation in *E. coli* while preserving rod shape. This reprogrammed growth mode bypasses the requirement for MreB filaments, highlighting a plasticity of the Rod system that suggests polar elongation may have emerged through the evolutionary loss of MreB.

Introduction

Most bacterial cells utilize the peptidoglycan (PG) cell wall as a major stress-bearing structure that determines cell shape and prevents cells from lethal rupture caused by high osmotic pressure [1, 2]. To grow in size, bacteria expand their existing PG structures using distinct patterns. In rod-shaped bacteria, two main modes of PG elongation are observed: polar elongation, where new cell wall material is added at one or both poles, and dispersed nonpolar elongation, which involves a more uniform insertion of PG along the lateral sides of the cell. These elongation strategies are typically species-specific, with each organism adopting either one mode or a combination of both [3, 4]. Although most well-studied rod-shaped bacteria exhibit dispersed elongation, polar elongation is notably characteristic of Gram-negative *Rhizobiales* and Gram-positive *Actinomycetales* [5-7].

PG of both Gram-positive and negative bacteria consists of glycan strands that are covalently crosslinked by short peptides [8, 9]. PG precursors are synthesized in the cytoplasm and flipped across the cytoplasmic membrane, where PG synthases, including glycosyltransferases (**GTases**) that polymerize them into glycan strands, which are then crosslinked into PG network by

transpeptidases (**TPases**). In most rod-shaped bacteria, two synthase systems assemble the PG during cell elongation, the Rod system and class A penicillin-binding proteins (**aPBPs**) [2, 10, 11]. The Rod system is a multiprotein complex that includes RodA, a shape, elongation, division, and sporulation (**SEDS**) family GTase; PBP2, a TPase of the class B penicillin-binding protein (**bPBP**) family; and the actin homolog MreB. This system defines rod shape and drives the majority of PG expansion during vegetative growth [11-13]. In contrast, aPBPs that possess both GTase and TPase activities do not depend on MreB, and the absence of single aPBPs rarely abolishes cell survival or rod-like morphology [12, 14-17].

MreB is essential for rod shape in the cells that elongate in dispersed nonpolar manner [13]. MreB polymerizes into antiparallel double filaments on the inner leaflet of the cytoplasmic membrane [18, 19]. Sensitive to membrane curvature, MreB filaments guide RodA and PBP2 to the cylindrical regions of the cell envelope, where PG elongation occurs [13, 20-23]. Depleting MreB or inhibiting MreB polymerization using A22, a specific inhibitor, causes cells to lose rod shape in a wide range of model organisms, including *Escherichia coli* [24, 25], *Caulobacter crescentus* [26], *Pseudomonas aeruginosa* [27], *Bacillus subtilis* [28], *Myxococcus xanthus* [29], etc. Conversely, MreB is either absent or nonessential in the bacteria that elongate at the poles [30]. Instead, these organisms repurpose cell division related proteins, such as FtsA and FtsZ in *Rhizobiales* and DivIVA in *Actinomycetales* to localize PG synthases to cell poles [31, 32]. Recent studies revealed that closely related bacterial species in the family *Caulobacteraceae* can adopt different PG elongation modes [3] and that Streptomyces can use both polar and dispersed elongation modes in a special growth stage called exploratory growth [4]. Nonetheless, it remains uncertain whether the PG elongation mode in rod-shaped bacteria can be entirely reprogrammed without compromising cell morphology.

E. coli (γ-proteobacterium) and *M. xanthus* (long assigned to δ-proteobacteria but recently reclassified into the newly established phylum Myxococccota [33]) both elongate their PG in dispersed manner using their respective Rod systems [17, 20, 34-36]. In this study, we found that the heterogeneous expression of *M. xanthus* MreB (**$\mathbf{MreB_{Mx}}$**) in wild-type *E. coli* caused the native MreB (**$\mathbf{MreB_{Ec}}$**) to form aggregates at cell poles and thereby redirect the Rod synthases to elongate PG at poles. Building on this observation, we further demonstrated that directly targeting PBP2 to cell poles is sufficient to switch *E. coli* PG elongation to poles. In both cases, the reprogrammed polar PG elongation maintains rod shape independent of MreB filaments. Given that MreB is an ancient protein believed to have existed prior to the emergence of *Rhizobiales* and *Actinomycetales* [37], it is plausible that polar growth may have evolved from dispersed elongation as a consequence of MreB loss.

Results

Heterologous expression of $\mathbf{MreB_{Mx}}$ in *E. coli* causes $\mathbf{MreB_{Ec}}$ to aggregate at cell poles

While cloning the *M. xanthus mreB* (***mreB*$_{\mathbf{Mx}}$**) gene into the plasmid pMAT3 [38] using the *E. coli* DH5α strain, we found that *E. coli* cells harboring the pMAT3-*mreB*$_{\mathrm{Mx}}$ plasmid grew slower and displayed dark aggregates near their cell poles under bright field microscopy (Fig. 1a). To determine whether this phenomenon is specific to the DH5α strain, we transformed the pMAT3-*mreB*$_{\mathrm{Mx}}$ plasmid into wild-type *E. coli* strains MG1655 and BW25113 and observed similar aggregates at cell poles (Fig. 1a). In contrast, the empty pMAT3 vector did not cause polar aggregation (Fig. 1a, insets). To maintain consistency, we performed all subsequent experiments in MG1655-derived strains.

Under a cryogenic electron microscope (**cryoEM**), these aggregates appeared as dense spheres that lack clear boundaries (Fig. 1b), similar to the inclusion bodies frequently observed in *E. coli* cells that express recombinant proteins [39]. Remarkably, in cells with polar aggregates, we

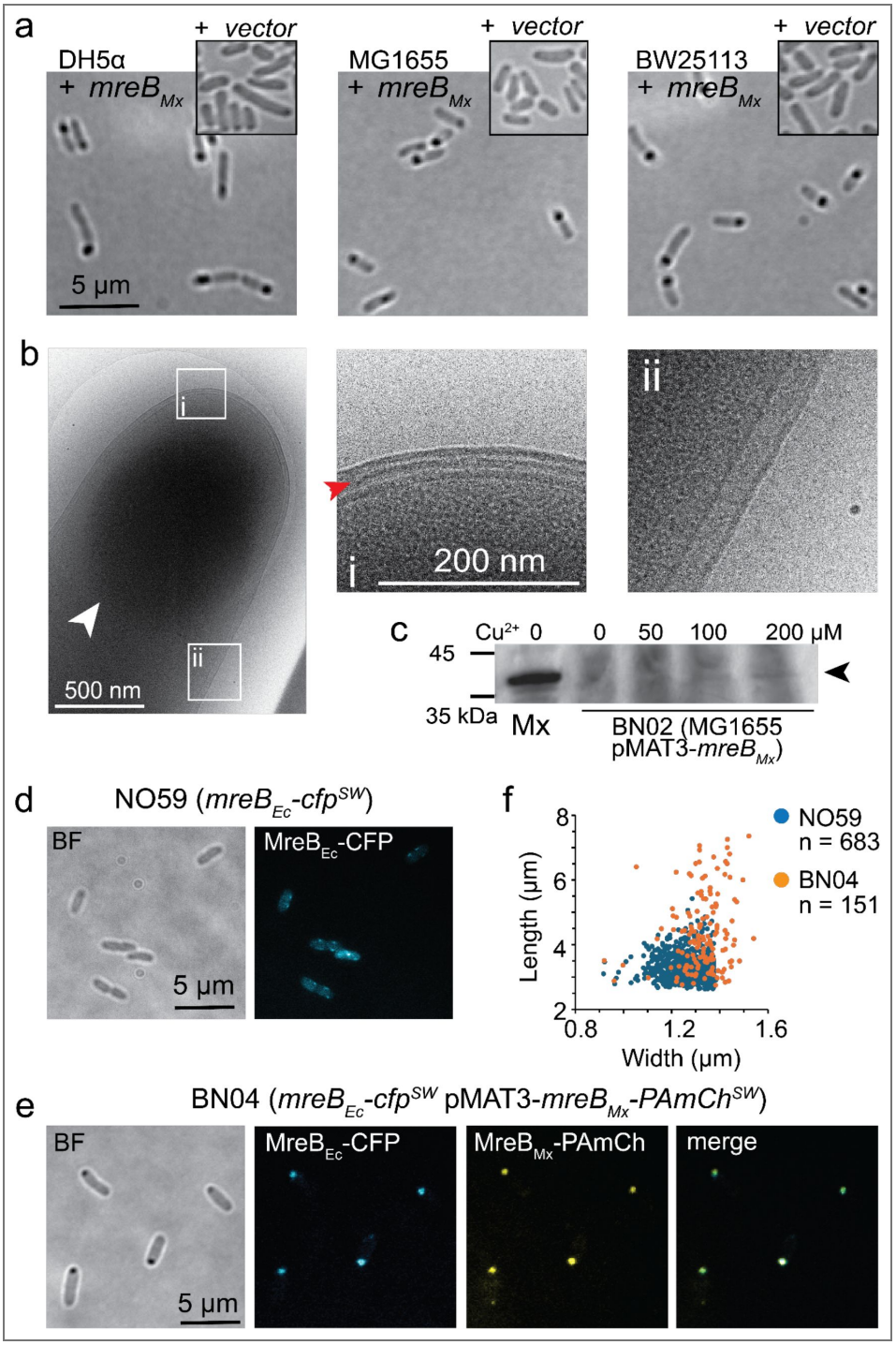

Fig. 1. Heterologous expression of $MreB_{Mx}$ in *E. coli* causes $MreB_{Ec}$ to aggregate at cell poles.

a) Heterologous expression of $MreB_{Mx}$ in *E. coli* strains DH5α, MG1655, and BW25113 causes *E. coli* cells to form dark aggregates at cell poles. Insets show the morphology of cells carrying the empty pMAT3 vector. **b)** CryoEM images of an *E. coli* MG1655 cell that expresses $MreB_{Mx}$. White arrow points to the polar aggregate. In the cells with polar aggregates, PG thickens at cell poles (inset i, red arrow), whereas PG is rarely visible in nonpolar regions (insert ii). **c)** $MreB_{Mx}$ expresses at very low levels, even when induced by high concentration of $CuSO_4$. The native expression of $MreB_{Mx}$ from the same number of *M. xanthus* (Mx) cells is shown in the first lane as a reference. Black arrow points to the bands detected by an anti-$MreB_{Mx}$ antibody. **d)** $MreB_{Ec}$-msfCFP^{SW} forms dispersed foci in the NO59 strain. **e)** In BN04 (NO59 pMAT3-*mreB*$_{Mx}$-*PAmCherry*^{SW}) cells, instead of forming filaments, $MreB_{Mx}$ and $MreB_{Ec}$ colocalize in polar aggregates. **f)** While BN04 strain retains rod shape, expressing $MreB_{Mx}$ increases both the length and width of cells. BF, bright field.

observed dark densities from the PG layers at the cell poles (Fig. 1b i), whereas PG was rarely visible in nonpolar regions (Fig. 1b ii). The correlation between polar aggregates and thickened PG suggests that these aggregates may enhance local PG synthesis.

