MECHANISMS OF POLAR GROWTH IN THE
ALPHAPROTEOBACTERIAL ORDER RHIZOBIALES

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ABSTRACT

All bacteria elongate and divide to faithfully reproduce their cell shape. Understanding the mechanisms that drive bacterial morphology requires an intimate knowledge of how the cell wall is synthesized. During cell division, most bacteria synthesize new cell wall at mid-cell and the mechanism underlying this process is highly conserved. In contrast, there is a high degree of diversity in bacterial growth patterning during elongation. Bacteria in the Rhizobiales exhibit an atypical form of unipolar elongation, and the molecular mechanisms of how new cell wall is synthesized during growth and division currently remains unexplored. Using microfluidics and fluorescent cell wall probes we first investigated whether polar growth is conserved in a morphologically complex bacterium, *Prosthecomicrobium hirschii*. We showed that *P. hirschii* has a dimorphic lifestyle and can switch between a long-stalked, non-motile form and a short-stalked, motile form. Furthermore, we found that all morphotypes of *P. hirschii* elongate using polar growth, suggesting the polar elongation is a widespread feature of bacteria in this order. Next, we used the rod-shaped bacterium *Agrobacterium tumefaciens* as a model to investigate the precise mechanisms that drive polar elongation. We characterized a comprehensive set of cell wall synthesis enzymes in *A. tumefaciens* and identified penicillin-binding protein 3a (PBP3a) and PBP3b as a synthetic lethal pair that function during cell division, and PBP1a as an essential enzyme required for polar growth and maintenance of rod shape.

Compositional analysis of the PBP1a depletion, suggested that LD-transpeptidase (LDT) enzymes may play an important role in polar growth. We identified three LDTs that likely function in polar growth. We also observed subpolar localization of LDTs, suggesting bacteria in the Rhizobiales may insert or remodel cell wall material in a subpolar zone during growth. Finally, we used RNA-seq to explore changes in gene expression during PBP1a depletion, revealing that that loss of PBP1a induces a lifestyle switch which mimics the switch from a free-living bacterium into a plant-associated state. The change in lifestyle is characterized by increased exopolysaccharide production and Type VI Secretion System activity and a decrease in flagella-mediated motility. This finding indicates that bacteria have a mechanism to sense changes in cell wall composition or integrity due to the loss of PBP1a and respond through changes in gene expression that impact physiology and behavior. This finding opens the door to future studies on the link between changes in cell wall composition and complex bacterial behaviors and lifestyles. Overall, this research provides mechanistic insights about the roles of cell wall synthesis during cell growth and division in the *A. tumefaciens*, which are conserved in other Rhizobiales, including agriculturally and medically species such as *Sinorhizobium* and *Brucella*.

CHAPTER 1

Diversity of Growth Patterns in the Alphaproteobacteria

Author Contributions

Williams M, and Kysela TD conducted the experiments and Williams M and Brown PB wrote and edited the paper.

ABSTRACT

Bacterial cell shape is highly variable in the Alphaproteobacteria, suggesting that multiple molecular mechanisms contribute to the generation of distinct morphologies. Although many growth patterns including lateral elongation, pre-septal elongation, polar elongation, budding and mid-cell growth have been observed among Alphaproteobacteria, only a few of the underlying mechanisms are well understood. We summarize the variations in growth patterning that determine morphological diversity in each order of the Alphaproteobacteria, highlighting the current understanding of pre-septal elongation and stalked budding in the *Caulobacteriales* and unipolar growth and prosthecae budding in the *Rhizobiales*. The Alphaproteobacteria have evolved novel growth patterns through the diversification and regulation of the cell elongation machinery. Ultimately, expanding studies of cell growth in the Alphaproteobacteria will deepen our understanding of the mechanisms that underlie morphological complexity in the domain Bacteria.

Keywords: Cell wall biogenesis, Bacterial budding, Peptidoglycan, Morphology, Penicillin-binding proteins, MreB, FtsZ

Ecological Diversity of Alphaproteobacteria

The breadth of environmental niches occupied by Alphaproteobacteria ranges from free-living bacteria in saltwater, fresh water, and soil environments, to

hydrothermal vents, and Antarctic sea ice. In addition to a wide range of geographical niches, the Alphaproteobacteria can also adopt various lifestyles including: plant symbionts, plant pathogens, facultative intracellular pathogens, and obligate intracellular pathogens. Given the assortment of life-styles and niches, it is not surprising that these bacteria have a high degree of phylogenetic, metabolic and morphological diversity [Figure 1a; 1, 2, 3]. The class Alphaproteobacteria is comprised of six highly diverse orders: *Rhizobiales*, *Caulobacterales*, *Rhodospirillales*, *Rhodobacterales*, *Sphingomonadales*, and *Rickettsiales* (Figure 1.1a) and contains bacteria with a striking array of buds, stalks, prosthecae, spirals, stars and asymmetric rods (Figure 1.1b). These orders are rich in morphological diversity, but we are just beginning to understand the mechanisms that generate such morphological complexity. Looking at cell shape and microcolony formation, early investigators surmised that many Alphaproteobacteria must grow fundamentally different from other rod-shaped bacteria [4, 5]. These initial observations provided foundational insights into the growth mechanisms required to faithfully reproduce unique bacterial shapes. Still, the underlying molecular mechanisms and evolution of bacterial shapes remains largely unknown for a majority of the Alphaproteobacteria.

Figure 1.1: Phylogeny and Morphology of Alphaproteobacteria. (A) A concatenated alignment of 37 conserved protein sequences was obtained using Phylosift [6]. The maximum likelihood phylogeny was estimated using RAxML [7] under the LG model of amino acid substitution with gamma-distributed rate

variation, and was formatted using iTOL [8]. Tree scale represents the branch length with 0.1 substitutions per site. Clade colors indicate the phylogenetic order: *Caulobacterales* (orange), *Rhizobiales* (green), *Rhodobacterales* (purple), *Sphingomonadales* (blue), *Rhodospirillales* (red), and *Rickettsiales* (pink). *Escherichia coli* was used as an outgroup. Icons represent the primary niche for each species as listed in the key. (B) Schematics of morphological diversity found within the Alphaproteobacteria grouped by mode of reproduction. Colored boxes represent the phylogenetic order corresponding to color of the bacterial orders in A. 1. *Hyphomonas neptunium* 2. *Hirschia baltica* 3. *Hyphomicrobium denitrificans* 4. *Rhodomicrobium vannielii* 5. *Asticcacaulis biprosthecum* 6. *Brevundimonas halotolerans* 7. *Caulobacter crescentus* 8. *Asticcacaulis excentricus* 9. *Agrobacterium tumefaciens* 10. *Brucella abortus* 11. *Prosthecomicrobium hirschii* 12. *Stella humosa* 13. *Sagittula stellata* 14. *Roseovarius tolerans* 15. *Rhodobacter sphaeroides* 16. *Magnetospirillum magnetotactum* 17. *Novosphingobium acidiphilum* 18. *Rickettsia typhi*

Methods to Visualize Bacterial Growth Patterning

Technological innovations and improvements in microscopy have enabled more detailed studies of bacterial growth patterning. Growth patterning in bacteria refers to the localized insertion of new cell wall (referred to as peptidoglycan) into the pre-existing wall material. The peptidoglycan is a stress-bearing meshwork of proteins and sugars that is part of the cell envelope. In Gram-negative bacteria, the cell wall is a thin layer sandwiched between the cytoplasmic and outer

membrane, while in gram-positive bacteria, the cell wall is thicker and connected by a myriad of proteins and polysaccharides. The cell wall has three main functions: first, to provide protection from osmotically stressful environments, second, provide support for cell envelope spanning structures such as flagella, pili and secretion systems, and third, maintain and faithfully reproduce the cell shape [9, 10]. To function properly, the peptidoglycan has a dedicated structure comprised of polysaccharide chains that are made up of two alternating subunits of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). Attached to the MurNAc subunit is a pentapeptide chain with D-amino acids. Typically, D-alanine makes up the terminal two amino acids. The discovery that bacteria can incorporate alternative D-amino acids into peptidoglycan [11] paved the way for pulse chase D-cysteine (D-cys) labeling experiments, in which it is possible to distinguish new and old peptidoglycan in purified sacculi [12]. Most recently, short pulse labeling experiments with fluorescent D-amino acids (FDAAs) in live bacterial cells have been completed [13-15]. These methods, along with improved microscopy techniques, have made detailed observations of cell wall growth in non-canonical bacteria relatively easy and have consistently demonstrated that the subcellular location of peptidoglycan insertion is precisely regulated during cell cycle progression [1, 16]. The ability to visualize sites of peptidoglycan insertion has rapidly improved our understanding of bacterial growth patterning during the cell growth cycle.

Growth Patterns During Elongation

The bacterial growth cycle is typically comprised of two distinct phases: elongation and division. During elongation, the new peptidoglycan is synthesized at a bacterial-specific location to enable cells to increase in length (Figure 1.2). The Alphaproteobacteria have evolved novel strategies for spatiotemporal regulation of peptidoglycan synthesis machinery during elongation, leading to the establishment of diverse growth patterns. Growth patterns during elongation include: insertion of peptidoglycan laterally along the sidewalls (Figure 1.2d,e), budding (Figure 1.2a,b,d), unipolar growth (Figure 1.2c), insertion of peptidoglycan near mid-cell (Figure 1.2e), and uncharacterized growth patterns (Figure 1.1b).

Figure 1.2: Growth Patterning in Representative Alphaproteobacteria. Colors represent type of growth patterning: polar elongation (blue), septal elongation (purple), lateral elongation (green) stalk elongation (orange), pre-septal elongation (red). Typical Alphaproteobacterial cell cycle for the species shown is as follows: a motile, swarmer cell differentiates into a non-motile sessile cell, elongates, synthesizes a flagellum at the daughter cell pole, and divides

producing a motile daughter cell and a non-motile mother cell that resumes growth.

The lateral insertion of peptidoglycan along the sidewalls of rod-shaped bacteria is best understood in *Escherichia coli* and *Bacillus subtilis*. Computational and high-resolution microscopy techniques have revealed that short filaments of the actin homolog MreB are targeted to areas of negative curvature and move circumferentially around the cell to maintain rod shape [17, 18]. Movement of MreB complexes is driven by cell wall synthesis [19, 20]. Cell wall synthesis during elongation requires enzymes including the monofunctional penicillin-binding protein (PBP)2 (DD-transpeptidase), the shape, elongation, division, and sporulation (SEDS) protein RodA (glycosyltransferase) and the bifunctional PBP1a (glycosyltransferase and DD-transpeptidase) [21-23] (See section 7). The integral membrane protein RodZ acts as a linker between peptidoglycan biosynthesis machinery in the periplasm and the cytoplasmic scaffolding complex MreB [24]. Thus, peptidoglycan insertion and cytoplasmic scaffolds are intrinsically linked, and work together to maintain proper rod shape. MreB-based cell elongation is well studied in canonical rod-shaped bacteria [25]. However, within the Alphaproteobacteria, some of the *Rhizobiales* maintain rod shape without MreB, and the molecular mechanisms underlying alternative modes of elongation are largely unresolved [26, 27].

Growth Patterns During Division

In contrast to the diversity of growth patterns observed during elongation, peptidoglycan insertion at positions near mid-cell prior to cell division is universally conserved (Figure 1.2; purple). Furthermore, the machinery required for cell division is well-conserved among the Alphaproteobacteria. Cell division begins with the sequential recruitment of ~30 proteins to mid-cell that assemble into a large complex called the divisome [28, 29]. The first protein to appear at mid-cell is the tubulin-like protein FtsZ that forms a ring-like structure called the Z-ring. The Z-ring is comprised of short filaments of FtsZ that treadmill at the division site [30, 31], and are tethered to the divisome complexes through C-terminal interactions with membrane-associated proteins such as FtsA and ZipA [32]. After assembly of the divisome is complete, peptidoglycan biosynthesis is activated [33-35]. As constriction proceeds, septal peptidoglycan is synthesized inwards to build the new poles of the daughter cells. Septal peptidoglycan synthesis requires the monofunctional PBP3 (DD-transpeptidase), the SEDS protein FtsW (glycosyltransferase), and the bifunctional PBP1b [36-38]. The order of recruitment and essentiality of divisome proteins may vary across species, but the overall mechanism of cell division appears to be broadly conserved [39]. In addition to peptidoglycan insertion for division and growth of the main cell body, some Alphaproteobacteria including *Caulobacter* and *Hyphomonas* use a specialized form of zonal peptidoglycan insertion at the cell body-stalk junction to enable stalk elongation (Figure 1.2a,d,e; orange).

Here, we will review the current models of growth patterning within each of the Alphaproteobacterial orders, with an emphasis on the orders *Rhizobiales* and *Caulobacterales*. We highlight more recent mechanistic studies of growth in a few model Alphaproteobacteria including: *Caulobacter*, *Hyphomonas*, and *Agrobacterium*, and consider outstanding questions and perspectives for future research on growth patterning within the Alphaproteobacteria.

Growth Patterns of Budding Bacteria in the Rhizobiales

One of the earliest indications of diversity in growth patterning of bacteria is evident by the abundance of budding bacteria within the order *Rhizobiales*. Classically defined budding bacteria insert new cell wall material into a small area of the mother cell to form a daughter cell that is typically smaller in size [5]. For the purposes of this review we will describe three types of budding: 1.) Budding from the tip of a stalk (stalked budding), 2.) budding from a stalked mother cell (prosthecae budding), and 3.) budding from one pole of a typical rod-shaped cell (unipolar elongation). All three types of budding are found within the *Rhizobiales* (Figure 1.1b; green boxes), resulting in a high degree of morphological complexity in this bacterial order. The prevalence of stalked budding, prosthecae budding, and unipolar elongation suggests that budding is an ancestral trait in the *Rhizobiales* and may have a common molecular mechanism.

