1 Building the bacterial cell wall at the pole

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9 **Abstract**

10 Polar growth is the predominant mode of cell wall extension in the Actinobacteria and
11 the alphaproteobacterial clade Rhizobiales. The observation of polar elongation in
12 taxonomically diverse bacteria suggests that polar growth may have evolved
13 independently. Indeed, the regulatory mechanisms governing the assembly of cell wall
14 biosynthesis machinery at the pole are distinct in the Actinobacteria and Rhizobiales.
15 Here we highlight recent advances in our understanding of polar growth mechanisms in
16 bacteria, with an emphasis on *Streptomyces* and *Agrobacterium*. This review illustrates
17 that common themes are emerging in the regulation of polar growth in diverse bacteria.
18 Emerging themes include the use of landmark proteins to direct growth to the pole and
19 coordination of polar growth with cell-cycle progression.

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21 **Introduction**

22 Across bacteria, an astonishing variety of morphologies have been observed. Bacterial
23 cell shapes range from relatively simple (rods and spheres) to increasingly complex
24 (vibrioid, helical, star-shaped, and branched). Over the last decade, understanding how
25 and why bacteria generate such a diverse range of cell shape has become an active
26 area of research [1-3]. The bacterial cell wall or peptidoglycan (PG) is a heteropolymer
27 of adjacent glycan strands crosslinked through peptide stems that is required to
28 maintain proper cell shape. While PG is a key shape determinant, it is the precise
29 targeting of cell wall biosynthesis machinery to specific locations that enables the
30 observed myriad of bacterial cell shapes to be formed. Indeed, the use of fluorescent D-

amino acids for high resolution imaging of patterns of cell wall biogenesis in diverse bacteria has confirmed the presence of many different growth patterns [4-8]. Although the biosynthetic machineries driving cell wall expansion are relatively well conserved among bacteria, the regulatory mechanisms that have allowed diverse growth patterns to emerge are only just beginning to be uncovered.

The regulation of cell wall growth is best-studied in rod-shaped bacteria. Many rod-shaped bacteria such as *Escherichia coli* and *Bacillus subtilis* elongate via insertion of new PG along the lateral sidewall [9,10]. This mode of growth requires precise spatial and temporal regulation of the elongasome, the complex of proteins responsible for PG biosynthesis during elongation. The bacterial actin homolog, MreB, is a major scaffolding protein that interacts with PG biosynthesis machinery and forms mobile patches that move perpendicular to the long axis of the cell [11-13]. In *E. coli*, MreB promotes local PG insertion in regions of negative curvature, straightening the cell and maintaining the proper cell shape [14]. Remarkably, not all rod-shaped bacteria use an MreB-dependent, dispersed mode of growth for cell extension. Some rod-shaped, budding, and filamentous bacteria, particularly within the Actinomycetales and Rhizobiales, utilize polar elongation as an alternative growth strategy [15-17]. Here, we will highlight recent advances in our understanding of the mechanisms that restrict PG synthesis to the pole during elongation, with an emphasis on *Streptomyces* and *Agrobacterium* species.

Spatial and temporal regulation of polar growth in *Streptomyces*

53 *How is polar growth directed to the proper location?*

54 In order for growth to be restricted to a specific subcellular location, the machinery
55 responsible for PG biosynthesis must be precisely targeted to the pole. A common
56 theme in targeting the PG machinery is the use of polymer-forming landmark proteins.
57 Cytoplasmic polymer-forming landmark proteins self-assemble at a specific location in a
58 cell-cycle dependent manner, enabling the PG biosynthesis to be synchronized with cell
59 cycle progression [18].

60 The filamentous Actinobacterium, *Streptomyces coelicolor*, utilizes at least three
61 polymer-forming proteins to guide growth at the pole. DivIVA may use negative
62 curvature as a cue to self-assemble into short filaments and form a matrix to establish
63 new growth poles where it recruits or activates PG biosynthetic machinery [19-21].
64 DivIVA also recruits the paralogous polymer-forming proteins, Scy and FlpP, to the
65 growth poles [22,23]. Scy colocalizes with DivIVA at both existing growth poles and
66 sites of future growth pole formation [23]. Scy is proposed to sequester DivIVA,
67 regulating the number of DivIVA foci and promoting the establishment of a limited
68 number of new growth zones [23]. FlpP is an intermediate filament-like protein that forms
69 a crosslinked network of fibers and contributes to maintaining rigidity and elasticity of *S.*
70 *coelicolor* hyphae [22,24]. During polar growth, FlpP forms an apical gradient and is
71 most concentrated at the growth pole, where it interacts with both DivIVA and Scy. The
72 increased density of FlpP at the cell pole provides a stress-bearing cytoskeletal network
73 which likely compensates for the inherent weakness of the PG at the growth pole [22].
74 Together, DivIVA, Scy, and FlpP form a tip-organizing center or polarisome which directs

PG biosynthesis to existing hyphal tips and newly established polarity centers to enable branching (Figure 1A).