The pMAT3 plasmid, which carries the ColE1 origin, was designed for gene expression under the control of a copper-inducible promoter [38]. However, cells carrying the pMAT3-*mreB*$_{Mx}$ plasmid formed aggregates even in the absence of added copper. We reasoned that the leaky expression from the promoter was unlikely to produce MreB$_{Mx}$ at levels high enough to form inclusion bodies. To assess how MreB$_{Mx}$ expression influences aggregate formation in *E. coli*, we performed immunoblotting. As shown in Fig. 1c, the highly sensitive anti-MreB$_{Mx}$ antibody [40] did not detect significant MreB$_{Mx}$ expression in the absence of copper, indicating that the leaky expression was extremely low and that the antibody did not cross-react with MreB$_{Ec}$ (Fig. 1c). Even at $CuSO_4$ concentrations as high as 200 μM, MreB$_{Mx}$ expression was only modestly induced in *E. coli*, to less than 5% of its native expression level in *M. xanthus* (Fig. 1c). Collectively, these findings suggest that even minimal levels of MreB$_{Mx}$ are sufficient to initiate aggregate formation. In this context, MreB$_{Mx}$ acts not as a primary structural component of the aggregates, but rather as a trigger for their formation. Therefore, in all subsequent experiments, we expressed MreB$_{Mx}$ from pMAT3 without the addition of copper. In this condition, among 799 cells we analyzed, 68.1% contained one aggregate, 3.4% contained two, while 28.5% did not show significant aggregates. We reason that this variability may stem from the heterogeneity of the leaky MreB$_{Mx}$ expression among individual cells and the limited sensitivity of bright field microscopy.

Given that MreB$_{Mx}$ and MreB$_{Ec}$ share 63.3% amino acid sequence identity [41], we hypothesized that even low levels of MreB$_{Mx}$ could interfere with MreB$_{Ec}$ filament assembly, causing MreB$_{Ec}$ to form inclusion body-like aggregates. In the MG1655-derived *E. coli* strain NO59 that expresses an internal monomer super-folder cyan fluorescence protein (**msfCFP^{SW}**)-labeled MreB$_{Ec}$ from its native locus and promoter [42], short MreB$_{Ec}$ filaments appear as foci on cell peripherals (Fig. 1d). To test our hypothesis, we generated the strain BN04 by expressing an internal photoactivatable mCherry (**PAmCherry^{SW}**)-labeled MreB$_{Mx}$ that is functional in *M. xanthus* [29] in NO59 using the pMAT3 vector. In bright field images, BN04 cells showed polar aggregates (Fig. 1e) similar to those observed in the MG1655 cells expressing the unlabeled MreB$_{Mx}$ (Fig. 1a). Thus, introducing fluorescent tags into both MreB homologs did not affect aggregate formation. When exposed to 405-nm excitation (0.2 kW/cm^2) for 2 s, the majority of MreB$_{Mx}$-PAmCherry^{SW} was photoactivated [29]. In the cells that contained aggregates, the emission signals of both MreB$_{Mx}$ and MreB$_{Ec}$ colocalized with the polar aggregates (Fig. 1e). Notably, MreB$_{Ec}$ filaments were never observed (Fig. 1e), indicating that heterologous expression of MreB$_{Mx}$ in *E. coli* causes the majority of MreB$_{Ec}$ to aggregate at cell poles.

Polar aggregation of MreB$_{Ec}$ causes *E. coli* to elongate PG at cell poles

Polar aggregation of MreB$_{Ec}$ clearly affected PG elongation: expression of MreB$_{Mx}$-PAmCherry^{SW} increased the generation time from 41.2 ± 3.0 min (n = 4) of NO59 to 127.6 ± 8.6 min (n = 3) of BN04 (Fig. S1) and led to a 33.11% and 5.70% increase in cell length and width, respectively (Fig. 1f). However, cells still retained rod shape.

To visualize newly synthesized PG, we used BODIPY-FL 3-amino-D-alanine (**BADA**), a fluorescently labeled D-alanine analog that incorporates into the PG scaffold during elongation [43, 44]. To visualize PG assembly on the entire cell surface, we used fluorescence microscopy to visualize a ~200-nm thick longitudinal section close to the coverslip under highly inclined and laminated optical sheet (HILO) illumination [29, 35, 45, 46] (Fig. 2a). In the NO59 strain, the incorporation of BADA appeared in a dispersed pattern, aligning with the distribution of MreB$_{Ec}$ filaments. Notably, the absence of BADA at the cell poles reinforces the nonpolar elongation mode (Fig. 2a, b). In contrast, in 71.81% (n = 674) of the BN04 cells, PG elongation occurred exclusively at cell poles, colocalizing with the aggregates that contained both MreB$_{Ec}$ and MreB$_{Mx}$ (Fig. 2d, e).

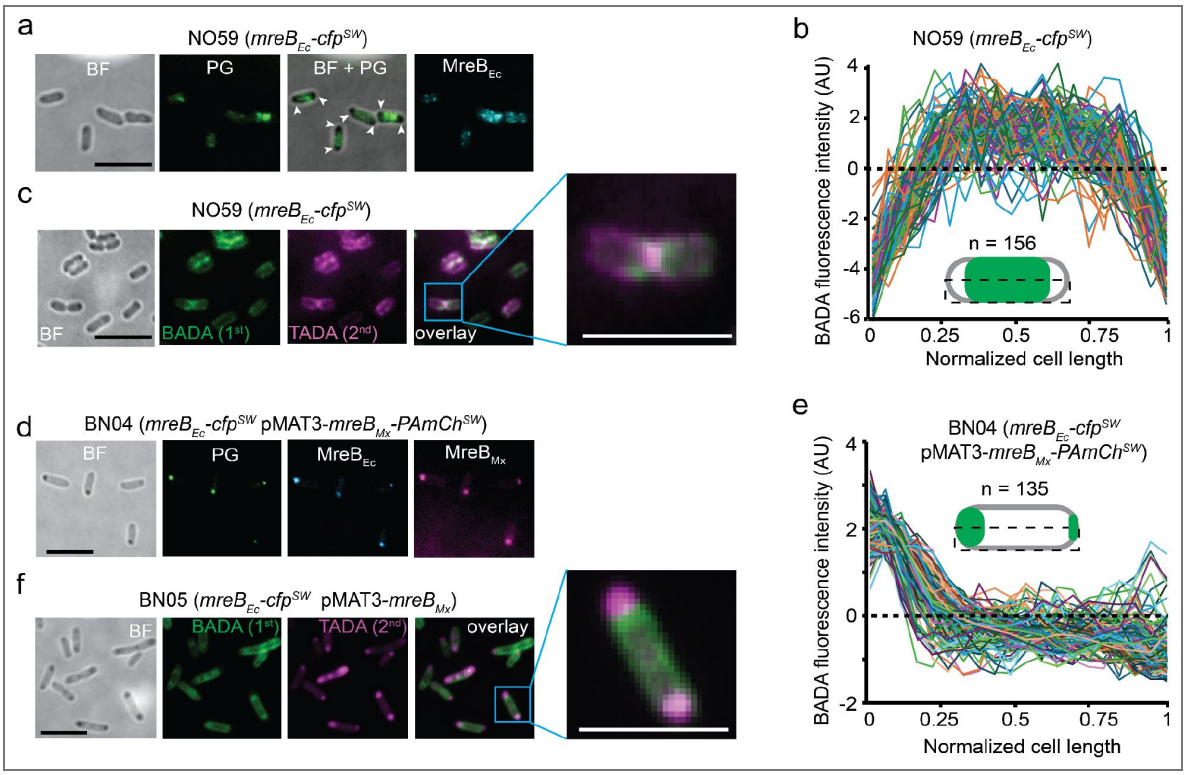

Fig. 2. Heterologous expression of $MreB_{Mx}$ causes *E. coli* to elongate PG at cell poles.

a) NO59 cells elongate PG at nonpolar regions in a dispersed manner, consistent with $MreB_{Ec}$ localization pattern. PG elongation was labeled by BADA. White arrows point to cell poles. **b)** Quantitative analysis of PG elongation across 156 NO59 cells. Cell lengths were normalized to 1 and BADA fluorescence intensity profiles were standardized using Z-score normalization (0 indicates the mean value for each cell). Insets here and in panel **e** illustrate patterns of PG elongation, with newly synthesized PG shown in green. Imaged cell regions are highlighted by dotted-line boxes. **c)** PG elongation mode in NO59 cells visualized by pause-chase sequential labeling with BADA and TADA, where both dyes were incorporated in a dispersed, nonpolar pattern. **d)** BN04 cells that express $MreB_{Mx}$-$PAmCherry^{SW}$ elongate PG at poles, consistent with the localization patterns of both $MreB_{Ec}$ and $MreB_{Mx}$. PG elongation was visualized by BADA. **e)** Quantitative analysis of PG elongation across 135 BN04 cells. For each cell, its BADA fluorescence intensity profile was measured starting from the only or brighter cell pole. **f)** Elongation mode in BN05 cells visualized by pause-chase sequential labeling with BADA and TADA. While the new growth (TADA) occurred at cell poles, the old growth (BADA) receded to nonpolar regions. BF, bright field. Scale bars, 5 μm.

To further validate this observation, we conducted a pulse-chase experiment to visualize PG elongation using sequential incorporation of two fluorescent D-alanine analogs. For the NO59 strain, we first stained PG elongation using BADA for 15 min, washed BADA out, then switched to the second dye, TAMRA 3-amino-D-alanine (**TADA**) for another 15 min. Consistent with its nonpolar elongation mode, both dyes were incorporated in the same dispersed pattern into lateral cell surfaces (Fig. 2c). To facilitate pause-chase labeling, we constructed a BN05 strain that expressed unlabeled $MreB_{Mx}$ in the NO59 background and visualized PG elongation using BADA and TADA sequentially. While the second dye, TADA, was incorporated exclusively at the cell poles, the first dye, BADA, receded to subpolar regions (Fig. 2f), indicating that newer PG elongation occurs exclusively at poles. These results suggest that some $MreB_{Ec}$ molecules in polar aggregates might be recruiting the Rod enzymes to poles and thus redirecting *E. coli* growth to a polar elongation mode.

The Rod system carries out the reprogrammed, polar PG elongation

To determine whether aPBPs or the Rod system drives polar elongation when $MreB_{Ec}$ aggregates at cell poles, we inhibited their activities by moenomycin (10 μg/ml) and mecillinam (100 μg/ml), respectively. While moenomycin blocks all aPBPs [47], mecillinam specifically inhibits PBP2 in the Rod system [48]. Under both treatments, the NO59 cells lost their rod shape but continued to incorporate BADA without a recognizable pattern (Fig. 3a), suggesting that although both aPBPs and the Rod system are required for rod shape in *E. coli*, either system is sufficient for PG elongation, albeit in disorganized manners. Notably, $MreB_{Ec}$ appeared diffusive under both conditions (Fig. 3a), indicating extensive disassembly of $MreB_{Ec}$ filaments, for which the mechanisms remain to be investigated.