Growth Patterns of Stalked Budding Bacteria

Hyphomicrobium (Figure 1.1b:3; Figure 1.2a) and *Rhodomicrobium* (Figure 1.1b:4) are the best characterized genera of stalked budding bacteria [40]. The stalk was classically termed a hypha, defined as an appendage from the main cell body required for reproduction. These stalked budding bacteria have dimorphic life cycles, and typically consist of an ovoid mother cell with a stalk extended from one pole that terminates in a daughter cell bud [5]. Based on observations of synchronized populations, four phases of growth have been described for *Hyphomicrobium* and *Rhodomicrobium*: swarmer cell maturation, stalk growth, bud formation, and bud detachment. Each phase occurs in a sequential manner in that growth from one phase will finish before growth of the next phase begins [Figure 2a; 41, 42]. The swarmer cell has a single polar flagellum that is shed before stalk biogenesis is initiated. The stalk is synthesized at one pole of the cell, and the cell wall is contiguous between mother cell and stalk. Stalk growth appears to occur at the mother cell-stalk junction, suggesting that the machinery required for stalk growth is spatially restricted to this junction. The stalk is required to produce a bud, which develops as a terminal swelling of the stalk. Whether the growth machinery is spatially restricted in the bud or diffuse within the bud remains to be explored. Before the daughter cell is released, the septum forms at the base of the stalk. Thus, the cell division machinery must be specifically targeted to the base of the stalk near the end of the cell cycle to enable septum formation. Finally, the bud is released from the tip

of the stalk producing two morphologically and functionally distinct cells; a motile swarmer cell and a stalked cell [40, 42].

In addition to the typical stalked budding cell cycle outlined above (Figure 1.2a), *Hyphomicrobium* species have been observed with more complex morphologies. For example, some species can adopt a branched and chained morphology, and helical stalks have been observed under different environmental conditions [43]. The morphological complexity of the stalked budding bacteria is a product of spatial and temporal regulation of growth machinery across the cell cycle to produce distinct areas of growth within the cell, but how is this machinery localized and regulated to produce more complex stalked morphologies? More in-depth studies are required to fully understand this mode of growth and the impact of changing environmental conditions on growth patterning. Future studies of *Hyphomicrobium* and other stalked budding bacteria belonging to the *Rhizobiales* may be hampered by long generation times, which can range from 6.5 to 14.5 hours [41], and the difficulty to culture these bacteria. Improved modelling and sequencing techniques may enable rapid prediction of growth requirements, and high-throughput culturing methods may help circumvent the challenge of studying cell wall biogenesis in slow-growing bacteria [44-46].

Like *Hyphomicrobium*, *Rhodomicrobium* can exhibit complex morphologies (Figure 1.1b:4) that are suggestive of a polymorphic lifestyle, in which it can adopt either a traditional stalked budding cell cycle or a more complex cell cycle. For example, *Rhodomicrobium* cells can form attached

mother-daughter cell pairs. Even though the cells remain attached, a peptidoglycan crossband is formed in the stalk nearest to the daughter cell completing compartmentalization of the cells [47]. Attached mother-daughter cell pairs continue to grow from multiple stalks, leading to a ball and chain morphology (Figure 1.1b:4). Remarkably, stalks may also terminate in an exospore rather than a daughter cell bud highlighting the complexity of *Rhodomicrobium* cell biology [4]. The intricate morphologies of *Rhodomicrobium* species present an interesting opportunity to ask what environmental conditions determine the switch to the chained morphology? How are these phenotypes regulated in a cell cycle dependent manner? How is the growth machinery spatially and temporally regulated to produce a single growth pole between a mother daughter pair that is part of a larger chain? Further studies of *Rhodomicrobium* cell cycle and growth are necessary to elucidate the regulation of more complex bacterial morphologies and lifestyles.

Morphological observations of other *Rhizobiales* species indicate that many have a dimorphic lifestyle and grow by stalked budding. For example, the cell cycle of *Rhodopseudomonas palustris* indicates that it grows by stalked budding [4] and this bacterium produces a unipolar polysaccharide (Fritts et al 2016). In addition, some *Pedomicrobium* species have multiple, lateral stalks rather than a single, polar stalk [40, 48]. Overall, the growth patterns of species within the genera *Rhodopseudomonas*, *Pedomicrobium*, and *Filomicrobium* are largely unstudied. Characterization of the essential genes in *R. palustris* predicted to function in

peptidoglycan biosynthesis [49], coupled with data mining from recent or ongoing genome sequencing projects of species in the genera *Pedomicrobium* and *Filomicrobium* [50], may enable additional studies about growth and development in these bacterial species.

Growth Patterns of Prosthecate Budding Bacteria

Unlike the stalked budding bacteria, stalks of the prosthecate budding bacteria are non-reproductive [Figure 2b, 51]. All prosthecate budding bacteria studied thus far appear to reproduce by budding directly from one pole of a stalked mother cell. In contrast, several phenotypes are variable within these bacteria including the number and length of stalks, presence of gas vesicles, and utilization of a dimorphic lifestyle. For example, *Prosthecomicrobium litoralum* is non-motile with multiple, short stalks [52], while *Ancalomicrobium adetum* is non-motile, contains gas vesicles, and has 1-20 long stalks that can be bifurcated at the ends [51]. Although, the mechanisms of stalk biosynthesis in the prosthecate budding bacteria remain wholly unexplored, it's intriguing to speculate that the long, bifurcated stalks of *A. adetum* may be a product of growth at the cell body-stalk junction to elongate the stalk and growth at the tip of the stalk to generate the bifurcated ends. Furthermore, how is the placement and arrangement of multiple long- or short-stalks determined? Studies exploring the synthesis and regulation of stalks in the prosthecate budding bacteria will contribute to our understanding of more specialized growth patterns.

Among the prosthecate budding bacteria, the cell cycle and growth pattern is best characterized in *Prosthecomicrobium hirschii* (Figure 1.1b:11; Figure 1.2b). *P. hirschii* can adopt a pleomorphic lifestyle, with both a non-motile, long-stalked form with 3-12 long stalks (Figure 1.2b, right) and a motile, short-stalked form with numerous, regularly placed stalks (Figure 1.2b, left). Both short-stalked and long-stalked forms persist in the same culture [Figure 1b:11; 51, 53], and short-stalked cells of *P. hirschii* have a dimorphic lifestyle [Figure 2b, 54]. Swarmer cells have a single polar flagellum that is shed during the transition into a sessile cell capable of elongating. Labeling of short-stalked cells with FDAAs confirmed that short-stalked cells grow by polar budding. Furthermore, prior to cell division new cell wall material is synthesized at mid-cell. Under the conditions tested, a short-stalked mother cell gave rise to a short-stalked daughter cell 99% of the time. Rarely, a short-stalked mother cell would give rise to a long-stalked daughter cell. The long-stalked morphotype is non-motile, and is equally likely to give rise to either another long-stalked cell or a short-stalked cell. Once differentiated into a long or short-stalked cell, the mother cell generally produces daughter cells of the same morphology for several generations. The variations in the Alphaproteobacterial growth cycle that allow for such morphological plasticity to emerge remains to be determined, and some key questions regarding *P. hirschii* cell cycle remain to be addressed. At what level is the switch between the short-stalked and long-stalked morphotypes regulated? Perhaps the transition is regulated at the genetic level via DNA methylation or gene silencing. Alternatively, the switch may be determined at the cellular level via quorum

sensing. Ultimately, identifying the trigger for morphological changes will allow for a better understanding of how the cell elongation machinery is regulated during short and long-stalked growth of *P. hirschii*, and will likely inform studies of other prosthecate budding bacteria with pleomorphic lifestyles.

Unipolar Elongation of Rod-shaped Bacteria

Bacteria with unusual morphologies such as stalks and prosthecae easily attracted scientific interest, and observations led to the hypothesis that these bacteria grow by budding; however, many genera in the *Rhizobiales* such as *Agrobacterium* (Figure 1.1b:9) and *Brucella* (Figure 1.1b:10) have a typical rod-shaped morphology. Despite their close relationship to other budding bacteria, it was assumed that the rod-shaped species in the *Rhizobiales* elongate using lateral insertion of peptidoglycan along the sidewall since this mechanism is shared by most rod-shaped bacteria such as *E. coli* [55].

An early indication that rod-shaped bacteria in the *Rhizobiales* may use an alternative mechanism of cell elongation is the atypical morphologies induced by blocks in cell division. When cell division is blocked, most rod-shaped bacteria such as *E. coli* form long, smooth filaments [56]. In contrast, blocking cell division in *Rhizobium* and *Agrobacterium* species induces branching and bulging phenotypes [57-59], indicating that these species may grow fundamentally different from other rod-shaped bacteria. Careful examination of *A. tumefaciens* branching mutants concluded that branches were the result of a tip splitting event

of the growing pole [60]. These observations along with genome sequences of several *Rhizobiales* confirms that these bacteria must use an alternative mechanism for cell elongation as the genes encoding the canonical elongation machinery including MreBCD, RodA, and PBP2 are absent from the genomes of all sequenced *Rhizobiales* [27, 61, 62].

A combination of time-lapse microscopy and labeling studies using Texas Red succinimidyl ester (TRSE), D-cys, or FDAAs led to the finding that *Agrobacterium tumefaciens* undergoes unipolar elongation [13, 27]. Since then, *A. tumefaciens* has emerged as a model bacterial system for mechanistic studies of unipolar elongation, which consists of three phases (Figure 1.2c). First, peptidoglycan is inserted at the new pole of the cell to allow cells to increase in length. Next, growth is terminated from the new pole and resumes near mid-cell. Finally, cell division occurs, producing two new poles which are primed for unipolar growth. While the growth pattern has been well-established, key questions remain. What enzymes are required for polar elongation? How is growth restricted to the pole during elongation? How is unipolar growth coordinated with other processes such as DNA replication, chromosome segregation, and cell division?

A bioinformatics approach to mine the *A. tumefaciens* genome for genes encoding enzymes capable of peptidoglycan biosynthesis revealed the presence of six genes encoding high molecular weight (HMW) PBPs [63]. A subset of these PBPs are likely to be recruited to the growth pole and function during

cellular elongation. Labeling of *A. tumefaciens* cells with a fluorescent antibiotic that binds PBPs confirms that at least some PBPs are localized to the growth pole, and citrine-PBP1a is observed at the growth pole [63]. The chemical composition of *A. tumefaciens* peptidoglycan also suggests a potential role for LD-transpeptidases (LDTs) in peptidoglycan biosynthesis [27]. LDTs are enriched in the genomes of *Rhizobiales* and at least one LDT localizes to the growth pole of *A. tumefaciens* supporting the hypothesis that these enzymes may contribute to unipolar growth [63]. Additional investigations are needed to determine the contributions of PBPs and LDTs to peptidoglycan insertion at the pole during elongation, at mid-cell prior to cell division, or to both processes. In addition, it will be necessary to identify and characterize the proteins that are responsible for spatial and temporal regulation of the activity of the biosynthetic enzymes.

While the candidate enzymes for polar peptidoglycan insertion have been identified, regulatory mechanisms which restrict growth to the pole during elongation remain elusive. Polarity determinants with potential to serve as scaffolding proteins including FtsZ and FtsA persist at the growth pole [63, 64]; however, these proteins likely primarily function in cell division. Indeed, depletion of FtsZ does not block unipolar growth [26]. The polar organizing protein, PopZ also persists at the growth pole [65], but deletion or depletion of PopZ does not block unipolar growth [66, 67]. Instead, the loss of PopZ severely impairs chromosome segregation [68]. Together these observations suggest that the growth pole is an important hub for the coordination of several important cell

cycle events including elongation, DNA replication, chromosome segregation, and cell division. The mechanisms required to enable peptidoglycan insertion strictly at the growth pole during elongation and to understand how cell cycle events are coordinated remain to be addressed.

While *A. tumefaciens* is emerging as a model for the study of unipolar growth in rod-shaped Alphaproteobacteria, bioinformatics searches illustrate that all *Rhizobiales* genomes lack the canonical machinery for dispersed lateral peptidoglycan insertion [27, 61, 62]. This observation suggests other rod-shaped bacteria within the *Rhizobiales* may be likely to exhibit unipolar growth. TRSE or D-cys labeling experiments in *Brucella abortus*, *Sinorhizobium meliloti*, and *Ochrobactrum anthropic* have confirmed unipolar growth is the main mode of elongation in these rod-shaped species as well [27]. Therefore, studies that elucidate the mechanisms of unipolar growth in the plant pathogen *A. tumefaciens* will likely be broadly applicable to other *Rhizobiales* species, including plant symbionts and human pathogens. Together, these data suggest that members of the order *Rhizobiales* elongate through different modes of budding that includes stalked budding, prosthecate budding, and unipolar growth. The prevalence of these growth modes indicates that budding is widespread in the *Rhizobiales*, and raises questions such as, what is the advantage of unipolar growth and why is one mode of budding preferred over another? Ongoing studies in the *Rhizobiales*, using a combination of in silico, genetic, and imaging approaches may shed light on these questions.

Diverse growth patterns of stalked bacteria in the Caulobacterales

The Alphaproteobacterial order *Caulobacterales* is morphologically diverse with many species capable of producing stalks. Some of the stalked bacteria reproduce by budding using the stalks as reproductive structures (Figure 1.1b:1-2). The stalked budding bacteria currently includes three genera: *Hyphomonas* (Figure 1.1b:1; Figure 1.2d), *Hirschia* (Figure 1.1b:2), and *Hellea*. In contrast, the stalks are not used as reproductive structures in a distinct subset of the stalked bacteria (Figure 1.1b:5-8), which includes the well-characterized *Caulobacter crescentus* (Figure 1.1b:7; Figure 1.2e). *C. crescentus* uses dispersed insertion of peptidoglycan along the lateral sidewalls during elongation of the main cell body and a specialized form of zonal growth to promote stalk elongation. Non-reproductive stalks are thought to function in nutrient uptake and starvation triggers stalk elongation [69]. Finally, some members of the *Caulobacterales* do not produce stalks. It is generally inferred that these bacteria elongate using the canonical MreB-dependent mechanism; however, few detailed studies of the growth pattern in these bacteria have been completed.