How are new branch sites selected?

During apical growth, *Streptomyces* cells exhibit a complex branching morphology. Remarkably, polarisome splitting is responsible for the formation of new polarity centers [25]. During elongation, the polarisome continues to remain associated with the growth pole; however, a daughter polarisome is left behind along the lateral hyphal wall and marks the future site of branch formation. The daughter polarisome must recruit additional proteins from the cytoplasm, presumably including Scy and FilP, and pass a critical size threshold before becoming competent to trigger PG biosynthesis and branch formation (Figure 1B-C).

How is polar growth terminated?

Streptomyces have a complex lifecycle in which reproductive hyphae must terminate polar elongation, establish multiple sites of cell division by forming FtsZ-dependent septa, and segregate many chromosomes prior to sporulation. Recent studies in *S. coelicolor* and *S. venezuelae* have illustrated that synchronization of key cell cycle processes must occur to enable a seamless transition from polar growth to sporulation.

In *Streptomyces*, the ParAB-*parS* partitioning system coordinates termination of polar growth and enables chromosome segregation prior to cell division and sporulation

[26,27]. Remarkably, ParA and Scy interact directly [23,26] suggesting a link between polar elongation and chromosome segregation. Scy inhibits ParA polymerization which is required for the initiation of chromosome segregation [26]. In relatively short hyphae, the growth poles contain strong foci of Scy, presumably preventing ParA polymerization. As the hyphae elongate, the *parAB* operon is developmentally induced [28] and ParA begins to accumulate at the growth poles [26]. Upon reaching the optimal length for sporulation, ParA levels are sufficiently high to escape Scy inhibition and ParA polymerizes along the hyphae. ParA polymerization results in the formation of evenly spaced ParB-*parS* complexes along the hyphae, enabling chromosome segregation, FtsZ-ring formation, and subsequent cell division [27] (Figure 1D). In addition, polymerization of ParA may trigger depolymerization of Scy, leading to termination of polar growth [26]. These observations suggest that balance of Scy and ParA may coordinate the termination of polar elongation with chromosome segregation.

The presence of a link between termination of polar growth and the initiation of chromosome segregation, cell division, and sporulation has been validated by characterization of the regulons of the WhiA and WhiB transcriptional regulators [29,30]. WhiA and WhiB co-control the same regulon and binding to target genes is codependent suggesting that these proteins function together to regulate the transition from growth to sporulation [30]. Mutants lacking WhiA or WhiB fail to terminate polar elongation suggesting that cessation of polar growth is a key process during the transition to sporulation [29-32]. The WhiA [29] and WhiB [30] regulons reveal that *filP* is repressed during the transition from growth to sporulation. Furthermore, when polar growth is terminated, FilP is redistributed evenly throughout the mycelium [22].

Together, these data lead to an attractive hypothesis in which the repression of *filP* may contribute to the disassembly of the polarisome during the transition to sporulation.

Disassembly of the polarisome is also triggered by phosphorylation of DivIVA [33]. AfsK is a Ser/Thr protein kinase that colocalizes with DivIVA at the growth poles; however, during normal growth only a low level of DivIVA phosphorylation is detected [33]. When the growth pole encounters complications with PG biosynthesis (such as antibiotic exposure) AfsK increases phosphorylation of DivIVA leading to disassembly of the polarisome. The bidirectional feedback between DivIVA (which directs PG biosynthesis [21]) and the PG biosynthesis machinery through AfsK-mediated phosphorylation of DivIVA may provide a mechanism for determining the timing of polar growth termination. Remarkably, in *Mycobacteria* phosphorylation of a peptidoglycan synthase governs the rate of polar elongation [34]. Thus, regulation of proteins involved in polar growth by phosphorylation is emerging as a common theme.

Polar growth of *Agrobacterium tumefaciens*

Many species of Alphaproteobacteria, particularly within the clade Rhizobiales, lack both MreB and DivIVA. Blocking cell division in some of these bacteria results in branching or bulging phenotypes rather than smooth filaments, leading to the hypothesis that these bacteria may exhibit polar growth [35]. Indeed, studies using both amine-reactive dyes and FDAAs have confirmed that *Agrobacterium tumefaciens* and other members of the rhizobiales elongate from the new pole [4,36,37]. The mechanisms underlying the spatial and temporal regulation of polar elongation in

Agrobacterium tumefaciens remain relatively poorly resolved (Figure 2A); however, this is an exciting area of ongoing research.