Under moenomycin treatment, the BN04 cells that expressed $MreB_{Mx}$ retained their rod shape and continued PG elongation at their poles, with both $MreB_{Ec}$ and $MreB_{Mx}$ remained at the poles (Fig. 3b). Thus, focused polar growth fully bypassed the requirement for aPBPs. In contrast, mecillinam inhibited PG growth and abolished rod shape in BN04 cells (Fig. 3b). As mecillinam is sufficient to block polar PG elongation, the Rod system is the sole player that drives polar PG elongation. Interestingly, the expression of $MreB_{Mx}$ did not cause $MreB_{Ec}$ to aggregate in the presence of mecillinam (Fig. 3b). As mecillinam blocks $MreB_{Ec}$ filament assembly (Fig. 3a), our observation suggests that $MreB_{Mx}$ may only cause assembled $MreB_{Ec}$ filaments to aggregate.

To drive focused PG elongation at cell poles, the Rod system enzymes RodA (GTase) and PBP2 (TPase) must localize to the poles. The TKL130 strain, derived from MG1655, expresses a PAmCherry-labeled PBP2 from its native locus and promoter [49]. Under a 405-nm excitation (0.2 kW/cm^2, 2 s) that activates the majority of PAmCherry, PBP2 localized in a dispersed pattern (Fig. 3c). To test if PBP2 switches its localization to cell poles while the Rod system drives polar PG elongation, we generated the BN06 strain by introducing the pMAT3-$mreB_{Mx}$ plasmid into the TKL130 background. Similar to our observation on BN04 and BN05 cells, heterologous expression of $MreB_{Mx}$ caused BN06 cells to form dark polar aggregates under bright-field microscopy, which colocalized with bright PBP2 foci (Fig. 3c).

To further test if $MreB_{Ec}$ aggregates recruit the Rod polymerases to cell poles, we investigated whether they still trigger polar PG elongation in the absence of RodZ, the connector between $MreB_{Ec}$ and Rod enzymes [42]. The NO56 cells that expressed $MreB_{Ec}$-$msfGFP^{SW}$ in a *ΔrodZ* background were near spherical (Fig. 3d). Consistent with the disconnection between $MreB_{Ec}$ and Rod enzymes, while $MreB_{Ec}$ still formed filaments, HCC-amino-D-alanine (**HADA**), another fluorescent D-amino acid, was incorporated near homogeneously on the entire cell surface (Fig. 3d). We introduced pMAT3-$mreB_{Mx}$ into the NO56 background to generate the BN07 strain. Similar to the parental NO56 cells, BN07 cells were near spherical, with imperfect local curvatures that mimicked cell poles (Fig. 3d). Strikingly, while $MreB_{Mx}$ expression caused $MreB_{Ec}$ to aggregate at such pole-like locations, these aggregates neither induced focused HADA incorporation, nor restored rod shape (Fig. 3d). Therefore, in the absence of RodZ, $MreB_{Ec}$

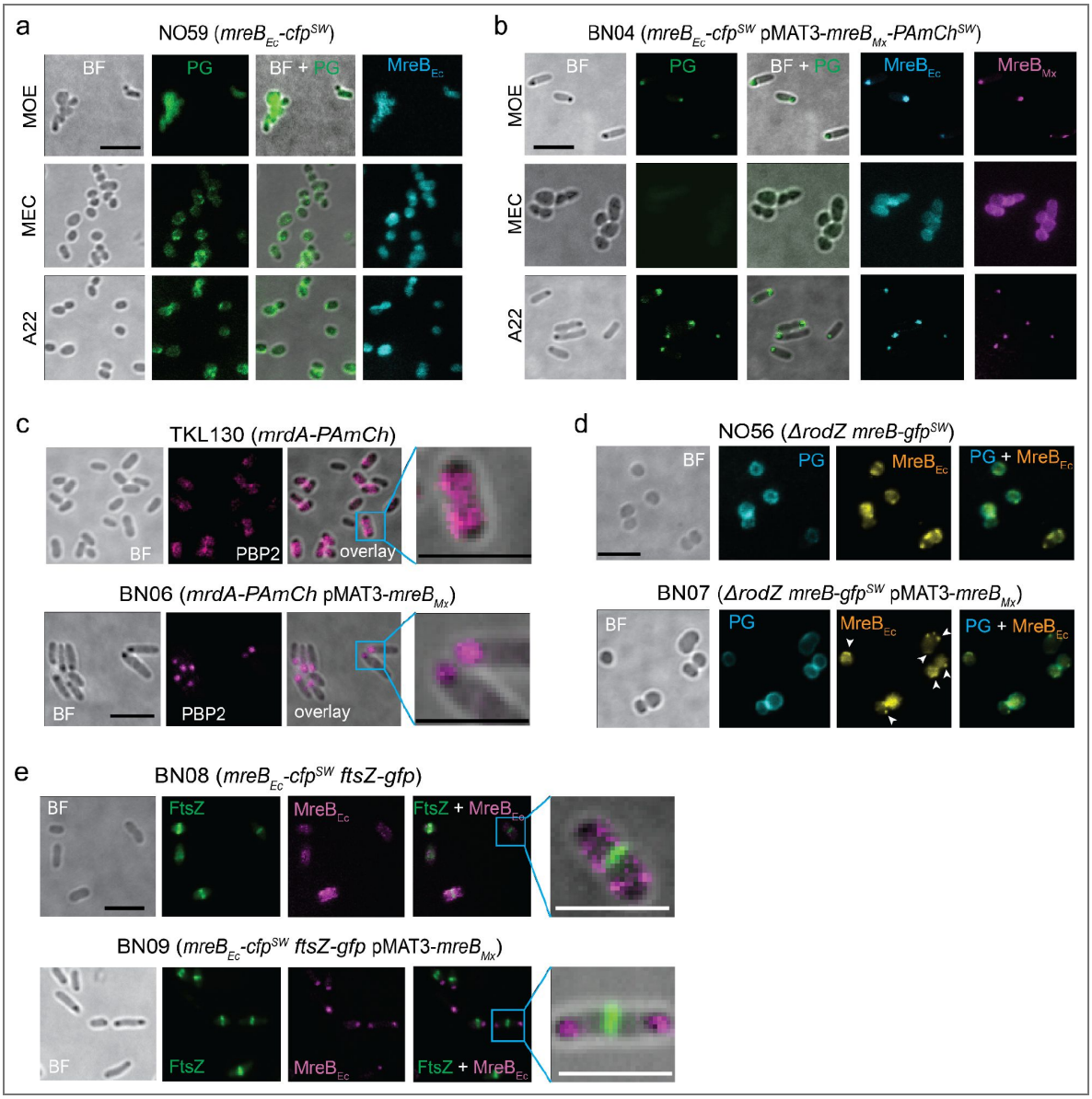

Fig. 3. The relocalized Rod system carries out the reprogrammed, polar PG elongation.

a) In the absence of heterologous MreB, *E. coli* cells elongate PG using both the Rod system and aPBPs. Neither moenomycin (MOE, 10 μg/ml) that inhibits all aPBPs, mecillinam (MEC, 100 μg/ml) that inhibits PBP2 in the Rod system, or A22 (10 μg/ml) that inhibits the polymerization of MreB filaments and thus disrupts the assembly of Rod complexes, abolishes PG elongation completely. PG elongation was visualized using BADA. **b)** *E. coli* cells expressing $MreB_{Mx}$ solely rely on the Rod system for polar PG elongation, as MEC alone is sufficient to abolish PG elongation. Such polar PG elongation is resistant to A22, suggesting that polar localized Rod enzymes elongate PG independent of MreB filaments. PG elongation was visualized using BADA. **c)** Heterologous expression of $MreB_{Mx}$ recruits *E. coli* PBP2 to cell poles. **d)** In the absence of RodZ, $MreB_{Ec}$ still forms aggregates at pole-like locations (white arrows) but fails to induce focused PG growth. PG elongation was visualized using BADA. **e)** The reprogrammed, polar PG elongation does not depend on the divisome as FtsZ does not colocalize with polar MreB aggregates.

aggregates fail to induce focused PG elongation. Taken together, in the reprogrammed, polar-elongating cells (BN04, BN05, and BN06), $MreB_{Ec}$ aggregates recruit the Rod polymerases to poles through RodZ.

Besides the Rod system and aPBPs, a third machinery, the divisome, specifically assembles PG at the division site. To further confirm that the divisome does not significantly contribute to polar PG elongation, we visualized the localization of FtsZ, the cytoskeletal element that orchestrates the PG synthases in the divisome, by expressing a GFP-labeled FtsZ as merodiploid [50] in the NO59 ($mreB_{Ec}$-cfp^{sw}) and BN05 ($mreB_{Ec}$-cfp^{sw} pMAT3-$mreB_{Mx}$) backgrounds. In both strains, FtsZ-GFP forms bright bands in the center of cells, marking the current or future division sites (Fig. 3e). Notably, in the BN05 background, FtsZ did not colocalize with the polar $MreB_{Ec}$ aggregates. Thus, the divisome does not contribute to the polar PG elongation. On the other hand, the reprogrammed PG elongation does not change the mode of cell division. Taken together, the polar localized Rod system is both sufficient and necessary for focused PG elongation at poles.

Targeting PBP2 to cell poles is sufficient to drive polar PG elongation

To determine whether *E. coli*'s elongation mode could be reprogrammed by directly targeting Rod polymerases to cell poles, we modified the PopZ-Linked Apical Recruitment (POLAR) system [51] to express a PopZ-H3H4-PBP2 fusion *in trans* in the NO59 strain with an arabinose-inducible promoter using the pHCL149 plasmid [51]. In this fusion, PopZ from *C. crescentus* has been proven to localize to *E. coli* cell poles while the H3H4 homo-oligomerization domain promotes targeting PBP2 to polar PopZ clusters [52]. Prior to arabinose induction, 95.93% (n = 123) of cells exhibited dispersed PG elongation and $MreB_{Ec}$ distribution (Fig. 4a). Upon induction, 92.06% (n = 126) of cells displayed polar PG elongation that colocalized with $MreB_{Ec}$ aggregates (Fig. 4a). These results indicate that targeting a critical amount of PBP2 to cell poles is sufficient to shift *E. coli* growth to polar elongation. In addition, the fact that $MreB_{Ec}$ forms polar aggregates following PBP2 indicates that PBP2 can regulate the localization of MreB.

Polar PG elongation does not require MreB filaments

Because cells continue to elongate PG at their poles despite most $MreB_{Ec}$ being sequestered in aggregates, polar PG elongation requires few, if any, $MreB_{Ec}$ filaments. To clarify the roles of MreB filaments in polar elongation, we first treated cells with A22 that inhibits MreB polymerization [18, 53]. In the NO59 cells that expressed $MreB_{Ec}$-$msfCFP^{SW}$, A22 (10 μg/ml) abolished rod shape and dispersed $MreB_{Ec}$ foci within 1 h, albeit cells continued to incorporate BADA, likely through aPBPs (Fig. 3a). These results confirm numerous reports that MreB filaments are essential for rod shape when cells adopt nonpolar PG elongation [13, 25, 54].