Stalked budding bacteria within the Caulobacterales

Among the genera of stalked budding bacteria in the *Caulobacterales*, the growth pattern is best characterized in *Hyphomonas* (Figure 1.2d). Based on its morphology and development, the stalked budding bacterium *Hyphomonas neptunium* was originally characterized as a member of the genus

Hyphomicrobium [70], but was later reclassified into the order *Caulobacterales* based on metabolic analysis, DNA hybridization and 16S rRNA sequencing [71, 72]. Despite the reclassification, *Hyphomonas* species have a similar cell cycle and morphology to the stalked budding bacteria in the *Rhizobiales* [Compare figures 2a and 2d, 73]. Stalk formation in *Hyphomonas* is essential for reproduction, and inhibition of DNA synthesis with nalidixic acid results in elongated stalks with no buds, suggesting bud formation is coupled to chromosome replication [74]. Additionally, the stalk cell membrane and cell wall is contiguous with the mother cell body, and a bud forms at the tip of the stalk [73]. The recent development of genetic tools for the manipulation of *Hyphomonas neptunium* [75] has opened doors to more in-depth studies of the growth and morphology of stalked budding bacteria.

Growth patterns during the *H. neptunium* cell cycle were revealed by FDAA labeling, and four distinct regions of peptidoglycan insertion were identified, which involves alternating rounds of dispersed and zonal peptidoglycan insertion [Figure 2D; 76]. First, dispersed peptidoglycan insertion occurs in the swarmer cell, followed by zonal peptidoglycan insertion at the base of the stalk to promote stalk growth. As the stalk forms, the cell becomes rounded near the old pole, and tapered at the stalked pole forming a tear drop shape. Upon completion of stalk elongation, another round of dispersed peptidoglycan insertion at the tip of the stalk occurs, which forms the daughter cell bud. Finally, zonal peptidoglycan insertion at the daughter cell bud neck drives cell division to release the newly

formed daughter cell. The initial time for a mother cell to grow a stalk and produce a bud is approximately 4 hours, but once the stalk is formed, new buds form at the tip of the stalk every 2.5 hours [76].

In contrast to the *Rhizobiales*, *H. neptunium* encodes genes for all the essential elongasome proteins including MreB and PBP2. Consistent with the zones of peptidoglycan insertion during growth, both MreB and PBP2 localize in a diffuse pattern in the mother cell, then to the base of the stalk during stalk elongation, and again in a diffuse pattern in the newly forming daughter cell [76]. Based on their localization patterns and the inability to construct deletion mutants, it appears that both MreB and PBP2 are essential members of the elongasome complex. Other peptidoglycan remodeling enzymes also likely contribute to elongation in *Hyphomonas*. For instance, *H. neptunium* contains three D-alanine carboxypeptidases, of which DacB is homologous to *E. coli* DacN, and DacH and DacL are specific to *H. neptunium*. While only the *dacB* mutant has morphological defects, DacL localizes to the base of the stalk during stalk elongation [76]. Identification and characterization of additional proteins unique to the stalked-budding bacteria and that localize to sites of elongation will help identify key features of the stalked budding mode of growth. Given that *H. neptunium* and *C. crescentus* encode similar elongasome proteins and display similar peptidoglycan profiles, it is likely that the differences in growth patterning are a product of novel binding partners that specifically regulate stalk synthesis and the transition between stalk and dispersed growth. Therefore, dissecting the

protein-protein interactions of elongasome machinery is an interesting avenue for future studies.

Other stalked budding genera in the *Caulobacterales* include *Hirschia* and *Hellea* [77, 78]. While detailed mechanistic studies of growth patterning have not been completed, both bacteria are reported to grow by budding from the end of a stalk. The completion of the genome of the type species *Hirschia baltica* [79] and *Hellea balneolensis* should enable comparative genomic approaches to uncover features that are common among the marine stalked budding bacteria. Since Alphaproteobacteria represent a majority of marine bacteria [3] these types of studies have the potential to identify proteins essential for stalked budding and may reveal the adaptations required for a dimorphic lifestyle in marine environments.

Caulobacterales with non-reproductive stalks

Bacteria with stalks are ubiquitous in water samples, and several genera of stalked bacteria have been identified including: *Caulobacter*, *Asticcacaulis*, and *Brevundimonas* (Figure 1.1b:5-8). *Caulobacterales* stalks can vary in their length, placement, and number depending on species. Stalks in the order *Caulobacterales* are non-reproductive extensions of the main cell body that are devoid of cytoplasmic proteins and DNA, and are composed of a cell envelope that is separated by protein cross-bands [80].

The stalk likely functions in nutrient uptake [81-87], and in phosphate-limiting conditions the *C. crescentus* stalk can elongate up to 10 times its normal length, which is thought to increase the rate of phosphate uptake [69, 88]. In *C. crescentus*, stalk lengthening may not only be a mechanism to increase nutrient uptake. Instead, phosphate levels could be used as a proxy for the amount of available nutrients and stalk lengthening enables the cell body to move away from the surface and into a more favorable nutrient environment [89]. This is a viable model for *C. crescentus* since the stalk is polarly localized (Figure 1.1b:7), and the tip of the stalk produces an adhesin used for attachment to surfaces [90-92]. In contrast, stalks in *Asticcacaulis excentricus* are typically sub-polar (Figure 1b:8), and the adhesin is present on the cell pole [90]. *Asticcacaulis biprosthecum* produces two lateral stalks on opposite sides of the cell body [Figure 1b:5; 93]. Thus, *Asticcacaulis* stalks are not capped with an adhesin and do not contribute to surface attachment, but the stalks still elongate in phosphate limiting conditions. Finally, most *Brevundimonas* species produce short, polar stalks that are capped with a polar adhesin [Figure 1b:6; 85]. A survey of 18 different *Brevundimonas* species showed that stalk formation is rare in nutrient rich conditions and that stalks may be produced on an as-needed basis [85]. This illustrates that stalk formation is highly variable between species, and under different nutrient conditions.

Although the exact function of the stalk remains elusive, the mechanism of stalk biosynthesis has been examined. In *C. crescentus*, stalk elongation requires the

localized insertion of peptidoglycan at the base of the stalk, which is dependent on the elongation-specific machinery MreB, RodZ and RodA [94]. In addition, the cytoskeletal bactofilins BacA/B localize to the stalked pole and act as a scaffold to recruit the DD-transpeptidase PBPC to the base of the stalk [95]. In turn, PBPC recruits the stalk-specific protein StpX [82, 96]. Several proteins required for stalk synthesis have been identified, and further investigations of stalk-specific proteins will likely lead to a more complete understanding of how the stalk is built. It is known that stalk synthesis is coupled to cell cycle progression in *C. crescentus*. In fact, stalk synthesis is transcriptionally regulated by a cytoplasmic phosphorelay signaling pathway, suggesting stalk growth can be controlled from intracellular signals [97, 98]. Regulation of stalk synthesis is likely multi-faceted, including external environmental signals such as phosphate levels and unknown internal signals. Given that stalk synthesis is not cell-cycle regulated in *Brevundimonas* and that there is little conservation of stalk-associated proteins between *Caulobacter* and *Brevudimonas* species [85], it will be necessary to explore the mechanisms of stalk biogenesis in multiple stalked bacteria.

In addition to studies detailing the mechanism of stalk biogenesis, labeling and localization studies in *C. crescentus* have shed light on the complex growth patterns that give rise to asymmetric growth and division. D-cys labeling of peptidoglycan and localization of the key elongation-specific proteins MreB and MurG, showed that cell elongation in *C. crescentus* occurs by two distinct mechanisms: dispersed growth in swarmer cells, and pre-septal elongation in

early and late-pre-divisional cells [Figure 2e; 99, 100]. The *Caulobacter* cell cycle begins with a motile swarmer cell that grows in a dispersed manner (Figure 1.2e green) characterized by diffuse patterns of MreB and MurG [99]. Based on conservation of the core cell elongation proteins, dispersed growth of swarmer cells in *C. crescentus* likely occurs by a similar mechanism to *E. coli* and *B. subtilis*. Next, the swarmer cell sheds its flagella and transitions into a sessile cell. In this early pre-septal cell, FtsZ is recruited to the mid-cell and in turn recruits MreB and MurG. Bands of FtsZ, MreB and MurG at mid-cell initiate an FtsZ-dependent mode of pre-septal elongation that is responsible for the majority of peptidoglycan biosynthesis during elongation (Figure 1.2e red). FtsZ is known to be a scaffold for peptidoglycan biosynthesis during cell division, but surprisingly it may also play a major role in cell elongation in many Alphaproteobacteria. Prior to cell division, MreB and MurG become dispersed again in the new pole daughter cell, while the stalked daughter cell resumes pre-septal elongation [99, 100]. Thus, growth patterning in *C. crescentus* has been well characterized, and shown to be regulated in part by specific scaffolding proteins. However, several questions on how the peptidoglycan biosynthesis machinery and cytoskeletal regulators interact and coordinate throughout the cell cycle remain to be explored. Since many aspects of the *Caulobacter* cell cycle are known to be under transcriptional, translational and post-translational control, how is growth patterning also regulated at each level? *C. crescentus* is a well-established model system that is poised to answer some of the detailed mechanistic questions regarding regulation of cell growth patterning in the

Alphaproteobacteria; however, the growth patterns of *Asticcacaulis* and *Brevundimonas* should also be experimentally determined as they may be distinct.

Cell growth in the Rhodospirillales

Across the Alphaproteobacteria, many species have adapted strategies that allow them to occupy diverse niches (Figure 1.1a). One of the most unique mechanisms is employed by magnetotactic bacteria in the order *Rhodospirillales*. Magnetotactic bacteria align themselves with the magnetic field of the earth to navigate toward microaerophilic environments mainly in fresh and salt water environments [101]. This type of motility is called magnetotaxis, and requires magnetotactic bacteria to synthesize magnetosomes, which are comprised of crystals of magnetite found in inner membrane invaginations [102]. The cell cycle of magnetotactic bacteria must be highly coordinated to ensure that both daughter cells inherit magnetosomes, but cell growth and division of magnetotactic bacteria is just beginning to be resolved. Magnetotactic bacteria exhibit diversity in cell shape, as spiral, cocci, and vibrioid morphologies have been described [103-105]. Relatively little is known about the growth and development of coccus and vibrio-shaped magnetotactic bacteria, however *Magnetospirillum gryphiswaldense* (Figure 1.1b:9) is emerging as model for mechanistic studies of cell cycle progression.

Studies in *M. gryphiswaldense* have primarily focused on the spatiotemporal regulation of magnetosomes during the cell cycle of magnetotactic bacteria [106-108]; however, these studies have also provided some indirect clues about the growth patterning in this bacterium. Tracking polyhydroxybutyrate (PHB) granules in elongating cells of *M. gryphiswaldense* revealed that PHB granules near the mid-cell separate as the cells grow in length, whereas PHB granules near the pole remain fixed [107]. This suggests a mechanism of growth reminiscent of pre-septal elongation in *C. crescentus* [99, 100]. Treatment of *M. gryphiswaldense* with cephalixin causes cells to filament, similar to *C. crescentus*, consistent with the possibility that a combination of lateral growth and pre-septal elongation may be used during elongation. Following cell division, the two daughter cells often have different sizes: one daughter cell is typically ~15% smaller than the other daughter cell. Uneven daughter cell length suggests the constriction sites are placed asymmetrically and is consistent with the presence of an Alphaproteobacterial cell cycle in *Magnetospirilla* [107]. The specific mechanisms of elongation and division in *M. gryphiswaldense*, and whether these mechanisms are conserved in non-magnetic *Rhodospirillales* such as *Rhodospirillum rubrum* awaits characterization.

Another group within the *Rhodospirillales* contains prosthecate bacteria with a unique, star-shaped morphology. *Stella humosa* is the type strain for this group and resembles a flat, six-pointed star with radial symmetry [Figure 1b, 12; 109]. Microscopic observations of *S. humosa* illustrate that this bacterium divides by

forming a cross-wall between its narrowest sides. After division each daughter cell has three prosthecae and must regenerate three new prosthecae to maintain its six-pronged morphology [109]. The star-shaped morphology only persists in low-nutrient conditions, and these bacteria can be asymmetrical or sphere-shaped under different environmental conditions [110]. *S. humosa* and other members of the prosthecate *Rhodospirillales* are a fascinating example of the breadth of bacterial morphologies that await further characterization.

The *Rhodospirillales* also includes the acetic acid bacteria, which are able to grow in ethanol and sugar-rich environments, and are often insect symbionts [111]. Although little is known about the mechanisms of elongation in the acetic acid bacteria, a recent analysis of 20 species representing the five major orders (*Rickettsiales* was not included) revealed that the peptidoglycan of the Alphaproteobacteria clusters into three groups based on similar peptidoglycan composition [112]. Group 1 was exclusive to the acetic acid bacteria of the *Rhodospirillales*, and peptidoglycan from group 1 was surprisingly distinct from the other two groups, containing novel, LD-(1-3) crosslinks and acylated mucopeptides. These novel modifications were shown to confer resistance to endopeptidases of co-habiting bacteria and evade recognition by host immune responses, respectively. Therefore, the acetic acid bacteria may have evolved novel mechanisms for the synthesis or modification of peptidoglycan. Characterization of growth patterning in the acetic acid bacteria remains an interesting area for future investigations.