Does A. tumefaciens use a landmark protein to coordinate polar growth?

In order to insert newly synthesized PG at the growth pole, the PG biosynthesis machinery must be targeted to the new cell pole, suggesting the presence of a polarisome-like complex. While the putative polarisome has not yet been defined, localization studies have revealed a number of attractive candidate proteins (Figure 2A), including PopZ [38]. *A. tumefaciens* PopZ (PopZ_{AT}) shares homology with the well characterized PopZ from *Caulobacter crescentus* (PopZ_{CC}). PopZ_{CC} is a polymer forming protein that exhibits a dynamic localization pattern and has key functions in both cell-cycle progression and chromosome segregation [39-43]. In contrast to the unipolar-to-bipolar localization of PopZ_{CC}, PopZ_{AT} marks the growth pole [38]. PopZ_{AT} localizes to the growth pole during elongation and reappears at new growth poles immediately following cell division. The localization pattern of PopZ_{AT} and its sequence similarity to PopZ_{CC} (particularly in the C-terminal domain required for interactions with the ParAB-*parS* partitioning system) make PopZ an intriguing candidate landmark protein to coordinate polar growth and chromosome segregation in *A. tumefaciens*.

Since *A. tumefaciens* lacks canonical elongation machinery and the growth poles originate from the site of cell division, it has been suggested that cell division machinery may function to regulate polar elongation [37,44]. Remarkably, canonical cell division proteins, FtsZ and FtsA, persist at the growth pole for at least a portion of the cell cycle

[16,38,44,45] and are predicted to form polymers suggesting that they may serve as bacterial scaffolds at the growth pole. The recent development of a depletion system for *A. tumefaciens* [46] will open the door for characterizing the role of these proteins in polar growth, cell division, or cell-cycle progression. Here, we show that depletion of FtsZ triggers multipolar growth and tip splitting (Figure 2B-C) suggesting that FtsZ is required not only for cell division but also for the termination of polar growth, highlighting the coordination of polar growth and cell division in *A. tumefaciens*.

Another non-polymer forming landmark protein, PodJ, plays an important role in coordination of polar growth with cell cycle progression [38,47]. PodJ has a complex localization pattern initially marking the old pole and later associating with both poles [38]. The arrival of PodJ at the new pole just before cessation of polar growth makes PodJ an attractive candidate to regulate polar growth. Deletion of PodJ results in multiple morphological defects such as elongated cells, branching, multiple constrictions, bent, and small rounded cells [47]. This phenotype suggests that PodJ is not strictly required for polar growth; however, PodJ may have a direct or indirect role in the transition of the growth pole into a non-growing old pole.

Concluding remarks

While many mechanistic insights remain to be uncovered, it is clear that *Streptomyces* and *Agrobacterium* have evolved distinct mechanisms to drive polar growth. Yet, a number of common themes are emerging among these phylogenetically distant polar-growing bacteria including the use of landmark proteins, tip splitting behavior, and coordination of growth with cell cycle progression. Ongoing and future studies of PG

185 biosynthesis and its regulation in polar growing bacteria will provide insights into a
186 number of outstanding questions.

187 *Is polar growth polar?* While many proteins involved in the regulation of polar
188 growth are present at the cell pole, the precise regions of PG insertion have not been
189 well defined in most polar growing bacteria. The combination of metabolic labeling of
190 cell surface components and superresolution microscopy is beginning to reveal the sites
191 of PG insertion at higher resolution [6]. This approach revealed that polar DivIVA directs
192 subpolar PG insertion in mycobacteria [48]. Future research should determine if
193 subpolar growth is common among the Actinobacteria or even more distant polar
194 growing bacteria.

195 *Is the growth machinery conserved?* The PG composition of polar growing
196 bacteria is distinctive. Both Actinomycetales and Rhizobiales PG is highly crosslinked
197 and enriched in muropeptides containing 3-3 crosslinks [36,49-51] suggesting a
198 potential role for DD-carboxypeptidases and LD-transpeptidases (Figure 3). While 3-3
199 crosslinks are typically found in PG from diverse bacteria, the abundance of
200 muropeptides containing 3-3 crosslinks is much higher in polar growing bacteria. For
201 example, in *E. coli* PG ~25% of the total muropeptides are crosslinked; however only
202 ~1-5% of crosslinked muropeptides contain 3-3 crosslinks [52]. In contrast ~64% of the
203 muropeptides from *A. tumefaciens* PG are crosslinked and 4-3 and 3-3 crosslinks are
204 equally abundant [36]. During stationary phase, *Mycobacterium* PG is highly crosslinked
205 (~65% of total muropeptides) and 3-3 crosslinks are highly abundant (70-80%)[50,51].
206 Loss of LD-transpeptidases in mycobacteria results in altered morphology [53,54] and at
207 least one LD-transpeptidases localizes to the growth pole in *A. tumefaciens* [37]. Thus, it

is intriguing to speculate that polar growing bacteria may use accessory enzymes including LD-transpeptidases as key components of the polar PG biosynthesis machinery.