In stark contrast, in the BN04 cells that expressed both $MreB_{Ec}$-$msfCFP^{SW}$ and $MreB_{Mx}$-$PAmCherry^{SW}$, both MreB homologs remained in polar aggregates in the presence of A22 and cells continued polar growth while maintaining rod shape (Fig. 3b). Similarly, when PBP2 was artificially targeted to the poles in the BN10 cells, cells displayed similar resistance against A22, with both $MreB_{Ec}$ and PG elongation focused on cell poles (Fig. 4a, b). Taken together, once cells switch their growth sites to cell poles, the Rod enzymes carry out PG elongation independent of MreB filaments.

Focused PG elongation depends on pre-established cell poles to preserve rod shape

To test whether the MreB protein is still required for focused PG elongation, we acquired the strain ATM1504 that carries an *mreB* deletion but expresses SdiA to suppress *ΔmreB* lethality [55]. The ATM1504 cells grew as spheres and incorporated BADA near homogeneously on their surfaces (Fig. 4c). We constructed the BN11 strain to express the PopZ-H3H4-PBP2 fusion *in trans* in the ATM1504 strain with an arabinose-inducible promoter. After 12 h of induction with 0.4% arabinose followed by 15 min of BADA staining, all cells remained spherical.

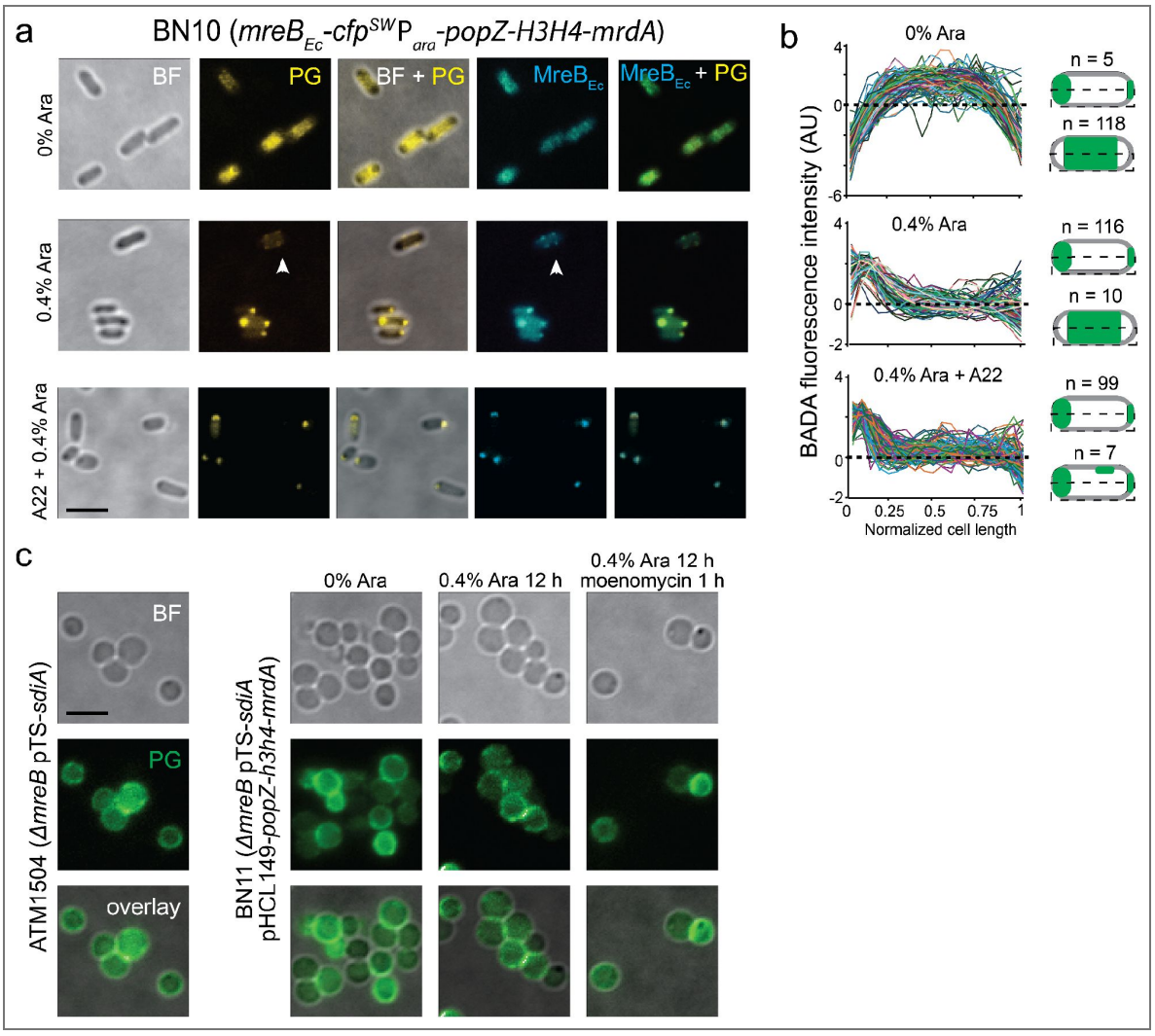

Fig. 4. Targeting PBP2 to cell poles is sufficient to drive polar PG elongation.

a) When PBP2 (encoded by *mrdA*) is ectopically expressed with a polar-targeting tag (PopZ-H3H4) by an arabinose-inducible promoter under 0.4% arabinose (Ara), $MreB_{Ec}$ aggregates at cell poles and cells switch to polar PG elongation and this reprogrammed growth mode is resistant to A22 (10 μg/ml). The white arrow points to a cell that still elongated PG at nonpolar sites. **b)** Quantitative analysis of PG elongation. Cell lengths were normalized to 1 and BADA fluorescence intensity profiles were standardized using Z-score normalization (0 indicates the mean value for each cell). Insets illustrate patterns of PG elongation, with newly synthesized PG shown in green. The numbers of cells that adopt each elongation pattern were presented above the insets. Imaged cell regions are highlighted by dotted-line boxes. **c)** Expressing pole-targeting PBP2 in the absence of $MreB_{Ec}$ neither induces focused PG elongation (labeled by BADA) nor restores rod-shape, despite that Rod enzymes still incorporate BADA. aPBPs were inhibited by moenomycin (MOE, 4 μg/ml) for 1 h before adding BADA for 15 min. Scale bars, 5 μm.

Two possibilities could account for these observations. First, Rod enzymes may be nonfunctional in the absence of MreB, such that the remaining aPBPs are insufficient to generate or maintain rod shape. Second, PopZ–H3H4–PBP2 does not form stable aggregates without pre-established poles. To test these possibilities, we induced PopZ– H3H4–PBP2 expression in BN11 cells with 0.4% arabinose for 12 h, followed by treatment with moenomycin (4 μg/ ml). After 1 h of inhibition, newly synthesized PG was labeled with BADA for 15 min. Despite inhibition of all aPBPs by moenomycin, induced BN11 cells continued to incorporate BADA (Fig. 4c), indicating that Rod enzymes remain active in the absence of $MreB_{Ec}$ and thereby ruling out the first possibility. Thus, without a mechanism that retains Rod enzymes to specific locations, cells cannot initiate focused PG elongation.

Discussion

Although spherical shapes are energetically efficient and favored by physical principles, growing evidence suggests that the last common ancestor of modern bacteria was rod-shaped [37], and that the evolution of rod-like morphology likely coincided with the emergence of PG [56]. The rigidity of PG is a double-edged sword. It provides sufficient mechanical support for cells but also poses a major hurdle for cell growth and division. Because the entire PG layer is a single molecule, cell growth and division rely on the modification on the existing PG scaffold. To preserve cell width homeostasis while continuously remodeling the cylindrical PG, the elongation machinery in rod-shaped bacteria must fine tune its activity in response to subtle variations in local curvature. Conserved in most rods but absent in most spheres, MreB is an ancient protein that plays critical roles in rod-like morphogenesis [13, 54]. MreB is essential in nearly all rod-shaped bacteria that utilize dispersed, nonpolar elongation because it functions as an effective curvature sensor and dynamic regulator. As MreB filaments move in concert with the Rod polymerases RodA and PBP2, they align along regions of highest membrane curvature, thereby refining the spatial positioning of the elongation machinery [20-22].

In this study, we reprogrammed *E. coli*, the most thoroughly studied rod-shaped bacteria that elongates its PG laterally in a dispersed fashion, to adopt polar elongation—a mode typically restricted to distantly related species. By targeting a single core component of the Rod system—MreB or PBP2—we successfully redirected the entire system to the cell poles, thereby shifting PG elongation to the poles. In this case, as the nonpolar region is no longer the growth zone, the established cylindrical PG structure is sufficient to maintain cell width, where MreB filaments become nonessential. This cross-phylum shift of growth mode highlights the evolutionary plasticity of bacterial morphogenetic systems and suggests that elongation modes, though often conserved within lineages, can be rewired through targeted manipulation of core components.

Due to technical limitations, we cannot exclude the possibility that the reprogrammed, pole-elongating *E. coli* strains still incorporate trace amounts of PG in nonpolar regions—just as we cannot rule out that bacteria growing in nonpolar mode also insert small amounts of PG at the poles. These elongation modes are not necessarily mutually exclusive; rather, individual bacteria may employ a specific blend of both polar and dispersed growth strategies such as recently discovered in *Caulobacteraceae* and *Streptomyces* [3, 4]. However, these two elongation modes may rely on distinct mechanisms to maintain rod shape. Dispersed, nonpolar elongation depends critically on MreB filaments. In *E. coli* and *B. subtilis*, PG-depleted spheroplasts can spontaneously regenerate rod shape through curvature-dependent localization of MreB filaments [21, 57]. Similarly, spherical, PG-free *M. xanthus* spores employ polarity regulators to monitor PG assembly and direct molecular motors that transport MreB filaments—and associated Rod enzymes—away from future poles [35, 36]. By contrast, we propose that in the absence of MreB-mediated modulation, focused PG elongation can sustain rod shape only when cell poles are already established. Without poles, the uniform membrane curvature of spherical cells cannot spatially constrain focused PG synthesis, necessitating alternative polarity determinants such as FtsA, FtsZ, or DivIVA.

An unexpected result is that moenomycin, which inhibits aPBPs, causes $MreB_{Ec}$ filaments to disperse (Fig. 3a). This effect is not likely due to the loss of rod shape because $MreB_{Ec}$ still forms filaments in the spherical *ΔrodZ* cells (Fig. 3d). Rather than affecting PG directly, moenomycin locks aPBPs in their glycan-charged conformation [58, 59]. Using *M. xanthus*, we discovered that one of the moenomycin-bound aPBPs activates the endopeptidase DacB, which in turn, modifies PG and turns cells into spheres [17]. As endopeptidases are space-makers for the TPases in both PBP2 in the Rod system and aPBPs [1, 60, 61], moenomycin-bound aPBPs could modulate PBP2 in the Rod system through DacB-like endopeptidase in *E. coli*. Such a mechanism also implies that, beyond determining the localization of the entire Rod system [62], PBP2 may also regulate MreB filamentation. In support of this hypothesis, we found that blocking PBP2 directly with mecillinam disperses MreB filament in a similar manner (Fig. 3a).