Cell growth in the Rhodobacterales

Like many Alphaproteobacteria, species in the order *Rhodobacterales* demonstrate features of asymmetry, including rosette formation, polar holdfast production, and motile and non-motile cells in the same culture [113-115]. Since budding typically results in a narrower, pointed, or tapered cell pole prior to bud development these morphologies are used to identify species that likely elongate by budding. Many *Rhodobacterales* have these characteristics [Figure 1b:13-15; 116, 117]. For example, both *Phaeobacter inhibens* and *Sagittula stellata* are clearly narrower at one pole and produce a unipolar polysaccharide at the opposite cell pole which enables rosette formation [118, 119]. Together, these observations suggest that many *Rhodobacterales* may utilize budding as a mode of cell growth although their growth patterning is largely unexplored. More studies, including time-lapse microscopy, FDAA, D-cys, or TRSE labeling, are needed to better characterize the mode of growth utilized by *Rhodobacterales*. Two candidates: *Rhodobacter sphaeroides* and members of the *Roseobacter* clade are emerging as candidate model systems for the study of growth patterning in the *Rhodobacterales*.

R. sphaeroides is emerging as a model for studies of the bacterial cell cycle and its regulation. In particular, studies of motility [120], quorum sensing [121], and chemotaxis [122] have been conducted. Morphological changes induced by antibiotic treatment suggest *R. sphaeroides* may utilize a MreB-dependent mode of pre-septal elongation. Treatment of cells with the PBP2 targeting antibiotic

amdinocillin causes mid-cell bulging, suggesting peptidoglycan insertion mainly occurs at or near mid-cell in elongating cells [123, 124]. Accordingly, PBP2, MreB and MreC fused to fluorescent proteins form bands or rings at mid-cell in elongating cells. Once constrictions form, PBP2 transits from mid-cell to the three-quarter site of each daughter cell. PBP2 localization to the future division site in cells with a septum may indicate that both elongation and division machinery are active in pre-divisional cells concurrently [123, 124]. Notably, the *R. sphaeroides* genome also contains a gene predicted to encode a PBP3 homolog, which presumably functions during division. It remains to be determined if both PBP2 and PBP3 function in discrete complexes for elongation and division simultaneously. In addition to the impact of antibiotic treatments, the environment can influence cell shape. For example, during aerobic growth, *R. sphaeroides* cells have a rod-shaped morphology with tapered ends, but adopt an ovococcal morphology with extensive membrane invaginations when growing photoheterotrophically [125, 126]. Thus, *R. sphaeroides* would be a promising model system to study cell growth patterning during rearrangements of cell shape in response to environmental conditions.

Marine niches, including open ocean, coastal waters and sea ice are largely colonized by species of the *Roseobacter* clade [127, 128]. Many marine *Roseobacters* are found in phytoplankton communities, in symbiosis with marine eukaryotes, and some can cause disease [128]. Interestingly, several *Prionitis* species were shown to form galls on marine algae [129],

reminiscent of crown galls induced by *A. tumefaciens* infection. The marine algae symbiont *Dinoroseobacter shibae* displays remarkable diversity in cell shape [130]. In this species, small ovoid or rod-shaped cells coexist with highly elongated and swollen cells in culture. Tracking growth of individual bacteria demonstrated that many *D. shibae* cells elongate by polar budding, and some cells alternate growth from pole-to-pole. In some cases, extreme asymmetric cell division events lead to the production of a large, swollen cell and a smaller, pleomorphic cell. Remarkably, in the same culture some ovoid cells undergo symmetric cell division events producing two identical ovoid-shaped cells. This phenotypic variation is controlled by quorum sensing (QS), and QS mutants grow as a population of homogenous ovoid cells [130]. The control of morphological variation by QS is an exciting and novel discovery that requires further investigation to determine its prevalence within the Alphaproteobacteria. In addition, mutation of the cell cycle control genes *ctrA*, *chpT* and *cckA* each result in a loss of phenotypic variation in *D. shibae*. Together, these results may indicate that the CtrA phosphorelay is integrated into the QS signaling pathway and contributes to the regulation of morphological heterogeneity [131]. Since many *Roseobacter* species display morphological heterogeneity and may grow by polar elongation, additional members of this bacterial family are prime candidates for the study of growth patterning in marine Alphaproteobacteria.

Cell Growth in Rickettsiales

Species in the order *Rickettsiales* have lost the ability to replicate outside of eukaryotic host cells, and they comprise many species that cause disease in humans. There are two families in the order *Rickettsiales*: *Rickettsiaceae* and *Anaplasmataceae*. The *Rickettsiaceae* are maintained through various animal reservoirs (Figure 1.1a), and can be spread to humans through arthropod vectors such as flea, tick, louse or mite [132, 133]. Two well-known species include *Rickettsia typhi*, which causes typhus and *Rickettsia rickettsii* which causes Rocky Mountain spotted fever. Both bacteria primarily replicate in the cytoplasm of vascular endothelial cells during human infections [133]. The *Anaplasmatceae* are generally transferred to humans through the bite of a tick, and can replicate in the vacuole of a variety of immune cells including erythrocytes, neutrophils, monocytes, macrophages, and endothelial cells [134].

Since the *Rickettsia* have adopted an obligate intracellular lifestyle, they have reduced genome sizes compared to closely related free-living bacteria. In addition, many *Rickettsiales* require less peptidoglycan since they replicate inside cells with an osmotically stable environment [135]. Given their obligate intracellular lifestyle, *Rickettsiales* are challenging to culture, which has hampered the study of cell growth patterning; however, comparative genomic analyses have provided some insight into the mechanisms of elongation. Species that do not encode genes for lipid II biosynthesis (MurA-G, MurY), SEDS proteins (FtsW, RodA), or class A (PBP1a/b) and class B PBPs (PBP2/3) do not synthesize a detectable peptidoglycan structure; this includes: *Anaplasma*

phagocytophilum, *Ehrlichia ruminantium*, and *Neorickettsia sennetsu* [136].

Based on the absence of these signature peptidoglycan proteins, several *Anaplasma*, *Ehrlichia* and *Neorickettsia* species may lack peptidoglycan.

Interestingly, a group of species that were classified as having low levels or incomplete peptidoglycan were shown to encode a subset of proteins involved in peptidoglycan biosynthesis [136]. The genomes of peptidoglycan intermediate species encode the full set of lipid II biosynthesis proteins, at least one of the SEDS proteins, and at least one class B PBP, but no class A PBPs. For example, *Anaplasma marginale*, *Wolbachia* species, and *Orientia tsutsugamushi* may all synthesize an intermediate peptidoglycan. Indeed, lipid II biosynthesis genes and the gene encoding PBP2 are expressed in *O. tsutsugamushi* and cells label with FDAAs, suggesting that peptidoglycan is indeed synthesized in this bacterium [137]. Peptidoglycan precursor pathways are also found in *Wolbachia* and lipid II is required for proper cell division, although mature peptidoglycan has not been detected [138].

Finally, several species in the genus *Rickettsia* encode homologs for the full set of peptidoglycan predictor proteins, and therefore are expected to synthesize classical peptidoglycan [136]. The notion that closely related bacteria have different categories of peptidoglycan may be supported by the fact that these species occupy different intracellular niches. For example, *A. phagocytophilum*,

which lacks peptidoglycan, localizes to monocytes and macrophages, while *A. marginale*, which synthesizes an intermediate peptidoglycan, inhabits erythrocytes. The bioinformatics approach has provided predictions for the likelihood of peptidoglycan biosynthesis within the *Rickettsiales* [136] which can be tested. Observations of cell growth patterning using FDAAs is a powerful tool to determine how the synthesis of intermediate and classical peptidoglycan is spatially and temporally regulated in the *Rickettsiales*.

Conservation of Core Elongation Machinery in the Alphaproteobacteria

The bacterial cell wall is a stress-bearing meshwork of proteins and sugars that is considered a major determinant of bacterial cell shape. Construction of peptidoglycan requires scaffolding, synthesis, and remodeling proteins that are well conserved across the domain Bacteria. The expansion of genes that encode PBPs and LDTs and the evolution of stalk-specific proteins are just a few examples of the diversification of the core cell elongation machinery that impacts bacterial morphology within the Alphaproteobacteria. Furthermore, the novel growth modes and phenotypic variation observed within the Alphaproteobacteria suggest that these bacteria must have evolved novel mechanisms to direct and regulate the activities of enzymes belonging to the core elongation machinery. Some regulators of peptidoglycan biosynthesis such as LpoA/B have been identified in *E. coli* [139, 140]; however, in the Alphaproteobacteria these regulatory elements remain largely unknown. Here, we provide a brief survey of the predicted peptidoglycan biosynthesis machinery found in genomes of

Alphaproteobacteria and consider some possible mechanisms to regulate peptidoglycan biosynthesis.

Survey of Peptidoglycan Biosynthesis Machinery

The rigid structure of the bacterial cell wall is largely responsible for cell shape, and expansion of new cell wall requires two main types of enzymes: glycosyltransferases to attach glycan chains, and DD-transpeptidases to polymerize peptide crosslinks. In *E. coli*, bifunctional (Class A) and monofunctional (Class B) PBPs either function during elongation (PBP1a, PBP2), or during division (PBP1b, PBP3), while the function of PBP1c is not well understood [141]. Since the high molecular weight (HMW) PBPs synthesize new peptidoglycan during elongation, division, and stalk growth, any gene loss, duplication or evolutionary divergence of HMW PBPs may impact cell growth patterning and morphology. Thus, we carried out a phylogenetic analysis comparing the HMW PBPs from representative species shown in Figure 1a, which encompasses all six orders of Alphaproteobacteria. Most PBPs group into clades with their respective *E. coli* homologs, suggesting that they may perform similar functions to the well-characterized *E. coli* enzymes (Figure 1.3a). A comparative analysis shows that homologs of PBP1a, PBP1b, PBP1c, PBP2 and PBP3 form distinct clades and PBPs largely group together based on bacterial order (Figure 1.3a). This analysis confirms impact of genome reduction on the presence of class A PBPs (PBP1a/b) within the *Rickettsiales* (Figure 1.3a) (See section 6).

Figure 1.3: Distribution and Domain Comparison of the High Molecular Weight (HMW) Penicillin-binding Proteins (PBPs). Phylogenetic trees from A and B were constructed using a ClustalW protein alignment [142] and creating a neighbor

joining tree using Geneious Tree Builder with 100 bootstrap datasets. Tree scales represent the branch length with 0.1 substitutions per site. (A). Phylogeny of HMW PBPs from 41 representative species of Alphaproteobacteria was formatted using Dendroscope [143]. PBPs are color coded by order as listed in the key. Leaves contain multiple species of the same order, and the size of a leaf represents the number of species in that leaf. The symbols next to the solid green leaves represent the species used to make the tree in B. (B) Phylogeny of PBP1b genes of 12 species of *Rhizobiales*, and *E. coli* PBP1a and PBP1b was formatted using iTOL [8]. The genus and NCBI Accession number for each gene are labeled. (C) Domain analysis of PBP1a and PBP1b genes. Transmembrane domain is purple, Glycosyltransferase (GT) is blue, Transpeptidase (TP) is orange, regions of intrinsic disorder are grey and were annotated using MobiDB [144], and BA14K-like Protein domain is red.

Remarkably, within the *Rhizobiales*, the distribution of PBPs is distinct from other Alphaproteobacterial orders. First, the elongation-specific PBP2 is universally absent (Figure 1.3a). This observation coupled with the absence of MreBCD and RodA is consistent with the hypothesis that a novel pathway for cell elongation has evolved in the *Rhizobiales*. Second, the gene encoding PBP1b has been universally duplicated. Phylogenetic analysis of the PBP1b proteins from *Rhizobiales* species indicates the presence of two distinct clades (Figure 1.3b). Each clade contains a PBP1b protein from each bacterial species, suggesting an ancient duplication event. Remarkably, the two PBP1b clades are more similar to

one another than either is to PBP1b from *E. coli* [Figure 3b; 63], perhaps suggesting functional divergence. Functional studies of the Alphaproteobacterium-specific PBP1b proteins may provide insights into novel species-specific or environment-specific growth patterns and morphologies. It will be of particular interest to determine if the Alphaproteobacterial PBP1b proteins are division-specific or have an alternative function in peptidoglycan biosynthesis. Finally, two PBP3 proteins are observed within a subset of *Rhizobiales* species (Figure 1.3a). Notably, most species with a duplication in the gene encoding PBP3 interact with plants (*Agrobacterium*, *Sinorhizobium*), raising the possibility that there may be a function for PBP3 when the bacterium is host-associated.

In addition to surveying the PBP distribution, it is also necessary to consider the distribution and function of other enzymes which may contribute to peptidoglycan biosynthesis. Currently, the number and distribution of peptidoglycan remodeling enzymes across the Alphaproteobacteria remains poorly characterized. There has been an obvious expansion of LD-transpeptidases (LDTs), particularly within the *Rhizobiales* and *Rhodobacterales* orders [63]. In *E. coli*, LDTs are typically considered to be peptidoglycan recycling enzymes with a limited role in peptidoglycan biosynthesis [145]. As well as homologs of *E. coli* LDTs, there are *Rhizobiales*- and *Rhodobacterales*-specific clades of LDTs [63]. Consistent with this observation, compositional analysis of mucopeptides from some species of *Rhizobiales* and *Rhodobacterales* reveals an increase in crosslinks formed by LDTs [27, 112]. These observations raise the possibility that LDTs may have a

more significant role in peptidoglycan biosynthesis in some Alphaproteobacteria. Furthermore, there is a *Rhizobiales*-specific clade of low molecular weight PBPs [LMW-PBPs; 63]. LMW-PBPs typically function as peptidoglycan hydrolases [146-148]; however, a functionally unique family of LMW-PBPs with transpeptidase activity has been described [149] raising the possibility of functional divergence of LMW-PBPs. Overall, there is a clear need for more bioinformatic and molecular studies to identify and characterize peptidoglycan remodeling enzymes in the Alphaproteobacteria. Certainly, understanding the landscape of peptidoglycan biosynthesis enzymes will help direct future studies of growth patterning.