What is the advantage of polar growth? Polar growth and subsequent cell division generates phenotypic variation which can increase fitness in unstable environments [17]. For example, differences in polar growth rates in *Mycobacterium* give rise to a heterogeneous population in which the slowest-growing bacteria exhibit increased resistance to antibiotics [55,56]. In *Agrobacterium*, FDAA labeling experiments show that the area of labeling increases with cell length [37], hinting that the elongation rate may be variable at the population level. While the potential advantages of polar growth remain to be elucidated, it is clear that distantly related bacteria have converged on a similar growth patterns. Future studies on diverse bacteria are needed to provide key insights into both unique and conserved mechanisms underpinning polar growth.

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Figure Captions

Figure 1. Polar growth of *Streptomyces*. A. During polar growth, PG synthesis is restricted to the growth pole. The growth pole is marked by the presence of the polarisome which is comprised of DivIVA, Scy, and FilP. The cell wall synthesis machinery is active at the growth pole. B. In order to initiate branching, the branch site is selected via the splitting of the polarisome. The small focus of polarisome marks the future site of PG biosynthesis. C. The polarisome matures into a larger protein complex and recruits the cell wall machinery to initiate branch formation. D. The transition to sporulation requires termination of polar growth. This transition includes the disassembly of the polarisome. ParA filaments form, enable compaction of DNA and chromosome segregation into pre-spore compartments.

Figure 2. Polar growth of *Agrobacterium*. A. During polar growth, PG synthesis is restricted to the growth pole. The growth pole is marked by the presence of a number of polymer-forming proteins including PopZ, FtsA, and FtsZ. The PG biosynthesis machinery is present at the growth pole and likely includes PBP1 and LD-transpeptidases. B. During the termination of polar growth, PodJ appears at the pole and likely functions to release PopZ from the pole. FtsZ, FtsA, and PG synthesis machinery relocate to the mid-cell as part of the transition from polar growth to cell

division. C. Time-lapse microscopy of wild type cells illustrates polar growth and typical cell division. Times of image acquisition are shown in hours on each image. D. Time-lapse microscopy of FtsZ-depleted cells reveals a role for FtsZ in both cell division and the termination of polar growth. When polar growth does not terminate, tip splitting behavior leads to the accumulation of growth poles. Depletion time in hours is shown on each image.

Figure 3. Representative PG crosslinking reactions catalyzed by DD- and LD-transpeptidases. Representative reactions were selected because D44 and D34D are the most abundant muropeptide dimers in *A. tumefaciens* PG [36]. PG is crosslinked through peptide stems to connect adjacent glycan strands and provide a mesh-like architecture. Most bacteria utilize the DD-transpeptidase activity associated with penicillin binding proteins to link glycan strands. In Gram negative bacteria, DD-transpeptidases link D-Alanine in position 4 of the peptide stem to the free amino group at the D-center of meso-diamino-pimelic acid (mDAP) at position 3 of the peptide stem, forming a characteristic 4-3 crosslink (black bar). The representative reaction illustrates the linkage of a monomeric muro-pentapeptide (M5) donor and a monomeric muro-tetrapeptide (M4) acceptor to generate a dimeric muropeptide containing a DD-DAla-mDAP crosslink (D44). In contrast, LD-transpeptidases link the carbonyl group at the L-center of mDAP at position 3 to the amino group at the D-center of mDAP in an adjacent peptide stem to generate 3-3 crosslinks (red bar). The representative reaction illustrates the linkage of a monomeric muro-tetrapeptide (M4) donor and a monomeric muro-tetrapeptide (M4) acceptor to generate a dimeric muropeptide containing a LD-mDAP-mDAP crosslink (D34D). DD-carboxypeptidases are responsible for the conversion of

476 monomeric muro-pentapeptides (M5) to monomeric muro-tetrapeptides (M4), which are
477 the substrate for LD-transpeptidases. Muropeptide naming conventions were taken from
478 Brown et al [36].

C Wild Type Cell Growth

D FtsZ-depleted Cell Growth

Graphical Abstract (for review)

1 Polymer-forming proteins mark site for PG synthesis

2 Scaffolding proteins and PG synthesis machinery are recruited to the pole

3 Polar growth must be coordinated with chromosome segregation and terminate prior to cell division