Members of the Rhizobiales and Actinomycetales orders, having lost MreB, maintain rod-like morphology. Strikingly, both orders elongate dominantly from cell poles. Similar to our polar-elongating *E. coli* strains, the established nonpolar regions of these bacteria are seldom modified and thus do not require MreB-like cytoskeleton to maintain cell width homeostasis. Given that MreB is an ancient protein believed to have existed prior to the emergence of *Rhizobiales* and *Actinomycetales* [37], it is plausible that polar growth may have evolved from dispersed elongation as a consequence of MreB loss, which in Rhizobiales and Actinomycetales allows the repurposing of FtsA and FtsZ in the former, and the acquisition of DivIVA in the latter to recruit PG elongation machineries to cell poles [31, 32]. Notably, *Streptomyces venezuelae* can combine MreB-independent polar growth and MreB-dependent dispersed growth [4], which may represent an evolutionary intermediate as reliance on MreB diminished.

Materials and Methods

Strains and growth conditions

Bacterial strains, plasmids, and primers used in this study are listed in Table S1. *E. coli* strains were grown overnight in liquid LB medium with the appropriate antibiotic at 37 °C.

For fluorescent D-amino acid (**FDAA**, including BADA, HADA, and TADA) staining experiments, cells were incubated for 20 min at 37 °C in LB media supplemented with 60 μM FDAA. Cells were collected (9,000 g, 1 min) washed three times with liquid LB and resuspended in ⅕ of the original volume. For sequential staining, cells were incubated with 60 μM of the first FDAA for 15 min, washed three times with liquid LB, incubated with 60 μM the second FDAA for 15 min, and washed three times with liquid LB before imaging. To determine the roles of aPBPs, the Rod system, and MreB in PG elongation, moenomycin (10 μg/ml), mecillinam (100 μg/ml), and A22 (10 μg/ml) was added, respectively, to the culture in liquid LB media 1h before adding FDAA.

Cryo-EM

E. coli cultures were grown in liquid LB medium to OD_{600} ~1. Cells were collected by centrifugation (3,000 g, 2 min) and resuspended in LB to a final OD_{600} of 12. 3 μl of the cell suspension was applied to a 1.2/1.3 200 mesh copper grid (Quantifoil) that were glow-discharged for 30 s at 15 mA. Grids were plunge-frozen in liquid ethane with an FEI Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C and 100% humidity, with a waiting time of 30 s, two-side blotting time of 2.5 - 3 s, and blotting force of 0. All subsequent grid handling and transfers were performed in liquid nitrogen. Images were acquired using a Thermo Fisher Scientific Titan Krios G4 transmission electron microscope.

Live cell imaging

Overnight *E. coli* cultures were diluted to OD_{600} 0.1 in fresh liquid LB medium and grown at 37 °C to OD_{600} 0.4 - 0.6 before imaging. For all imaging experiments, we spotted 5 μl of culture on agar (1.5%) pads. For the treatments with antibiotics, antibiotics were added to both the cell suspension and agar pads. The length and width of cells were determined from differential interference contrast (**DIC**) images using a MATLAB (MathWorks) script [17, 29, 35, 64]. The fluorescence of CFP,

Strain	Description	Source
DH5α	*E. coli* strain for molecular cloning	Lab collection
MG1655	Wild-type *E. coli* strain	Lab collection
BW25113	Wild-type *E. coli* strain	Lab collection
NO59	Kan^R, MG1655 *mreB-msfcfp*^{SW}	[42]
NO56	Kan^R, cam^R, MG1655 *ΔrodZ mreB-msfcfp*^{SW}	[42]
TKL130	MG1655 *mrdA (pbp2)-PAmCherry*	[49]
BN01	Tet^R, DH5α pMAT3-*mreB*$_{Mx}$	This study
BN02	Tet^R, MG1655 pMAT3-*mreB*$_{Mx}$	This study
BN03	Tet^R, BW25113 pMAT3-*mreB*$_{Mx}$	This study
BN04	Kan^R, Tet^R, NO59 pMAT3-*mreB*$_{Mx}$-*PAmCherry*^{SW}	This study
BN05	Kan^R, Tet^R, NO59 pMAT3-*mreB*$_{Mx}$	This study
BN06	Tet^R, TKL130 pMAT3-*mreB*$_{Mx}$	This study
BN07	Tet^R, NO56 pMAT3-*mreB*$_{Mx}$	This study
BN08	Cam^R, NO59 pXY027 (*ftsZ-gfp*)	This study
BN09	Tet^R, Cam^R, BN08 pMAT3-*mreB*$_{Mx}$	This study
BN10	Kan^R, Cm^R, NO59 pHCL149-*popZ-h3h4-mrdA (pbp2)*	This study
ATM1504	MG1655 *ΔmreB::kan*/pTS-*sdiA*	[55]
BN11	Kan^R, Cm^R, ATM1504 pHCL149-*popZ-h3h4-mrdA (pbp2)*	This study
DZ2	Wild-type *M. xanthus* strain	[63]
Plasmid	**Description**	**Source**
pMAT3	Tet^R, the plasmid for protein expression in *M. xanthus*	[38]
pHCL149	Cm^R, the plasmid for directing proteins to *E. coli* cell poles	[51]
pMAT3-*mreB*$_{Mx}$	Tet^R, pMAT3-*mreB*$_{Mx}$	This study
pMAT3-*mreB*$_{Mx}$-*PAmCh*^{SW}	Tet^R, pMAT3-*mreB*$_{Mx}$-*PAmCherry*^{SW}	This study
pXY027	Plasmid carrying *ftsZ-gfp*	[50]
pHCL149-*mreB*$_{Ec}$	Cm^R, pHCL149-*popZ-h3h4-mreB*$_{Ec}$	This study
pHCL149-*rodA*	Cm^R, pHCL149-*popZ-h3h4-mrdB (rodA)*	This study
pHCL149-*pbp2*	Cm^R, pHCL149-*popZ-h3h4-mrdA (pbp2)*	This study
Primers	**Sequence**	**Source**
Cloning *mreB*$_{Mx}$ into pMAT3	tctagaTTTGACTGGCTTCACACCCTCTTC aagctTCAGCCCGGCTGGCAGACCTGCC	This study
Cloning *pbp2* (*mrdA*) into pHCL149	ggatccATGAAACTACAGAACTCTTTTC aagcttTTAATGGTCCTCCGCTG	This study

Table S1. **Bacterial strains, plasmids, and primers used in this study.**

BADA, and TADA was excited by different lasers: CFP and HADA at 405 nm, GFP and BADA at 488 nm, and mCherry and TADA at 561 nm. For $MreB_{Mx}$-$PAmCherry^{SW}$ and PBP2-PAmCherry, PAmCherry was activated using a 405-nm laser (0.3 kW/cm^2, 2 s), excited and imaged using a 561-nm laser (0.2 kW/cm^2). DIC and fluorescence images of cells were captured using an Andor iXon Ultra 897 EMCCD camera (effective pixel size 160 nm) on an inverted Nikon Eclipse-Ti™ microscope with a 100× 1.49 NA TIRF objective under HILO illumination [29, 35, 45, 46].

Immunoblotting

The expression of $MreB_{Mx}$ was determined by immunoblotting following SDS-PAGE using a polyclonal anti-$MreB_{Mx}$ serum [40] and a goat anti-Rabbit IgG (H+L) secondary antibody, HRP (Thermo Fisher Scientific, catalog # 31460). The blots were developed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific REF 32109) and a MINI-MED 90 processor (AFP Manufacturing).

Data availability

Source data will be provided for Fig. 1f, 2b, 2e, 4b.

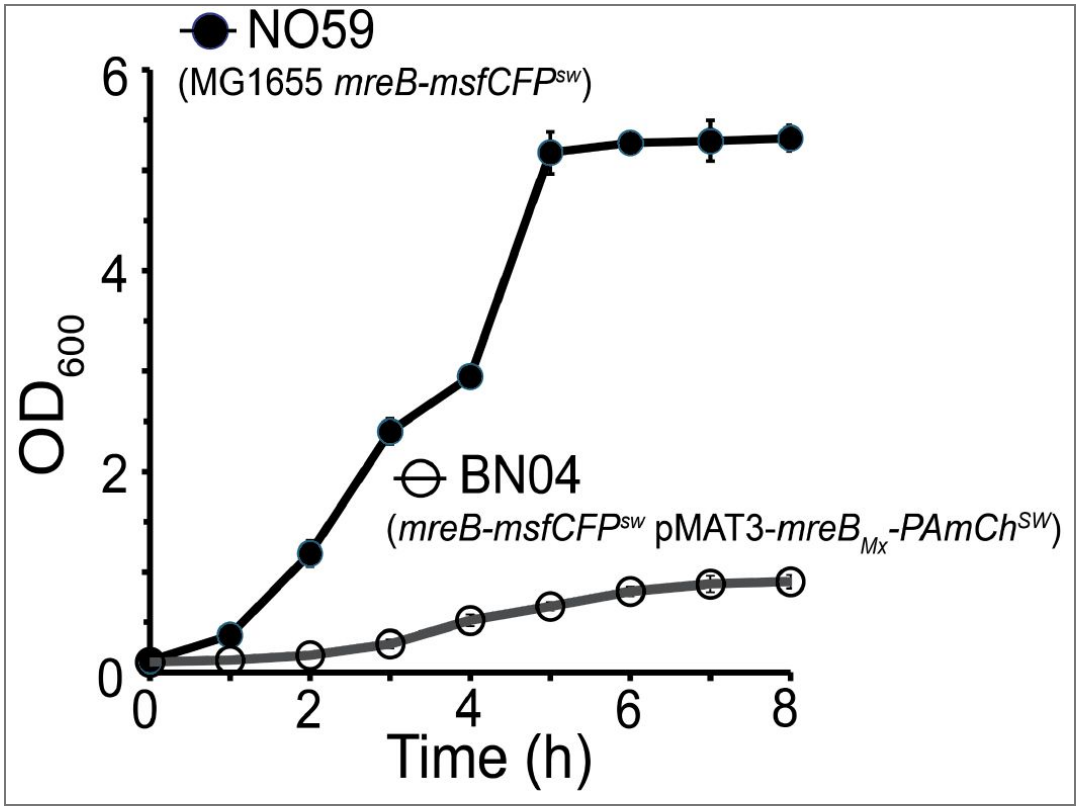

Fig. S1. BN04 cells grow slower than its parental strain NO59. Averages and standard deviations were calculated from three technical repeats.

Acknowledgements

We thank Drs. Michael VanNieuwenhze and Yen-Pang Hsu for providing TADA and HADA, Drs. Randy Morgenstein, KC Huang, and Anthony Maurelli for sharing the strains NO59, NO56, TKL130, and ATM1504 and Dr. Gaya Yadav for the assistance on cryoEM imaging. Part of this work was supported by the National Institutes of Health grants GM129000 to B. N.. We received financial support from Dr. David R. Zusman, who played no role in the design, execution, or presentation of this work.