Regulation of peptidoglycan biosynthesis machinery

In addition to considering the function of enzymes involved in peptidoglycan synthesis, it is necessary to identify and characterize the proteins that regulate the activity of these enzymes. The presence of species-specific domains in peptidoglycan synthesis and remodeling enzymes suggests that the activity of some of these enzymes may be regulated directly. The crystal structure of *Acinetobacter baumannii* PBP1a revealed a non-catalytic oligonucleotide–oligosaccharidebinding (OB)-fold domain near the TP domain [150] that is also present in some Alphaproteobacterial PBP1a proteins (Figure 1.13c, green). The functional significance of this domain remains to be explored, but it is intriguing to speculate that this domain may be involved in regulation of PBP1a activity. Furthermore, intrinsically disordered (ID) domains exhibit conformational

flexibility [151, 152] and as such are candidate regions to facilitate the regulation of PBP activity. Alphaproteobacterial PBP1a and PBP1b proteins are enriched for regions of intrinsic disorder (Figure 1.3c, grey). While ID domains are present mainly at the C-terminus of PBP1a, the location of ID domains in PBP1b genes is more diverse (Figure 1.3c). The presence of ID domains before the transmembrane domain suggests that PBP1b may have novel binding partners in the cytoplasm and N-terminal ID domains may bind periplasmic proteins. The majority of ID domains in PBPs are uncharacterized in the Alphaproteobacteria, and a better understanding of these domains has the potential to further our understanding of the regulation of PBP activity.

The localization of PBPs to specific sites of growth by interactions with a widely conserved set of core cytoskeletal proteins is a common theme in peptidoglycan regulation [153]. During *E. coli* elongation, MreB targets PBP2 to patches along the lateral wall. Similarly, during division, FtsZ filaments provide a scaffold for PBP3 and other cell division machinery. Bactofilins are widely distributed albeit less well-characterized cytoskeletal elements that may play a role in spatial regulation of peptidoglycan biosynthesis. In *Caulobacter*, BacA and BacB localize to the stalked cell pole during the swarmer-to-stalked cell transition, and assemble into polymers that recruit an Alphaproteobacterium-specific PBP1b homolog (PBPC) to the pole [95]. Bactofilins are widely distributed across the Alphaproteobacteria with most species having 1 homolog. In contrast, the *Rhizobiales* typically have no bactofilin homologs with the exception of the

stalked budding bacteria *Hyphomicrobium* and *Rhodomicrobium*, which have 4 and 3 bactofilins, respectively. Understanding how the distribution of core cytoskeletal elements contributes to the localization and regulation of peptidoglycan biosynthesis enzymes may provide insight into the microbe-specific patterns of peptidoglycan insertion that generate diversity in cell shape. Several key questions regarding the regulation of peptidoglycan biosynthesis remain open-ended. For example, how are peptidoglycan biosynthesis enzymes targeted to the pole in the *Rhizobiales*, which lack both MreB and bactofilin cytoskeletal elements? Do species with multiple bactofilin homologs have additional regulation of peptidoglycan biosynthesis in time and space, or are their functions largely redundant? Exploring the role of bactofilins, intermediate filaments, and polar landmark proteins will likely shed light on the mechanisms of subcellular targeting of peptidoglycan synthesis and regulation.

The Alphaproteobacteria have evolved novel growth patterns through the diversification of the peptidoglycan biosynthesis machinery. Alphaproteobacteria-specific proteins, novel domains, and novel pathways for regulation are all factors that contribute to diverse cell shapes. Future studies should seek to understand how the core peptidoglycan machinery is regulated in time and space.

Concluding Remarks

The molecular basis that determines bacterial shape has been mainly studied in rods and cocci. Expanding the study of cell growth to non-canonical bacteria will deepen our understanding of morphological complexity. The Alphaproteobacteria have evolved several novel mechanisms of cell growth patterning. Novel growth patterns of budding bacteria include: stalked budding, prosthecate budding and unipolar elongation. Additional types of novel growth patterns have also been characterized, which include stalk elongation and pre-septal elongation. Each type of growth patterning correlates to a unique morphology that is specifically adapted to its environment. However, the evolutionary pressures and advantages that drive diversity of shape remain poorly understood. Thus, expanding the repertoire of sequenced genomes will allow for comparative genomic analysis to identify novel growth modes. Evidence suggest multiple mechanisms for arriving at similar morphologies have evolved. As such, a multidisciplinary approach, including imaging, genetic, genomic, and molecular investigations will be required to fully understand the evolution of cell growth patterning. As more diverse species of Alphaproteobacteria continue to be characterized, novel growth patterns may be discovered. For bacteria such as *C. crescentus*, *H. neptunium*, and *A. tumefaciens* we have begun to identify some key mechanistic features that regulate growth patterning. In the *Caulobacteriales*, the core elongation machinery is conserved in many species and its activity is regulated in time and space by protein-protein interactions to ensure that peptidoglycan is made at the right time and place. In contrast, in the *Rhizobiales* the core

elongation machinery remains elusive. Furthermore, detailed studies of growth patterning in many Alphaproteobacteria such as *Sphingomonadales* and acetic acid bacteria remain wholly unexplored highlighting the need to survey growth patterns in diverse Alphaproteobacteria. Finally, detailed studies of elongation in model Alphaproteobacteria are necessary to shed light on the evolution of growth patterning in bacteria. Ultimately, it is an exciting time to study bacterial cell growth patterning in such a morphologically diverse class of bacteria that occupy a wide-range of habitats and include microbes of agricultural, environmental and medical significance.

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CHAPTER 2

Short-stalked *Prosthecomicrobium hirschii* cells have a *Caulobacter*-like cell cycle

Author Contributions

Williams M, Hoffman MD, Daniel JJ, Madren SM, Dhroso A, Giva SA conducted the experiments. Williams M, Hoffman MD and Brown PB designed the experiments. Williams M, Hoffman M, Korkin D and Brown PB analyzed data and Williams M, Hoffman MD and Brown PB wrote and edited the paper.

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ABSTRACT

The dimorphic Alphaproteobacterium *Prosthecomicrobium hirschii* has both short-stalked and long-stalked morphotypes. Notably, these morphologies do not arise from transitions in a cell cycle. Instead, the maternal cell morphology is typically reproduced in daughter cells, which results in microcolonies of a single cell type. In this work, we further characterized the short-stalked cells and found that these cells have a *Caulobacter*-like life cycle in which cell division leads to the generation of two morphologically distinct daughter cells. Using a microfluidic device and total internal reflection fluorescence (TIRF) microscopy, we observed that motile short-stalked cells attach to a surface by means of a polar adhesin. Cells attached at their poles elongate and ultimately release motile daughter cells. Robust biofilm growth occurs in the microfluidic device, enabling the collection of synchronous motile cells and downstream analysis of cell growth and attachment. Analysis of a draft *P. hirschii* genome sequence indicates the presence of CtrA-dependent cell cycle regulation. This characterization of *P. hirschii* will enable future studies on the mechanisms underlying complex morphologies and polymorphic cell cycles.

INTRODUCTION

The Alphaproteobacteria comprise a diverse group of bacteria, including important pathogens of animals (*Brucella* spp. and *Rickettsia* spp.) and plants (*Agrobacterium* spp.), plant symbionts (*Rhizobium* spp., *Sinorhizobium* spp., and

Mesorhizobium spp.), photosynthetic bacteria (*Rhodobacter* spp.), freshwater bacteria (*Caulobacter* spp.), and marine bacteria (*Hyphomonas* spp.). Despite the diverse habitats and lifestyles of these bacteria, many alphaproteobacterial species have a characteristic life cycle that culminates in asymmetric cell division [1-4]. *Caulobacter crescentus* has served as a model bacterial system for the study of cell cycle regulation, and decades of research have provided insights into the mechanism underlying the precise cell cycle control that allows the production of two morphologically and functionally diverse daughter cells, a motile swarmer cell and an adherent stalked cell [5]. A polar adhesin, termed holdfast, is found at the tip of the stalk and is required for permanent attachment to a surface [6].

Bioinformatic analysis of alphaproteobacterial genomes suggests that the core architecture of the regulatory genes that govern *Caulobacter* cell cycle progression is broadly conserved within at least two clades of Alphaproteobacteria, Rhizobiales and Caulobacterales [7, 8]. Furthermore, genes shown to be essential for cell cycle progression in *C. crescentus* have also been shown to have important functions in cell cycle regulation of *Agrobacterium tumefaciens* [9-12], *Sinorhizobium meliloti* [3, 13-16], and *Brucella abortus* [14, 17-20]. Notably, the essentiality and regulons of cell cycle regulators are varied in Alphaproteobacteria, suggesting that mechanisms of cell cycle regulation are plastic and may provide a means of adapting to a particular environment [12, 15, 21].

The ability to produce a polar polysaccharide appears to be a conserved trait among the Alphaproteobacteria. Polar polysaccharides are described for a number of species within the Caulobacterales and Rhizobiales clades [4, 6, 10, 22-26][4, 6, 10, 22–26). A comparison of the genes responsible for the synthesis of the polar polysaccharide among *C. crescentus* and several Rhizobiales species suggests that these genes are largely conserved (see Figure 2.S1 and Table 2.S1 in the Supplementary material) [27]. The production of polar polysaccharides is stimulated by contact with a surface in three alphaproteobacterial species, *C. crescentus*, *Asticcacaulis biprosthecum*, and *A. tumefaciens*, indicating that this may be a general phenomenon [28].

Asymmetric cell division and polar polysaccharide production are likely ancestral traits within the Caulobacterales and Rhizobiales clades, and yet, there are many alphaproteobacterial species for which these traits have not been described. In this work, we have characterized *Prosthecomicrobium hirschii*, a member of the Rhizobiales clade (see Figure 2.S2 in the Supplementary material), which at first glance does not appear to use a regulated cell cycle to generate distinct morphotypes or produce a polar polysaccharide [29]. *P. hirschii* cells adopt one of two morphologies: (i) numerous short stalks or (ii) 3 to 12 markedly longer stalks (Figure 1A) [29]. The short-stalked morphotype of *P. hirschii* has a single polar or subpolar flagellum (Figure 1E) [29], which raises the possibility that this bacterium undergoes asymmetric cell division.

Figure 2.1: *P. hirschii* has two distinct morphotypes. (A) Scanning electron microscope image of *P. hirschii* cells highlights the short- and long-stalked morphologies. The image was acquired at 60,000 magnification. Scale bar 1 μm . (B) Fluorescent D-amino acid staining of cells reveals polar and mid-cell peptidoglycan synthesis. Scale bar 2 μm . (C) Time-lapse differential interference contrast (DIC) images taken every 60 min on MMB agar pads show a short-stalked mother cell giving rise to a short-stalked daughter cell (top) and a short-stalked mother cell giving rise to a long-stalked daughter cell (bottom). The white arrowhead indicates the formation of a long stalk. Scale bar 2 μm . (D) A long-stalked mother cell gives rise to a long-stalked daughter cell (top), and a long-stalked mother cell gives rise to a short-stalked daughter cell (bottom). Scale bar 2 μm . (E) Transmission electron micrograph of an individual short-stalked *P.*

hirschii cell with a single polar flagellum. Scale bar 1 μm . (F) Montage showing a nonmotile mother cell producing a motile daughter cell. The white arrowheads indicate the stationary mother cell. The red arrowheads indicate the position of the motile daughter cell in each image. The images shown were acquired at 20-min intervals. Scale bar 2 μm .

Traditional microscopy techniques are integral to better understand the division of this bacterium. However, there are advantages to smaller-scale platforms that reduce medium consumption, precisely control the cell environment, and automate analysis. In addition, significant advances have been made in the development of microfluidic systems for cell biology [30-33]. Of particular importance to this work, precise fluid handling on microfluidic devices provides more control over the microenvironment of the cells [34, 35]. The integration of automated valves and pumps on-chip has enabled the addition of sample preparation and analysis on one device [36-38]. A recent work on the development of a microfluidic device for bacterial synchronization and high-resolution analysis [39] of *C. crescentus* highlights the utility of these devices. Swarmer cell enrichments on the microfluidic device are comparable to standard synchronization techniques, but synchronized populations are collected on-demand over 4 days, and cells are tracked in downstream microchannels postsynchronization to observe single-cell behavior.

In this study, we carefully examined the short-stalked morphotype of *P. hirschii* with traditional microscopy techniques, and our observations indicate that these cells may have a *Caulobacter*-like cell cycle in which cell division gives rise to two functionally distinct daughter cells. Although the two cells are similar in appearance, one cell is motile, and the other cell is nonmotile. Furthermore, we observed that short-stalked *P. hirschii* cells produce a polar adhesin. The presence of a polar adhesin renders this bacterium a suitable organism to integrate into our previously developed microfluidic platform, due to the ability of the cell to permanently adhere to the microchannel walls. With our device, we were able to synchronize *P. hirschii* cells and observe the behavior of newborn cells, confirming that short-stalked *P. hirschii* cells exhibit a *Caulobacter*-like cell cycle. Finally, a study of the *P. hirschii* genome confirmed the presence of a complex regulatory circuit consistent with the presence of a CtrA-regulated cell cycle.

MATERIALS AND METHODS

Strain and culture conditions *P. hirschii* (ATCC 27832T) was obtained from James Staley (University of Washington, Seattle, WA) and grown in minimal medium broth (MMB) or peptone yeast extract (PYE) medium at 26°C with shaking.

Materials We purchased glass slides (50 mm by 75 mm by 1.5 mm) from Corning Incorporated; poly(dimethylsiloxane) (Sylgard 184) from Dow Corning, Inc.; no. 1 Gold Seal cover glass (48 by 60 mm) from VWR International, LLC; Shipley 1813 photoresist, SU-8 2010, and Nano PG Developer from MicroChem Corp.; Alexa Fluor 488-wheat germ agglutinin lectin from Life Technologies; and all other chemicals from SigmaAldrich.

Scanning electron microscopy *P. hirschii* cells were grown to an optical density at 600 nm (OD_{600}) of 0.3 in PYE medium, centrifuged, and washed in sterile water. Cell suspensions were spotted onto poly-L-lysinecoated cover glass, fixed with 3% glutaraldehyde for 5 min and 1% osmium tetroxide for 5 min, washed twice in deionized water, and dehydrated with sequential washes of 50, 70, 90, 95, and 100% ethanol. The cover glass was dried in a CPD 030 (Balzers) critical point drying apparatus with liquid CO₂, mounted on a stub, sputter coated in an E5100 (Polaron Equipment) vacuum evaporator with a palladium-gold (80%–20%) target, and examined with a JEOL JSM-5800 LV scanning electron microscope at 15 kV.

Transmission electron microscopy Droplets of exponentially growing cultures were deposited onto a piece of Parafilm, and electron microscopy (EM) carbon-Formvar-coated copper grids (200 mesh) were floated onto the droplets for 2 min. Excess liquid was removed with filter paper, and the grids were quickly

washed four times by floating onto droplets of double-distilled water, transferred to droplets of 1% uranyl acetate in water for 2 min, washed once in double-distilled water, dried, and examined with a JEOL JEM-1010 transmission electron microscope.

Light microscopy and FDAA staining Epifluorescence microscopy was performed with an inverted Nikon Eclipse TiE, a QImaging Rolera EM-C2 1K EMCCD camera, and the Nikon NIS-Elements imaging software. Short pulses of fluorescent D-amino acid (FDAA) labeling were completed as described previously [40]. Briefly, exponentially growing cells (OD_{600} , ~0.3) were stained with 1 mM HADA (7-hydroxycoumarin-3-carboxylic acid-amino-D-alanine; emission maximum, 450 nm; blue) for 5 min and then washed three times in PYE to remove excess dye prior to imaging.

Initial attachment and polar adhesion detection Wheat germ agglutinin lectin conjugated to Alexa Fluor 488 (WGA-AF488) (Life Technologies) was used to detect polar polysaccharide in *P. hirschii*. In all experiments, a concentration of 0.5 μ g/ml WGA-AF488 was used. Epifluorescence microscopy was performed with a Nikon Eclipse 90i equipped with a Photometrics Cascade 1K EMCCD camera and MetaMorph imaging software (Molecular Devices, LLC). An inverted optical microscope, equipped with a total internal reflection fluorescence (TIRF) system (cell[^]TIRF; Olympus, Inc.) and a highsensitivity CCD camera (C9100-13;

Hamamatsu, Inc.), was used to visualize the polar holdfast in both wide-field and TIRF modes. To achieve a higher signal-to-noise ratio for images in TIRF mode, cover glass (no. 1) was cleaned in a solution of NH₄OH, H₂O₂, and H₂O (2:1:2) at 70°C for 20 min and rinsed with ultrapure water (18 MΩ · cm). The glass substrates were then sonicated in ultrapure water, rinsed again, dried in a stream of nitrogen, and heated for 10 min in a 70°C oven to remove excess water. A mixed culture of *P. hirschii* in MMB was dispensed onto the clean slides and placed in a humid chamber overnight to promote permanent attachment. After overnight incubation, cover glass with the attached cells was washed gently with a syringe filled with 50 µg/ml WGA-AF488 in MMB. Approximately 2 ml of the lectin-containing medium was added to the cover glass, incubated for 10 min to stain the holdfast, and rinsed with MMB to remove any unbound lectin. The holdfast-bound lectin was excited with a 491-nm laser, and images in wide-field and TIRF modes were collected to optically characterize the polar holdfast.

Biofilm formation and LIVE/DEAD staining Bacteria were grown to mid-log phase in MMB and diluted to an OD₆₀₀ of 0.05. Three milliliters of diluted bacteria was placed in the wells of 12-well polystyrene plates containing polyvinyl chloride coverslips placed vertically in each well. The plates were incubated at 26°C for the indicated times (see Figure 2.2E). After incubation, coverslips were removed and rinsed with distilled water (dH₂O) to remove planktonic cells. Half a microliter of LIVE/DEAD stain mixture (LIVE/DEAD BacLight bacterial viability kit L7007; Life Technologies) was added to 500µl of dH₂O, placed on the top of the

coverslip, and incubated in the dark for 15 min at room temperature. The stained coverslips were rinsed with dH₂O and observed by epifluorescence microscopy.

Microfluidic device fabrication Polydimethylsiloxane (PDMS)-glass microfluidic devices were fabricated, as previously described [39], from two layers (control and fluid) of PDMS and one layer of glass. Briefly, the fluid and control layers were cast from SU-8 masters created by standard photolithography. An initial 15- μ m-thick layer of SU-8 created a robust film, on top of which the fluid and control channels were created. This SU-8 layer was prebaked on a programmable hot plate (65°C for 3 min and 95°C for 1 min), exposed to UV light (270 mJ/cm²), and postbaked in a manner similar to the prebake. For the fluid control master, a 20- μ m thick SU-8 layer was spin-coated onto the initial layer and exposed to 270 mJ/cm² through the first photomask. For the control layer master, a 40- μ m-thick layer of SU-8 was spin-coated in two steps onto the substrate and exposed to 350 mJ/cm² of UV light through a second photomask. The masters were developed for 2 min in Nano PG Developer, rinsed with 2-propanol, and dried with nitrogen. Control layer replicas were prepared by spreading 9 ml of uncured PDMS onto the master. The PDMS was degassed under a vacuum for 30 min and cured for 2 h, at which point the pressure and vacuum access holes were punched. A fluid layer was created by spreading 3 ml of uncured PDMS across the master and degassing the PDMS under a vacuum for 10 min. The glass substrate was spun at 1,000 rpm for 35 s to achieve a 100- μ m-thick layer suitable for the lifting-gate valve architecture and was partially cured for 15 min.

The control and fluid layers were aligned, and the two substrates were brought together and cured for 2 h to permanently bond the layers. All PDMS replicas were cured at 65°C to minimize any shrinking in the layers that would compromise optimal alignment and valve operation. The bonded control and fluid layers were slowly peeled off the glass substrate, and fluid-access holes were punched through the PDMS. Number 1 cover glass was cleaned in a solution of NH_4OH , H_2O_2 , and H_2O (2:1:2) at 70°C for 20 min, rinsed with ultrapure water, and dried in a stream of nitrogen. The PDMS valves were covered with a mask prior to plasma cleaning to prevent irreversible adhesion that would render the valves nonfunctional. Both the bonded control and fluid layers and the glass coverslip were cleaned of plasma, the mask was removed, and the substrates were brought into direct contact with each other for an irreversible bond between the PDMS and the coverslip.

Device operation The fluidic device was coupled to an AssayMark system (Microfluidic Innovations, LLC). The AssayMark system interfaces with the microfluidic device through a stainless-steel manifold that contains pressure (35,000 Pa) and vacuum (-89,000 Pa) connections, which align with the pressure and vacuum holes in the control layer of the device. The control software allows the user to operate the valves in manual and programmed modes. The programmed mode automates the actuation of pumps and valves over multiple days with no user input. The lifting-gate valve architecture in this device permits thin cover glass (no. 1) to be used as a cover plate for the fluid layer, and as a

consequence, high-resolution imaging through the thin cover glass in both wide-field and TIRF modes is possible. Images were taken with high-numerical aperture (NA) objectives in the analysis channel at 100 magnification.

Cell culture, seeding, and synchronization The microfluidic device was inoculated by pumping a mixed culture from the cell reservoir through the incubation region to the waste reservoir. After the incubation region was filled with culture, the pumps were stopped to initiate a gentle hydrostatic flow. During this time, permanent adhesion of the bacteria to the microchannel wall was observed. Fresh culture was pumped in every 10 min to replenish the population of swarmer cells. Device seeding was terminated after 1 h, and PYE medium was pumped from the incubation reservoir through the incubation region to flush away any unadhered cells. The biofilm in the incubation region was grown over a period of 3 days. The doubling time of *P. hirschii* (~6 h) is much slower than that of *C. crescentus* synchronized on this same platform [39]. As a result, 3 days of growth was necessary to establish a biofilm large enough to produce a number of motile progeny. During this growth period, the incubation region was rinsed every 30 min to remove unadhered cells and to perfuse fresh medium over the established biofilm. To collect a synchronized population, the incubation region was flushed with medium for 15 min. The exit valve to the incubation region was closed to prevent flow, and newborn cells were released into the fresh medium for 20 min. Pumps 1 and 3 were used to route the medium containing the synchronized cell population to the analysis channel for downstream

experiments. The inverted optical microscope was used to collect images from the microfluidic device experiments. WGA-AF488 was added to the medium prior to synchronization to observe the fluorescence associated with rapid cell attachment to the analysis channel surface. Video data were collected with the MetaMorph Advanced imaging software (Molecular Devices, LLC), and cell tracks were obtained from the video data with the ImageJ ParticleTracker plug-in.

***P. hirschii* genome sequencing, assembly, and annotation** *P. hirschii*

genome sequencing and assembly were completed as described previously [41]. The annotation used in this analysis was completed by the University of Missouri Informatics Research Core Facility (IRCF) and is publicly available on the IRCF genomics website (<http://genomics.ircf.missouri.edu/genomes/>). The various scripts and files associated with the bacterial annotation pipeline are available for download from GitHub (<https://github.com/sgivan/BGA>). A custom genome annotation pipeline that progresses through four components was used: gene prediction, gene refinement, functional data collection (FDC), and function prediction (FP). Glimmer3 predicts initial gene boundaries in the DNA sequence of the genome assembly [42]. Gene refinement proceeds through two phases. The first phase refines the gene start position based on potential upstream ribosome binding site (RBS) motifs obtained with RBSfinder [43]. A consensus RBS is defined by DREME [44]. The second phase of gene refinement follows FDC. During FDC, every gene identified by Glimmer3 is conceptually translated and subjected to BLASTP searches against two databases, Swiss-Prot and

KEGG, and hidden Markov model searches of Pfam [45-47]. Following FDC, gene start positions are confirmed or modified based on likelihood models of potential upstream and downstream start locations constructed from comparisons to similar database sequences. The data from FDC are also used to resolve significant overlaps between neighboring coding regions. Automated FP relies on a scoring matrix generated dynamically for every gene. The scoring matrix is constructed by parsing database hit descriptions to score words and phrases weighted by the quality of the database hits. Subsequently, database hits are ranked by the scoring matrix, and the highest-scoring hit is used to create a functional description of the genomic element. The distribution of scores provides a confidence estimate of the quality of the function assignment. The automated FP is an unsupervised process. A final component of the pipeline searches for conserved coding potential in intergenic regions of the genome. If identified, these open reading frames (ORFs) are subjected to FDC and FP.

Ortholog identification Orthologs of genes described to have an important function in cell cycle regulation or holdfast biogenesis of *C. crescentus* or *A. tumefaciens* were identified in *P. hirschii*, following searches for bidirectional best hits (BBH). Two query data sets composed of proteins from *C. crescentus* and *A. tumefaciens* were used for the BBH analysis. In all cases in which the protein was encoded in both query genomes, the same BBH was identified in the translated coding sequence (CDS) from the *P. hirschii* genome. An E value of 0.01 and sequence identity of 30% were used as thresholds in the analysis.

Genome-wide motif searches The *P. hirschii* genome was searched for the presence of conserved motifs involved in the cell cycle regulation of Alphaproteobacteria, including CtrA-binding sites (TTAAN₇TTAA) and CcrM methylation sites (GANTC) [7]. Our RelaxedMotifSearch (RMS) algorithm provides a fast and simple search of the patterns of short sequences of form “XYZ,” in which prefix X and suffix Z are to be matched, and Y is a variable part of the pattern of a predefined length N. The search can be done at the whole-genome scale and allows for a single-base-pair substitution in the prefix X and/or suffix Z. The input includes a specific motif(s) of interest, and the outputs are the locations in which the motif(s) is found. The RMS guarantees to find a comprehensive list of the motifs in both DNA strands. The search scans the motifs by consecutively trying to match subsequences of the genome sequence with a shift of one nucleotide. In the event the motif is found, RMS shifts $|X| + |Z| + N$ nucleotide positions and continues the search. Searches were completed on both the total genome sequence and the predicted CDS.

Phylogenetic tree construction A phylogenetic tree was constructed with *gyrA* sequences from representative species. Sequences were aligned with MUSCLE [48]. RAxML [49] reconstructed the maximum-likelihood phylogeny with the JTT amino acid substitution matrix, a fourcategory discrete gamma distribution, and single invariant category of sequence rate variation among sites. Analysis of 100

bootstrapped data sets determined support for clades occurring in the maximum-likelihood phylogeny.