Additional information

Author ORCID iDs

Beiyan Nan: https://orcid.org/0000-0002-0326-9529

References

1. **Holtje JV** (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. *Microbiol Mol Biol Rev* **62**:181-203 https://doi.org/10.1128/mmbr.62.1.181-203.1998 | PubMed
2. **Rohs PDA,** Bernhardt TG (2021) Growth and Division of the Peptidoglycan Matrix. *Annu Rev Microbiol* **75**:315-336 https://doi.org/10.1146/annurev-micro-020518-120056 | PubMed
3. **Delaby M,** et al. (2025) Phenotypic plasticity in cell elongation among closely related bacterial species. *Nat Commun* **16**:5099 https://doi.org/10.1038/s41467-025-60005-y | PubMed
4. **Zambri MP,** et al. (2025) Streptomyces uses both polar and dispersed cell wall synthesis during exploratory growth. *Nat Microbiol* https://doi.org/10.1038/s41564-025-02080-x | PubMed
5. **Brown PJ,** et al. (2012) Polar growth in the Alphaproteobacterial order Rhizobiales. *Proc Natl Acad Sci U S A* **109**:1697-701 https://doi.org/10.1073/pnas.1114476109 | PubMed
6. **Cameron TA,** Zupan JR, Zambryski PC (2015) The essential features and modes of bacterial polar growth. *Trends Microbiol* **23**:347-53 https://doi.org/10.1016/j.tim.2015.01.003 | PubMed
7. **Chung ES,** et al. (2024) Single-cell imaging of the Mycobacterium tuberculosis cell cycle reveals linear and heterogenous growth. *Nat Microbiol* **9**:3332-3344 https://doi.org/10.1038/s41564-024-01846-z | PubMed
8. **Vollmer W,** Blanot D, de Pedro MA (2008) Peptidoglycan structure and architecture. *FEMS Microbiol Rev* **32**:149-67 https://doi.org/10.1111/j.1574-6976.2007.00094.x | PubMed
9. **de Pedro MA,** Cava F (2015) Structural constraints and dynamics of bacterial cell wall architecture. *Front Microbiol* **6**:449 https://doi.org/10.3389/fmicb.2015.00449 | PubMed
10. **Egan AJF,** Errington J, Vollmer W (2020) Regulation of peptidoglycan synthesis and remodelling. *Nat Rev Microbiol* **18**:446-460 https://doi.org/10.1038/s41579-020-0366-3 | PubMed
11. **Meeske AJ,** et al. (2016) SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* **537**:634-638 https://doi.org/10.1038/nature19331 | PubMed
12. **Cho H,** et al. (2016) Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. *Nat Microbiol* 16172 https://doi.org/10.1038/nmicrobiol.2016.172 | PubMed
13. **Garner EC** (2021) Toward a Mechanistic Understanding of Bacterial Rod Shape Formation and Regulation. *Annu Rev Cell Dev Biol* **37**:1-21 https://doi.org/10.1146/annurev-cellbio-010521-010834 | PubMed
14. **Vigouroux A,** et al. (2020) Class-A penicillin binding proteins do not contribute to cell shape but repair cell-wall defects. *eLife* **9** https://doi.org/10.7554/elife.51998 | PubMed

15. **Denome SA,** et al. (1999) Escherichia coli mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. *J Bacteriol* **181**:3981-93 https://doi.org/10.1128/jb.181.13.3981-3993.1999 | PubMed

16. **McPherson DC,** Popham DL (2003) Peptidoglycan synthesis in the absence of class A penicillin-binding proteins in Bacillus subtilis. *J Bacteriol* **185**:1423-31 https://doi.org/10.1128/jb.185.4.1423-1431.2003 | PubMed

17. **Zhang H,** et al. (2023) Coordinated peptidoglycan synthases and hydrolases stabilize the bacterial cell wall. *Nat Commun* **14**:5357 https://doi.org/10.1038/s41467-023-41082-3 | PubMed

18. **van den Ent F,** et al. (2014) Bacterial actin MreB forms antiparallel double filaments. *eLife* **3**:e02634 https://doi.org/10.7554/eLife.02634 | PubMed

19. **Salje J,** et al. (2011) Direct membrane binding by bacterial actin MreB. *Mol Cell* **43**:478-87 https://doi.org/10.1016/j.molcel.2011.07.008 | PubMed

20. **Ursell TS,** et al. (2014) Rod-like bacterial shape is maintained by feedback between cell curvature and cytoskeletal localization. *Proc Natl Acad Sci U S A* **111**:E1025-34 https://doi.org/10.1073/pnas.1317174111 | PubMed

21. **Hussain S,** et al. (2018) MreB filaments align along greatest principal membrane curvature to orient cell wall synthesis. *eLife* **7** https://doi.org/10.7554/elife.32471 | PubMed

22. **Wong F,** Garner EC, Amir A (2019) Mechanics and dynamics of translocating MreB filaments on curved membranes. *eLife* **8** https://doi.org/10.7554/elife.40472 | PubMed

23. **Bratton BP,** et al. (2018) MreB polymers and curvature localization are enhanced by RodZ and predict E. coli's cylindrical uniformity. *Nat Commun* **9**:2797 https://doi.org/10.1038/s41467-018-05186-5 | PubMed

24. **Kruse T,** et al. (2003) Dysfunctional MreB inhibits chromosome segregation in Escherichia coli. *Embo J* **22**:5283-92 https://doi.org/10.1093/emboj/cdg504 | PubMed

25. **Maharjan S,** et al. (2026) Alanine-scanning mutagenesis library of MreB reveals distinct roles for regulating cell shape and viability. *PLoS Genet* **22**:e1012070 https://doi.org/10.1371/journal.pgen.1012070 | PubMed

26. **Figge RM,** Divakaruni AV, Gober JW (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus. *Mol Microbiol* **51**:1321-32 https://doi.org/10.1111/j.1365-2958.2003.03936.x | PubMed

27. **Robertson GT,** et al. (2007) A Novel indole compound that inhibits Pseudomonas aeruginosa growth by targeting MreB is a substrate for MexAB-OprM. *J Bacteriol* **189**:6870-81 https://doi.org/10.1128/jb.00805-07 | PubMed

28. **Jones LJ,** Carballido-Lopez R, Errington J (2001) Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell* **104**:913-22 https://doi.org/10.1016/s0092-8674(01)00287-2 | PubMed

29. **Fu G,** et al. (2018) MotAB-like machinery drives the movement of MreB filaments during bacterial gliding motility. *Proc Natl Acad Sci U S A* **115**:2484-2489 https://doi.org/10.1073/pnas.1716441115 | PubMed

30. **Kysela DT,** et al. (2013) Biological consequences and advantages of asymmetric bacterial growth. *Annu Rev Microbiol* **67**:417-35 https://doi.org/10.1146/annurev-micro-092412-155622 | PubMed

31. **Cameron TA,** et al. (2014) Peptidoglycan synthesis machinery in Agrobacterium tumefaciens during unipolar growth and cell division. *mBio* **5**:e01219-14 https://doi.org/10.1128/mbio.01219-14 | PubMed

32. **Letek M,** et al. (2008) DivIVA is required for polar growth in the MreB-lacking rod-shaped actinomycete Corynebacterium glutamicum. *J Bacteriol* **190**:3283-92 https://doi.org/10.1128/jb.01934-07 | PubMed

33. **Waite DW,** et al. (2020) Proposal to reclassify the proteobacterial classes Deltaproteobacteria and Oligoflexia, and the phylum Thermodesulfobacteria into four phyla reflecting major functional capabilities. *Int J Syst Evol Microbiol* **70**:5972-6016 https://doi.org/10.1099/ijsem.0.004213 | PubMed

34. **de Pedro MA,** et al. (1997) Murein segregation in Escherichia coli. *J Bacteriol* **179**:2823-34 https://doi.org/10.1128/jb.179.9.2823-2834.1997 | PubMed

35. **Zhang H,** et al. (2020) Establishing rod shape from spherical, peptidoglycan-deficient bacterial spores. *Proc Natl Acad Sci U S A* **117**:14444-14452 https://doi.org/10.1073/pnas.2001384117 | PubMed

36. **Zhang H,** Venkatesan S, Nan B (2021) Myxococcus xanthus as a Model Organism for Peptidoglycan Assembly and Bacterial Morphogenesis. *Microorganisms* **9** https://doi.org/10.3390/microorganisms9050916 | PubMed

37. **Yulo PRJ,** Hendrickson HL (2019) The evolution of spherical cell shape; progress and perspective. *Biochem Soc Trans* **47**:1621-1634 https://doi.org/10.1042/bst20180634 | PubMed

38. **Gomez-Santos N,** et al. (2012) Comprehensive set of integrative plasmid vectors for copper-inducible gene expression in Myxococcus xanthus. *Appl Environ Microbiol* **78**:2515-21 https://doi.org/10.1128/aem.07502-11 | PubMed

39. **Rinas U,** et al. (2017) Bacterial Inclusion Bodies: Discovering Their Better Half. *Trends in Biochemical Sciences* **42**:726-737 https://doi.org/10.1016/j.tibs.2017.01.005 | PubMed

40. **Mauriello EM,** et al. (2010) Bacterial motility complexes require the actin-like protein, MreB and the Ras homologue, MglA. *Embo J* **29**:315-26 https://doi.org/10.1038/emboj.2009.356 | PubMed

41. **Aramayo R,** Nan B (2022) De Novo Assembly and Annotation of the Complete Genome Sequence of Myxococcus xanthus DZ2. *Microbiol Resour Announc* e0107421 https://doi.org/10.1128/mra.01074-21 | PubMed

42. **Morgenstein RM,** et al. (2015) RodZ links MreB to cell wall synthesis to mediate MreB rotation and robust morphogenesis. *Proc Natl Acad Sci U S A* https://doi.org/10.1073/pnas.1509610112 | PubMed

43. **Hsu YP,** et al. (2017) Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem Sci* **8**:6313-6321 https://doi.org/10.1039/c7sc01800b | PubMed

44. **Kuru E,** et al. (2012) In Situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent D-amino acids. *Angew Chem Int Ed Engl* **51**:12519-23 https://doi.org/10.1002/anie.201206749 | PubMed

45. **Nan B,** et al. (2015) The polarity of myxobacterial gliding is regulated by direct interactions between the gliding motors and the Ras homolog MglA. *Proc Natl Acad Sci U S A* **112**:E186-93 https://doi.org/10.1073/pnas.1421073112 | PubMed

46. **Toyonaga T,** et al. (2021) Chained Structure of Dimeric F(1)-like ATPase in Mycoplasma mobile Gliding Machinery. *mBio* **12**:e0141421 https://doi.org/10.1128/mbio.01414-21 | PubMed

47. **Emami K,** et al. (2017) RodA as the missing glycosyltransferase in Bacillus subtilis and antibiotic discovery for the peptidoglycan polymerase pathway. *Nat Microbiol* **2**:16253 https://doi.org/10.1038/nmicrobiol.2016.253 | PubMed