RESULTS

Short-stalked *P. hirschii* cells are motile

A mixed culture of *P. hirschii* cells grown in PYE or MMB contained cells of two distinct morphologies (Figure 2.1A) [29]. The population has cells with many short stalks that often appear to be positioned regularly around the cell surface (short-stalked cells). The population also has cells with stalks that are much longer but fewer in number (long-stalked cells). When individual cells are spotted on agarose pads and allowed to grow, two morphologies do not arise by a morphological transition in the cell cycle. Indeed, the morphologies of 367 mother cells remained fixed during 20 h of observation. This result is consistent with previous observations and presumably occurs because *P. hirschii* cells grow by tip elongation [29, 50]. Staining of live *P. hirschii* cells with fluorescent D-amino acids (FDAA) [40] confirmed that new peptidoglycan is synthesized at one pole during elongation and at mid-cell in late-predivisional cells (Figure 2.1B). When grown in PYE or MMB, the short-stalked morphology appears to be favored, and a short-stalked mother cell gave rise to a short-stalked daughter cell 99% of the time (n 339) (Figure 2.1C). In contrast, long-stalked mother cells were about equally likely to produce a short-stalked daughter cell (54% [n = 15]) or a long-stalked daughter cell (46% [n = 12]) (Figure 2.1D). Time-lapse microscopy

illustrated that the short- and long-stalked morphotypes reproduce at similar rates, with division times of approximately 6 h (Figure 2.1C and D). Irrespective of the daughter-cell morphology, a mother cell typically produced daughters of the same morphology for many generations, resulting in the formation of microcolonies dominated by cells of a single morphology (see Figure 2.S3 in the Supplementary material) [29]. The observation that microcolonies are composed of either long- or short-stalked cells revealed a clear functional difference in the two cell types. Microcolonies of long-stalked cells were tightly compacted due to the nonmotile nature of these cells (see Figure 2.S3 in the Supplementary material). In contrast, microcolonies of short-stalked cells were capable of spreading on the agar (see Figure 2.S3 in the Supplementary material), which suggests that cells of this morphology are motile by means of a polar or subpolar flagellum (Figure 2.1E) [29]. Notably, when a mixture of long- and short-stalked cells was observed under light microscopy, only a small fraction (~10%) of the short-stalked cells appeared to be actively swimming, and rosettes were frequently observed (Figure 2.1B, top, and 2.2A). Furthermore, when cells were observed in 0.5% agarose that was sufficiently wet for motile cells to swim, nonmotile mother cells were observed to give rise to motile daughter cells (Figure 2.1F). These observations are consistent with the hypothesis that short-stalked *P. hirschii* cells may have a *Caulobacter*-like cell cycle during which asymmetric cell division will result in the formation of two functionally distinct daughter cells, a motile cell and a sessile cell (Figure 2.1F).

Figure 2.2: Short-stalked *P. hirschii* cells produce a holdfast. (A) A polar polysaccharide is detected by labeling with WGA-AF488 (green) in short-stalked cells but is absent in long-stalked cells. Scale bar 2 μm . (B) Short-stalked cells with a holdfast attach polarly to a glass surface. A DIC image (left) and fluorescence image of WGA-AF488 signal (right) of attached cells are shown.

Scale bar 2 μm . (C) Wide-field (left) and TIRF (right) images of a holdfast reveal that the holdfast is located at the surface-cell interface and forms a cap around the cell pole. Schematics indicate the focal plane imaged for the wide-field and TIRF images. Scale bar 1 μm . (D) Schematic of the experimental setup used in the static biofilm assay in panel E. (E) Biofilm attached to polyvinyl chloride (PVC) coverslips stained with the BacLight LIVE/DEAD stain at different times of a static biofilm assay. The images represent overlays of green (live cells) and red (dead cells) collected by epifluorescence microscopy from the meniscus and bottom of the coverslip. Scale bar 5 μm . (F) Genetic organization of the putative unipolar polysaccharide (upp) gene cluster (C1_5514 to C1_5522). The locus tag numbers and predicted functions are listed below each arrow. Black arrows indicate the positions of hypothetical proteins. AT, acetyltransferase; GT, glycosyltransferase; MPA-1, membrane-periplasmic auxiliary protein.

Short-stalked *P. hirschii* cells produce a polar polysaccharide

The ability of the cells to form rosettes is often indicative of the presence of a polar polysaccharide. Wheat germ agglutinin lectin conjugated to Alexa Fluor 488 (WGA-AF488), which binds to N-acetylglucosamine, was used to determine if the polar polysaccharide is produced in planktonic cultures of *P. hirschii*. Lectin bound to one pole of some short-stalked cells but was never observed to bind to long-stalked cells (Figure 2.2A, left). In addition, lectin bound at the center of rosettes (Figure 2.2A, right), which indicates that short-stalked *P. hirschii* cells are capable of producing a holdfast-like polar polysaccharide. To determine if the

polar polysaccharide of *P. hirschii* mediates surface attachment, *P. hirschii* cells were incubated with cover glass for 24 h. The cover glass was washed to remove unattached cells, the polar polysaccharide of attached cells was labeled with WGA-AF488, and attached cells were visualized with epifluorescence microscopy. *P. hirschii* cells bound to the coverslip in an end-on configuration, and each cell attached to the surface also bound WGA-AF488 (Figure 2.2B). To ensure that the polar polysaccharide is present at the cell surface interface, microscopy in wide-field and TIRF modes was conducted to detect WGA-AF488-labeled polar polysaccharides of cells attached to cover glass. In images collected in wide-field mode, the polar polysaccharide appeared to be a ring, which suggests that the polar polysaccharide forms a cap around the pole of the cell (Figure 2.2C, left). In TIRF mode, which only visualizes fluorescence signal approximately 100 nm deep into the sample, the WGA-AF488 was detected as a single uniform spot, which suggests that the polar polysaccharide is present at the cell pole-to-surface interface and likely promotes attachment to surfaces (Figure 2.2C, right). Because *P. hirschii* produces a polar polysaccharide and attaches to surfaces, we hypothesized that this bacterium should be capable of robust biofilm formation. Under static conditions, we observed that *P. hirschii* cells readily attach to a submerged vertical coverslip (Figure 2.2D), with an enrichment of cells near the liquid-air interface (Figure 2.2E). After 4 days, we observed robust biofilm formation on the coverslip along the meniscus of the coverslip, and LIVE/DEAD staining indicated that the attached cells were largely viable (Figure 2.2E, right). Taken together, these results suggest that *P. hirschii*

produces a unipolar polysaccharide capable of mediating cell-to-surface and cell-to-cell attachment. This notion is supported by the presence of a nearly complete *wzy*-type polysaccharide gene cluster with similarity to the *C. crescentus* holdfast and *A. tumefaciens* unipolar polysaccharide (UPP) biosynthesis clusters in the *P. hirschii* genome (Figure 2.2F; see also Figure 2.S1 and Table 2.S1 in the Supplementary material).

Short-stalked *P. hirschii* cells can be synchronized

The presence of the *P. hirschii* polar adhesin and its ability to form a biofilm suggested that this bacterium could be synchronized on a microfluidic platform previously used to synchronize *C. crescentus* [39]. Figure 2.3A is a schematic of the microfluidic device design. Using this device, we collected bright-field and fluorescence images of the biofilm. The bright-field image of the cells (Figure 2.3C, left) suggests the biofilm develops as a monolayer on the surface of the microchannel. During the 3 days of biofilm growth, cells adhered to the microchannel wall by the polar adhesin, which was confirmed by the fluorescence of WGA-AF488 added to the medium to indicate the presence of the polar polysaccharide (Figure 2.3C, middle and right). The biofilm density increased over time, and cells remained attached to the biofilm, even when the pumps perfused fresh medium over the biofilm every 30 min. The cells were adhered to the surface and remained attached during medium perfusion. In addition to attachment, the cells were confined to the incubation region by the valves (Figure 2.3B), and this confinement kept the downstream analysis channel

free from cells prior to analysis. Using the automated operation of the microchip device by the AssayMark system, we synchronized cells, and newborn motile cells were released into the fresh medium in the incubation region for 20 min. During this time, the activity in the biofilm was monitored to ensure that cells in the biofilm were dividing. Motile cells accumulated in the biofilm region during the 20-min release time (Figure 2.3D, left). After the 20-min release period, cells were pumped to the analysis channel where motile cells were highly enriched (~75%), as evidenced by the cell tracks for the synchronized population in the analysis channel (Figure 2.3D, right). In addition to the release of newborn motile cells, we observed the rapid attachment of newborn cells to the microchannel walls (Figure 2.3E; see also Figure 2.S4 in the Supplementary material). Within 5 min after the synchrony, a cell had attached to the microchannel wall and developed a unipolar polysaccharide (Figure 2.3E, left). After an additional 5 min, two more cells had attached to the microchannel in the field of view (Figure 2.3E, right). Over time, the number of motile cells in the microchannel decreased, and the number of adhered cells increased. After 10 min, the proportion of cells attached to the microchannel increased from ~30% to 55% (see Figure 2.S4 in the Supplementary material). These data suggest that polar adhesin-mediated attachment is rapid, and our observations are consistent with the possibility that surface contact stimulates polar polysaccharide production, as described for other Alphaproteobacteria [28]. We monitored the swim behavior of the synchronized cells over the course of 3 h in the analysis channel and observed that the newly adhered cells in the microchannel continued to grow (Figure 2.3F;

see also Figure 2.S5 in the Supplementary material). We observed that the cells attached to the microchannel wall were similarly sized predivisional cells, suggesting that these cells are tightly synchronized by our microfluidic platform (Figure 2.3F; see also Figure 2.S5 in the Supplementary material). The majority of cells were synchronized based on the observation that predivisional septa increased from 8.33% ($n = 108$) shortly after synchronization to 70.86% ($n = 127$) at ~200 min post-synchrony (see Figure 2.2.S5 in the Supplementary material). The observation that a synchronized motile population transitions to a sessile state is further evidence for an asymmetric *Caulobacter*-like cell cycle.

Figure 2.3: *Synchronization of short-stalked P. hirschii cells in the microfluidic device.* (A) Fluid layer design of the microfluidic device. A biofilm is formed in the incubation region over 3 days, and the synchronized population is pumped to the analysis channel for observation. (B) Bright-field image of cell confinement in the microfluidic device. The area above the valve seat has cells grow up to, but not beyond, the valve seat. No cells were observed on the opposite side during biofilm growth. Scale bar 50 μm . (C) Biofilm formation within the incubation region of the microfluidic device. Biofilm is labeled with WGA-AF488, which indicates the presence of the polar polysaccharide. White-light (left), fluorescent (middle), and overlay (right) images are provided. Scale bar 5 μm . (D) Newborn cells released from the biofilm are motile. Tracks of motile cells were generated with ImageJ ParticleTracker and are overlaid on the false-colored white-light images in the incubation region (left) and the analysis channel (right). Scale bar 5 μm . (E) Newborn cells rapidly attach to the analysis channel. White-light (left) and fluorescent (right) images of cells labeled with WGA-AF488 attached to the analysis channel 5 (left) and 10 (right) min after collection. The arrowheads indicate the position of a cell that attached within 5 min of collection. Scale bar 1 μm . (F) Attached cells grow in the analysis channel. Three hours after collection, predivisional cells were observed attached to the analysis channel. Scale bar 1 μm

Conservation of cell cycle regulators and binding sites

Many members of the Alphaproteobacteria, including *C. crescentus* and *A. tumefaciens*, have a complex life cycle resulting in the formation of two distinct cell types, a motile cell and a nonmotile sessile cell [5, 10, 27, 51]. Notably, the regulatory components underlying the alphaproteobacterial cell cycle are well conserved, and transcriptional control has been hypothesized to be a conserved mechanism to govern cell cycle progression in *C. crescentus*, *A. tumefaciens*, and *S. meliloti* [1, 7, 16]. Because short-stalked *P. hirschii* cells exhibit features of a similar cell cycle (Figure 1F), the newly sequenced genome of *P. hirschii* [41] was searched for hallmarks of regulatory networks governing the alphaproteobacterial cell cycle. Like other Alphaproteobacteria, the *P. hirschii* genome contains orthologs of proteins predicted to function in cell cycle regulation (see Table 2.S2 in the Supplementary material). The *P. hirschii* genome contains orthologs of the global regulators GcrA, CcrM, SciP, and CtrA. In *C. crescentus*, these global regulators function in regulatory modules to control progression through the cell cycle: the CtrA/MucR, GcrA/CcrM, and CtrA/SciP modules regulate sequential transcription of target genes during the G1, S, and G2 phases, respectively [8, 52]. The *P. hirschii* genome also contains orthologs of proteins predicted to regulate CtrA function, including CckA, ChpT, DivL, and DivK. In *C. crescentus*, the CckA/ChpT phosphorelay is required for the phosphorylation and activation of CtrA [51]. The activity of the hybrid histidine kinase CckA is modulated by the tyrosine kinase DivL and the single-domain response regulator DivK. Phosphorylated DivK can inhibit the activity of the DivL-CckA complex, resulting in the inhibition of CtrA activity. The phosphorylation

state of DivK is regulated by the PdhS family of histidine kinases, which includes PleC and DivJ in *C. crescentus* and additional kinases in other Alphaproteobacteria. [1, 10, 13, 51, 53] The *P. hirschii* genome encodes four PdhS proteins with similarity to the PleC, DivJ, PdhS1, and PdhS2 proteins of *A. tumefaciens* (see Table 2.S2 in the Supplementary material). In *C. crescentus*, CtrA activity is also modulated by proteolysis by the ClpPX protease, along with CpdR and RcdA. The proteolysis machinery is conserved in *P. hirschii* (see Table 2.S2 in the Supplementary material). The presence of genes encoding key components known to function in cell cycle regulation of Alphaproteobacteria suggests that *P. hirschii* may utilize temporal transcriptional control as a means to control cell cycle progression. The CtrA protein sequence of *P. hirschii* is remarkably well conserved, with 94.2% protein sequence identity with CtrA from *A. tumefaciens* (see Figure 2.S6A in the Supplementary material). Based on the high degree of CtrA protein conservation and successful complementation of *C. crescentus* CtrA mutants with CtrA from *Rickettsia prowazekii* [54], it is reasonable to hypothesize that CtrA-binding sites are highly conserved among Alphaproteobacteria [7]. To determine which genes might be directly regulated by CtrA, we searched for the consensus CtrA-binding site (TTAAN7TTAA [7]) in the *P. hirschii* genome. Twenty exact matches to the consensus CtrA-binding site were identified, and 90% (18/20) of these sites were found in intergenic regions (Figure 2.4A, closed circles). Seven of these putative CtrA-binding sites were found upstream of genes involved in flagellum biosynthesis or methyl-accepting chemotaxis proteins (Figure 2.4A and D; see also Figure 2.S6B in the