48. **Spratt BG** (1975) Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12. *Proc Natl Acad Sci U S A* **72**:2999-3003 https://doi.org/10.1073/pnas.72.8.2999 | PubMed

49. **Lee TK,** et al. (2014) A dynamically assembled cell wall synthesis machinery buffers cell growth. *Proc Natl Acad Sci U S A* **111**:4554-9 https://doi.org/10.1073/pnas.1313826111 | PubMed

50. **Buss J,** et al. (2015) A multi-layered protein network stabilizes the Escherichia coli FtsZ-ring and modulates constriction dynamics. *PLoS Genet* **11**:e1005128 https://doi.org/10.1371/journal.pgen.1005128 | PubMed

51. **Lim HC,** Bernhardt TG (2019) A PopZ-linked apical recruitment assay for studying protein-protein interactions in the bacterial cell envelope. *Mol Microbiol* **112**:1757-1768 https://doi.org/10.1111/mmi.14391 | PubMed

52. **Laloux G,** Jacobs-Wagner C (2013) Spatiotemporal control of PopZ localization through cell cycle-coupled multimerization. *J Cell Biol* **201**:827-41 https://doi.org/10.1083/jcb.201303036 | PubMed

53. **Bean GJ,** et al. (2009) A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB. *Biochemistry* **48**:4852-7 https://doi.org/10.1021/bi900014d | PubMed

54. **Shi H,** et al. (2018) How to Build a Bacterial Cell: MreB as the Foreman of E. coli Construction. *Cell* **172**:1294-1305 https://doi.org/10.1016/j.cell.2018.02.050 | PubMed

55. **Ranjit DK,** Liechti GW, Maurelli AT (2020) Chlamydial MreB Directs Cell Division and Peptidoglycan Synthesis in Escherichia coli in the Absence of FtsZ Activity. *mBio* **11** https://doi.org/10.1128/mbio.03222-19 | PubMed

56. **Errington J** (2013) L-form bacteria, cell walls and the origins of life. *Open Biol* **3**:120143 https://doi.org/10.1098/rsob.120143 | PubMed

57. **Billings G,** et al. (2014) De novo morphogenesis in L-forms via geometric control of cell growth. *Mol Microbiol* **93**:883-96 https://doi.org/10.1111/mmi.12703 | PubMed

58. **Lovering AL,** et al. (2007) Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. *Science* **315**:1402-5 https://doi.org/10.1126/science.1136611 | PubMed

59. **Sung MT,** et al. (2009) Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from Escherichia coli. *Proc Natl Acad Sci U S A* **106**:8824-9 https://doi.org/10.1073/pnas.0904030106 | PubMed

60. **Singh SK,** et al. (2012) Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of Escherichia coli K12. *Mol Microbiol* **86**:1036-51 https://doi.org/10.1111/mmi.12058 | PubMed

61. **Dorr T,** et al. (2013) Substrate specificity of an elongation-specific peptidoglycan endopeptidase and its implications for cell wall architecture and growth of Vibrio cholerae. *Mol Microbiol* **89**:949-62 https://doi.org/10.1111/mmi.12323 | PubMed

62. **Ozbaykal G,** et al. (2020) The transpeptidase PBP2 governs initial localization and activity of the major cell-wall synthesis machinery in E. coli. *eLife* **9** https://doi.org/10.7554/elife.50629 | PubMed

63. **Campos JM,** Zusman DR (1975) Regulation of Development in Myxococcus-xanthus - Effect of 3' Doublebond 5'-Cyclic Amp, Adp, and Nutrition. *Proceedings of the National Academy of Sciences of the United States of America* **72**:518-522 https://doi.org/10.1073/pnas.72.2.518 | PubMed

64. **Zhang H,** et al. (2023) Coordinated peptidoglycan synthases and hydrolases stabilize the bacterial cell wall. *Nat Commun* https://doi.org/10.1038/s41467-023-41082-3 | PubMed

Peer reviews

Reviewer #1 (Public review):

This is an interesting paper, with the primary finding being that localizing MreB or PBP2 to the cell poles in E. coli is primarily demonstrated via an aggregate formed by expressing M. xanthus MreB.

I have 2 main concerns:

(1) First, the authors should clarify and adjust their interpretation of FDAA incorporation: As written, the authors interpret FDAA incorporation as being caused by incorporation during PG polymerization. However, in E. coli, FDAA incorporation does not result from the elongation of PG strands or their initial 4-3 crosslinking by DD transpeptidases following polymerization, but rather by the remodeling of L,D-transpeptidases.

Thus, it is not accurate to refer to the FDAA incorporation as PG elongation, but rather the modification of crosslinks from 4-3 to 3-3 crosslinks at that location. To claim a link to PG polymerization, other experiments or substantial explanations are needed.

(E. coli Cells Incorporate FDAAs by L,D-TPases in a Growth Independent Manner) - *https://doi.org/10.1021/acschembio*.

(2) Second, the evidence provided that this is polar elongation is not sufficient to prove elongation. In all images claiming polar growth, the FDAA focus appears as a single spot, which corresponds to the MreB aggregate visible in bright-field. That might indicate incorporation, but it does not demonstrate polar elongation. To prove this, the authors should do different-length pulses of FDAAs and demonstrate an increasing length of labeled PG along the cell. Cells with one focus should show increasing length; polar foci should elongate from both ends. I find the current 2-color labeling insufficient, as BADA labels the entire cell.

Small points:

(3) Lines 350- 302: "In this case, as the nonpolar region is no longer the growth zone, the established cylindrical PG structure is sufficient to maintain cell width, where MreB filaments become nonessential." Does polar elongation give robustness to rod shape? The authors should include an analysis of cell width and its variation within a cell and between cells.

(4) In the abstract: "This reprogrammed growth mode bypasses the requirement for MreB filaments, highlighting a plasticity of the Rod system that suggests polar elongation may have emerged through the evolutionary loss of MreB." This argument should not be made without evolutionary analysis or reference to such work indicating this is the case.

(5) 365-367 "PG-depleted spheroplasts can spontaneously regenerate rod shape through curvature-dependent localization of MreB filaments [21, 57]". This should be amended. The Billing paper did indeed study spheroplasts, but the Hussain paper used teichoic acid-depleted cells that still had a cell wall.

https://doi.org/10.7554/eLife.112612.1.sa3

Reviewer #2 (Public review):

Summary:

Based on observations of localisation of MreBEc at the poles within an aggregate-like structure, upon heterologous expression of MreBMx, the authors set out to investigate how this non-canonical localisation of MreBs leads to a reprogramming of peptidoglycan synthesis to the poles. This is analogous to the polar growth observed in phyla which are not dependent on dispersed growth of PG, but only at the poles, and are MreB independent.

The authors proceed to establish that PG synthesis is MreB-dependent, Rod enzyme-dependent, and requires the prior establishment of a pole.

Strengths:

(1) It is a very interesting idea to design experiments to demonstrate reprogramming of non-polar to polar growth based on the observation of localisation of a heterologously expressed MreB.

(2) The experiments to demonstrate the factors that determine polar growth and the observation of the PG in each of these experimental situations are convincing.

(3) I find the observation of an extra layer of PG in the heterologously expressed system very intriguing. It will be interesting to see if this layer merges with the other PG layer at some stage or branches from the non-polar growth near the poles.

Weaknesses:

(1) It is not clear what exactly the identity of the polar aggregates is and how much of this activity is an artefact of partially functional MreBs.

(2) I find it intriguing that the localisation and growth are predominantly at one pole only. It is unclear to me how this can be reconciled with growth and shape maintenance, and an increase in length and width. Is the increase in length and width a consequence of misshapen cells that are bulged in the absence of a normal PG layer?

(3) The authors do not follow up on the observations in the first figure on the length and width changes and the extra peptidoglycan layer (which I feel are the most interesting aspects), and how this can be connected to the polar growth observed in the later sections of the manuscript.

(4) The claim that this could be a precursor of an MreB-independent polar growth mechanism appears to be a bit far-fetched, because the system is still dependent on having an established pole for PG synthesis to occur in the new place.

https://doi.org/10.7554/eLife.112612.1.sa2

Reviewer #3 (Public review):

Summary:

Most rod-shaped bacteria grow by one of two mechanisms: growth from the pole or growth from the midcell. It is rare for a single species to utilize both modes of growth, although a few examples do exist. Here, the authors have artificially induced E. coli cells to grow from the poles, either by expressing mreB from Myxoccocus xanthus in E. coli, leading to the mislocalization of MreB to the poles in large aggregates, or by forcing the localization of major cell wall synthesis proteins to the cell pole. The fact that cells switched modes of growth suggests an evolutionary pathway from midcell to polar growing cells as well as suggests that there might be unknown conditions in nature when cells may switch growth modes.

Strengths:

(1) The authors use a strain that has replaced mreB with a functional fluorescent version at the native site. This eliminates any effects of having two copies of mreB. Because MreB is fluorescently tagged, they can monitor its localization when mreB from M. xanthus is expressed in E. coli. They notice that MreBec now forms bright polar foci and that there appear to be changes to the cell wall at the pole.

(2) D-amino acids are specific to the cell wall, and fluorescent versions (FDAA) have been used to mark sites of new cell wall insertion. The authors use these FDAAs to determine how cell wall synthesis correlates to MreB and if that changes when MreBmx is expressed. Again, there is pretty clear evidence that cell wall synthesis follows MreB localization to the pole.

(3) MreB itself does not synthesize the cell wall, but localizes the proteins, such as PBP2, that do. Using a published method to force proteins to the pole, the authors show that when they target PBP2 to the pole, they can phenocopy the polar growth seen when MreB is polar. Interestingly, these cells become resistant to A22, a drug that targets MreB, suggesting that localized growth at the pole does not require MreB and is sufficient to maintain rod shape.

Weaknesses:

(1) The authors do not show what the poles of control cells look like, making it difficult to determine if there is a change when MreBmx is expressed. However, the localization of both MreBec and MreBmx clearly forms bright foci at the pole.

(2) While more quantification is needed, the authors show some evidence that RodZ, an MreB interaction partner, is needed for this polar growth, as cells lacking rodZ still form foci at pole-like regions when MreBmx is in the cell; however, these cells remain spherical and do not elongate from these foci.

When MreB is deleted, and cells become spherical, the authors were unable to cause the polar growth mode. They suggest that this is due to the lack of a preexisting pole; however, experimental evidence to test this is missing.

Conclusion:

Overall, the authors do a good job of showing that E. coli can grow with a polar method rather than a midcell method of cell wall insertion. It is unclear why MreBec forms at poles when MreBmx is present and even if this MreB is functional. The foci look similar to inclusion bodies, which are normally aggregates of misfolded proteins that migrate to the poles. Past work has shown that when MreB is more polarly localized, branches form, which is not seen here. Importantly, the authors also show that there is feedback between the localization of MreB and PBP2 as both appear to regulate the localization of the other.

https://doi.org/10.7554/eLife.112612.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *This is an interesting paper, with the primary finding being that localizing MreB or PBP2 to the cell poles in E. coli is primarily demonstrated via an aggregate formed by expressing M. xanthus MreB.*
>
> *I have 2 main concerns:*
>
> *(1) First, the authors should clarify and adjust their interpretation of FDAA incorporation: As written, the authors interpret FDAA incorporation as being caused by incorporation during PG polymerization. However, in E. coli, FDAA incorporation does not result from the elongation of PG strands or their initial 4-3 crosslinking by DD transpeptidases following polymerization, but rather by the remodeling of L,D-transpeptidases.*
>
> *Thus, it is not accurate to refer to the FDAA incorporation as PG elongation, but rather the modification of crosslinks from 4-3 to 3-3 crosslinks at that location. To claim a link to PG polymerization, other experiments or substantial explanations are needed.*
>
> *(E. coli Cells Incorporate FDAAs by L,D-TPases in a Growth Independent Manner) - https://doi.org/10.1021/acschembio.*

We appreciate the reviewer for bringing up this important question. We will address this concern by further explaining our results in the revised manuscript.

However, we respectfully disagree with the reviewer for two reasons:

First, the paper by Kuru et al. (mentioned by the reviewer) revealed that (exact quote): “Our *in vitro* and *in vivo* data unequivocally demonstrate that these bacteria incorporate FDAAs using two extra cytoplasmic pathways: through activity of their D, D-transpeptidases, and, if present, by their L, D-transpeptidases…These mechanistic findings enabled development of a new, FDAA-based, *in vitro* labelling approach that reports on subcellular distribution of muropeptides, an especially important attribute to enable the study of bacteria with poorly defined growth modes” (Kuru et al., 2019).

Thus, the paper by Kuru et al. identified two FDAA labeling patterns, a D, D-transpeptidase-dependent, concentrated labeling for PG growth and an L, D-transpeptidase-dependent, growth-independent labeling for PG modification along the entire cell envelope (Kuru et al., 2019). The polar FDAA foci we presented do not match the reported pattern of L, D-transpeptidase-dependent incorporation.

Second, we provided the evidence in Fig. 3b that the polar FDAA labeling is due to the activity of PBP2, a D, D-transpeptidase, because mecillinam that inhibits PBP2 is sufficient to abolish polar FDAA incorporation.

> *(2) Second, the evidence provided that this is polar elongation is not sufficient to prove elongation. In all images claiming polar growth, the FDAA focus appears as a single spot, which corresponds to the MreB aggregate visible in bright-field. That might indicate incorporation, but it does not demonstrate polar elongation. To prove this, the authors should do different-length pulses of FDAAs and demonstrate an increasing length of labeled PG along the cell. Cells with one focus should show increasing length; polar foci should elongate from both ends. I find the current 2-color labeling insufficient, as BADA labels the entire cell.*

We appreciate this comment and totally agree with the reviewer. The wide BADA labeling band could indeed come from L, D-transpeptidases. We will follow the reviewer's recommendation to address this comment with additional staining experiments.

> *Small points:*
>
> *(3) Lines 350- 302: "In this case, as the nonpolar region is no longer the growth zone, the established cylindrical PG structure is sufficient to maintain cell width, where MreB filaments become nonessential." Does polar elongation give robustness to rod shape? The authors should include an analysis of cell width and its variation within a cell and between cells.*

We appreciate this comment. Judging from the bright-field images we presented, we believe that polar elongation does give robustness to rod shape. We will follow the reviewer's recommendation and provide the said analysis.

> *(4) In the abstract: "This reprogrammed growth mode bypasses the requirement for MreB filaments, highlighting a plasticity of the Rod system that suggests polar elongation may have emerged through the evolutionary loss of MreB." This argument should not be made without evolutionary analysis or reference to such work indicating this is the case.*

We appreciate this comment and will remove this statement from our revised manuscript.

> *(5) 365-367 "PG-depleted spheroplasts can spontaneously regenerate rod shape through curvature-dependent localization of MreB filaments [21, 57]". This should be amended. The Billing paper did indeed study spheroplasts, but the Hussain paper used teichoic acid-depleted cells that still had a cell wall.*

We thank the reviewer for pointing out this mistake. We will amend this statement in our revised manuscript.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Based on observations of localisation of MreBEc at the poles within an aggregate-like structure, upon heterologous expression of MreBMx, the authors set out to investigate*

> *how this non-canonical localisation of MreBs leads to a reprogramming of peptidoglycan synthesis to the poles. This is analogous to the polar growth observed in phyla which are not dependent on dispersed growth of PG, but only at the poles, and are MreB independent.*
>
> *The authors proceed to establish that PG synthesis is MreB-dependent, Rod enzyme-dependent, and requires the prior establishment of a pole.*
>
> *Strengths:*
>
> *(1) It is a very interesting idea to design experiments to demonstrate reprogramming of non-polar to polar growth based on the observation of localisation of a heterologously expressed MreB.*
>
> *(2) The experiments to demonstrate the factors that determine polar growth and the observation of the PG in each of these experimental situations are convincing.*
>
> *(3) I find the observation of an extra layer of PG in the heterologously expressed system very intriguing. It will be interesting to see if this layer merges with the other PG layer at some stage or branches from the non-polar growth near the poles.*
>
> *Weaknesses:*
>
> *(1) It is not clear what exactly the identity of the polar aggregates is and how much of this activity is an artefact of partially functional MreBs.*

We appreciate this comment and will address it by further explaining our results in the revised manuscript. While we can only say that the polar aggregates resemble inclusion bodies, we do believe that they cause polar PG growth because in the cells that express $MreB_{Mx}$, polar PG growth does not occur at the poles that lack MreB aggregates.

> *(2) I find it intriguing that the localisation and growth are predominantly at one pole only. It is unclear to me how this can be reconciled with growth and shape maintenance, and an increase in length and width. Is the increase in length and width a consequence of misshapen cells that are bulged in the absence of a normal PG layer?*

We appreciate this comment. Judging from the bright-field images we presented, we believe that polar elongation does not generate bulges and is thus sufficient for maintaining rod shape. We will follow the reviewer's recommendation to clarify this.

> *(3) The authors do not follow up on the observations in the first figure on the length and width changes and the extra peptidoglycan layer (which I feel are the most interesting aspects), and how this can be connected to the polar growth observed in the later sections of the manuscript.*

We appreciate this comment. We believe that the thickened PG patches are integral parts of the polar PG, rather than an extra layer, which is, however, technically challenging to prove. Thus, we will relay on fluorescence microscopy to visualize polar PG growth.

> *(4) The claim that this could be a precursor of an MreB-independent polar growth mechanism appears to be a bit far-fetched, because the system is still dependent on having an established pole for PG synthesis to occur in the new place.*

We appreciate this comment and will remove such speculations from our revised manuscript.

> ***Reviewer #3 (Public review):***
>
> *Summary:*

> *Most rod-shaped bacteria grow by one of two mechanisms: growth from the pole or growth from the midcell. It is rare for a single species to utilize both modes of growth, although a few examples do exist. Here, the authors have artificially induced E. coli cells to grow from the poles, either by expressing mreB from Myxoccocus xanthus in E. coli, leading to the mislocalization of MreB to the poles in large aggregates, or by forcing the localization of major cell wall synthesis proteins to the cell pole. The fact that cells switched modes of growth suggests an evolutionary pathway from midcell to polar growing cells as well as suggests that there might be unknown conditions in nature when cells may switch growth modes.*
>
> *Strengths:*
>
> *(1) The authors use a strain that has replaced mreB with a functional fluorescent version at the native site. This eliminates any effects of having two copies of mreB. Because MreB is fluorescently tagged, they can monitor its localization when mreB from M. xanthus is expressed in E. coli. They notice that MreBec now forms bright polar foci and that there appear to be changes to the cell wall at the pole.*
>
> *(2) D-amino acids are specific to the cell wall, and fluorescent versions (FDAA) have been used to mark sites of new cell wall insertion. The authors use these FDAAs to determine how cell wall synthesis correlates to MreB and if that changes when MreBmx is expressed. Again, there is pretty clear evidence that cell wall synthesis follows MreB localization to the pole.*
>
> *(3) MreB itself does not synthesize the cell wall, but localizes the proteins, such as PBP2, that do. Using a published method to force proteins to the pole, the authors show that when they target PBP2 to the pole, they can phenocopy the polar growth seen when MreB is polar. Interestingly, these cells become resistant to A22, a drug that targets MreB, suggesting that localized growth at the pole does not require MreB and is sufficient to maintain rod shape.*
>
> *Weaknesses:*
>
> *(1) The authors do not show what the poles of control cells look like, making it difficult to determine if there is a change when MreBmx is expressed. However, the localization of both MreBec and MreBmx clearly forms bright foci at the pole.*

We appreciate this comment and totally agree with the reviewer. We will provide reference images in the revised manuscript.

> *(2) While more quantification is needed, the authors show some evidence that RodZ, an MreB interaction partner, is needed for this polar growth, as cells lacking rodZ still form foci at pole-like regions when MreBmx is in the cell; however, these cells remain spherical and do not elongate from these foci.*

These results strongly support our conclusions. In the cells that express $MreB_{Mx}$, $MreB_{Mx}$ causes $MreB_{Ec}$ to mislocalize, and mislocalized $MreB_{Ec}$ recruits Rod enzymes (RodA and PBP2) to poles through the connector protein RodZ. Here when we delete *rodZ*, while $MreB_{Ec}$ still forms aggregates, it is unable to recruit Rod enzymes to those aggregates.

In contrast, when we directly relocalize PBP2 to cell poles through the PopZ tag, polar PG growth can bypass the requirement for $MreB_{Ec}$ filaments (Fig. 4).

> *When MreB is deleted, and cells become spherical, the authors were unable to cause the polar growth mode. They suggest that this is due to the lack of a preexisting pole; however, experimental evidence to test this is missing.*

We appreciate this comment. We plan to localize PBP2 to cell poles in an *mreB* depletion strain to test if cells still remain rods when *mreB* is depleted.

> *Conclusion:*
>
> *Overall, the authors do a good job of showing that E. coli can grow with a polar method rather than a midcell method of cell wall insertion. It is unclear why MreBec forms at poles when MreBmx is present and even if this MreB is functional. The foci look similar to inclusion bodies, which are normally aggregates of misfolded proteins that migrate to the poles. Past work has shown that when MreB is more polarly localized, branches form, which is not seen here. Importantly, the authors also show that there is feedback between the localization of MreB and PBP2 as both appear to regulate the localization of the other.*

Because CFP-labeled $MreB_{Ec}$ is still fluorescent in the aggregates, we believe that some $MreB_{Ec}$ molecules are still correctly folded there, at least on the surfaces of those aggregates.

Reference

Kuru, E., Radkov, A., Meng, X., Egan, A., Alvarez, L., Dowson, A., Booher, G., Breukink, E., Roper, D.I., Cava, F., Vollmer, W., Brun, Y., and VanNieuwenhze, M.S. (2019) Mechanisms of incorporation for D-amino acid probes that target peptidoglycan biosynthesis. ACS Chem Biol.

https://doi.org/10.7554/eLife.112612.1.sa0