Supplementary material), suggesting that CtrA may regulate the motility of *P. hirschii*. Three putative CtrA-binding sites were upstream of genes encoding penicillin binding proteins (PBPs) and the min genes, suggesting a role in the regulation of cell growth and division, as in *S. meliloti* [16]. CtrA-binding sites also occupied the regions upstream of regulatory genes, including *dgcB* and *pdhS1*. Relaxing the search for CtrA-binding sites to allow one substitution in the binding site resulted in the identification of additional candidate CtrA-binding sites in the genome. Many of these putative binding sites were found upstream of genes with predicted functions in chemotaxis, motility, adhesion, cell growth and division, and regulation (Figure 2.4A, open circles, and 2.4D; see also Figure 2.S6B and C in the Supplementary material). In addition, putative CtrA-binding sites were identified in the origin of replication (Figure 2.4C), suggesting a role for CtrA in integrating DNA replication with the cell cycle, as described for *Caulobacter* species [55, 56]. Further support for the hypothesis that DNA replication is integrated with the *P. hirschii* cell cycle was provided by the presence of four consensus CcrM methylation sites (GANTC [7]) (Figure 4C). In *C. crescentus*, the methylation of DNA by CcrM plays an important role in the regulation of cell cycle progression [57-59], and methylated sites are subject to transcriptional regulation by GcrA [14]. Methylation by CcrM is conserved in other Alphaproteobacteria, including *B. abortus* [18], *A. tumefaciens* [9], and *S. meliloti* [60]. Furthermore, *ccrM* genes from *C. crescentus* and *S. meliloti* are able to cross-complement [60], suggesting that the methylation site may be conserved. Finally, methylation-dependent binding of GcrA from other Alphaproteobacteria

has been shown in vitro, suggesting that methylation-dependent recruitment of GcrA to promoters may be conserved [14]. Because orthologs of both CcrM and GcrA are present in the *P. hirschii* genome (see Table 2.S2 in the Supplementary material), the genome was scanned for potential methylation sites. In the *P. hirschii* genome, there are a total of 7,538 GANTC sites potentially methylated by CcrM. Notably, the number of possible methylation sites (7,538) is far lower than would be predicted by the expected frequency of this sequence (16,996 occurrences) in the *P. hirschii* genome, suggesting that these sites are underrepresented in the genome. The proportion of intergenic regions in the genome is 11.95%, and nearly a quarter (24.6%) of these methylation sites are found in intergenic regions, suggesting that these sites might be targets for transcriptional regulation. Putative methylation sites are enriched upstream of genes encoding proteins with function in DNA replication, cell division, and cell cycle regulation (Figure 2.4B and D). This observation is consistent with the described role of CcrM-mediated methylation in facilitating cell cycle progression, DNA metabolism, and cell division in *C. crescentus* [57, 58].

Figure 2.4: *P. hirschii* genome reveals signatures of cell cycle regulation. (A) Relative distances of CtrA-binding sites to the ATG of predicted targets. The predicted targets of CtrA regulation in chemotaxis, motility, adhesion, cell growth and division, and regulation are shown. For chemotaxis, all predicted targets are methyl-accepting chemotaxis proteins. Closed circles indicated a perfect match to the TTAAN7TTAA consensus sequence. Open circles have one substitution in the predicted CtrA-binding site. (B) Relative distance of CcrM methylation sites to the ATG of predicted targets in DNA replication, cell division, and regulation. All predicted methylation sites match the GANTC consensus sequence. (C) The *P. hirschii* origin of replication contains CtrA binding sites with one substitution (open circles) and multiple CcrM methylation sites (stars). (D) Schematic of genes that may be regulated via putative CtrA binding (solid arrows) or via putative CcrM methylation (dashed arrows), based on the findings in this study. Genes are grouped together according to function. Ori, origin of replication.

DISCUSSION

The results presented in this work suggest that the short-stalked cells of *P. hirschii* have a *Caulobacter*-like cell cycle in which cell division results in the release of a motile cell (Figure 2.1F). The motile cell is capable of rapid attachment to surfaces mediated by a polar polysaccharide (Figure 2.3E). Following surface attachment, the cells undergo a period of cell growth, leading to the production of a predivisional cell with a polar polysaccharide at one cell

pole and a flagellum at the opposite pole. Cell division ultimately leads to the production of two functionally distinct daughter cells, a motile cell and a sessile cell (Figure 2.5). Asymmetric cell division is proposed to be broadly conserved in the *Caulobacteriales* and *Rhizobiales* clades of the Alphaproteobacteria [1, 7], and our results support this notion.

Figure 2.5: Schematic of the short-stalked cell cycle. Sessile cells (S) are attached to a surface by means of a polar polysaccharide (gold) and are capable of elongation. Late predivisional cells (PD) produce a polar or subpolar flagellum. Cell division produces two distinct daughter cells: a motile cell (M) with a flagellum and a sessile cell (S) with a polar polysaccharide.

Rapid attachment to surfaces by means of a polar polysaccharide has been characterized in *C. crescentus* and other Alphaproteobacteria [28]. We have observed a polar polysaccharide in *P. hirschii* (Figure 2.2) that shares key

features with the well-studied *C. crescentus* holdfast and *A. tumefaciens* UPP. First, we observed labeling of the polar polysaccharide with WGA-AF488 (Figure 2.2A to C and 2.3C). WGA is an N-acetylglucosamine-specific lectin and is known to bind both the holdfast and the *A. tumefaciens* UPP, suggesting that the polar polysaccharides of *C. crescentus*, *A. tumefaciens*, and *P. hirschii* all contain N-acetylglucosamine. Second, we observed that newborn *P. hirschii* cells rapidly attach to surfaces and produce a polar polysaccharide (Figure 2.3E). Both *C. crescentus* and *A. tumefaciens* have been shown to extrude a polar polysaccharide within minutes of contacting a surface [25, 28, 61], and *P. hirschii* cells have a similar response to surface contact. Third, we observed that the *P. hirschii* cells bound to surfaces within a biofilm produce the UPP (Figure 2.3C). Both holdfast and UPP play essential roles in attachment and biofilm formation [25, 26, 62, 63]. Finally, we have identified a gene cluster with similarity to the upp biosynthesis cluster (Figure 2.2E; see also Figure 2.S2 in the Supplementary material) in the *P. hirschii* genome. The *P. hirschii* upp gene cluster contains putative CtrA-binding sites (Figure 2.4A and D; see also Figure 2.S6C in the Supplementary material), which are suggestive of cell cycle regulation of polysaccharide biosynthesis, as has been described for holdfast [64, 65] and suggested for *A. tumefaciens* UPP [11]. Because the putative *P. hirschii* UPP biosynthesis cluster is missing a flippase (see Figure 2.S2 in the Supplementary material), we hypothesize that additional proteins involved in UPP biosynthesis and export remain to be identified. Indeed, a putative flippase at another genomic locus has been identified (see Figure 2.S1 and Table 2.S1 in the Supplementary

material). Taken together, these observations suggest that *P. hirschii* is capable of producing a unipolar polysaccharide that enables rapid attachment to surfaces and likely promotes biofilm formation. Additional characterization of *P. hirschii* genes predicted to function in UPP biosynthesis will be necessary to understand the mechanisms underlying attachment and biofilm formation in this bacterium; however, we have been hindered by a lack of a genetic system and an inability to generate mutants in this bacterium, despite testing a number of approaches.

In this work, we provide evidence that the short-stalked cells of *P. hirschii* have a *Caulobacter*-like cell cycle and explore the potential role of global regulators in mediating cell cycle progression. Studies indicate that the *P. hirschii* genome contains genes that encode candidate cell cycle regulators, including CtrA and CcrM, suggesting that transcriptional control may play an important role in regulation of the *P. hirschii* cell cycle (Figure 2.4; see also Table 2.S2 in the Supplementary material). The high degree of conservation of regulatory proteins in the Alphaproteobacteria has led to the suggestion that transcriptional control of the cell cycle is conserved among Alphaproteobacteria [1, 7]. The *Caulobacter* paradigm of transcription regulation of cell cycle progression has recently been extended to *S. meliloti*, providing additional support for this notion [15]. CtrA-binding sites are found upstream of cell cycle regulated genes in both *C. crescentus* and *S. meliloti*; however, there is very little overlap between the CtrA regulons [15]. In contrast, CtrA-binding sites are strongly conserved among

Alphaproteobacteria closely related to *S. meliloti*, suggesting that the functions regulated by CtrA may be influenced by the lifestyle(s) of the bacteria [15].

The regulation of motility and flagellum biosynthesis by CtrA is posited to be an ancestral trait ; thus, our observation of CtrA-binding sites upstream of genes involved in motility and chemotaxis is to be expected. Remarkably, in *P. hirschii*, the putative mechanism of CtrA regulation of motility is more similar to that of *C. crescentus* than to that of *S. meliloti*. CtrA-mediated regulation of motility is largely indirect in *S. meliloti*, in which CtrA is thought to regulate Rem, an important regulator of flagellar gene expression [15, 66]. In contrast, CtrA directly binds to the promoter regions of the *flg*, *fli*, *flm*, and *flj* genes, representing all four tiers of the flagellar regulatory hierarchy, and many *mcp* genes in *C. crescentus* [67]. Putative CtrA-binding sites are present upstream of presumptive class II flagellar genes (*flgBC*, *fliLM*, *flhA*, and *fliI*), class III flagellar genes (*flgI* operon), class IV flagellar genes (*fljM* and *fljNO*), and numerous chemotaxis genes in the *P. hirschii* genome (Figure 2.4A; see also Figure 2.S6B in the Supplementary material). These observations suggest that CtrA may have a direct role in the regulation of flagellum biosynthesis and chemotaxis in *P. hirschii*. In *P. hirschii*, CtrA-binding sites are also observed upstream of genes encoding a putative unipolar polysaccharide biosynthesis cluster (Figure 2.4A; see also Figure 2.S6C in the Supplementary material). We are tempted to speculate that because *P. hirschii* and *C. crescentus* share an environmental niche (freshwater), common mechanisms of cell cycle regulation may be beneficial. Indeed, simulations have

shown that in patchy nutrient environments, such as freshwater, a developmental cell cycle with both sessile and motile cell types may confer a survival advantage [68].

Although the potential regulation of motility by CtrA in *P. hirschii* is similar to that of *C. crescentus*, this trend is not universal. We found that the predicted regulation of cell division in *P. hirschii* by CtrA resembles what is observed in *S. meliloti*, in which CtrA represses the min operon [16]. Likewise, in *P. hirschii*, CtrA binding sites are found upstream of the genes encoding the Min system, which regulates the placement of the septum, and upstream of *ftsK*, which encodes an essential cell division protein. These results support the notion that CtrA has a critical role in controlling the timing of min expression and subsequent cell division in the Rhizobiales [1, 15, 16]. This finding may reflect a common mechanism for the regulation of cell division among bacteria exhibiting polar elongation [50]. Additional research is necessary to test the hypothesis that *P. hirschii* exhibits cell cycle dependent transcription and to understand the influence of global regulators on processes, such as cell division, biofilm formation, and motility.

Notably, the *Caulobacter*-like cell cycle is observed only among short-stalked *P. hirschii* cells. Long-stalked *P. hirschii* cells are nonmotile and do not produce an observable polar polysaccharide (Figure 2.2A; see also Figure 2.S3 in the

Supplementary material). The long-stalked cells are capable of cell elongation and division; however, when long-stalked mother cells produce long-stalked daughter cells, the progeny are not morphologically or functionally distinct from the mother cells (Figure 2.1D, top). Thus, *P. hirschii* exhibits polymorphic cell cycles in which two morphologically different cell types with distinct cell cycles persist in the same culture. Other Alphaproteobacteria, particularly *Rhodomicrobium* and *Hyphomicrobium* spp., are described to have polymorphic cell cycles in which one cell morphology routinely utilizes a *Caulobacter*-like cell cycle, which suggests that the complex elaboration of the dimorphic cell cycle may be widespread [2, 69].

The ability to synchronize short-stalked *P. hirschii* cells in a microfluidic device provides an opportunity to better understand the interplay among the two morphologies of *P. hirschii*. Under standard laboratory conditions, the short-stalked morphology is dominant and appears to be stable, with very few short-stalked cells giving rise to long-stalked daughter cells (Figure 2.1C). In contrast, long-stalked cells frequently gave rise to short-stalked daughter cells (Figure 2.1D, bottom). These observations raise the possibility that the different morphologies provide an advantage under certain nutrient conditions, as described for *Rhodomicrobium* species [2]. In the microfluidic device, synchronous populations of short-stalked *P. hirschii* cells could be exposed to a variety of nutrient conditions in an effort to determine if a particular condition triggers short-stalked cells to produce long-stalked daughter cells. The

identification of the conditions that trigger such a switch would certainly provide insights into the complex polymorphic cell cycles of *P. hirschii*.

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SUPPLEMENTARY MATERIAL

Figure 2.S1 Conservation of polar polysaccharide synthesis genes. Genetic organization of the holdfast biosynthesis gene cluster in *C. crescentus* encodes genes that function as a glycosyltransferase (*HsfE*, dark blue arrow and *HsfG*, red arrow), flippase (*HsfF*, orange arrow), deacetylase (*HsfH*, purple arrow), polymerase (*HsfC*, light green arrow and *HsfI*, pink arrow), secretin (*HsfD*, brown arrow), autokinase (*HsfB*, light blue arrow), and membrane periplasmic axillary protein (*HsfA*, grey arrow). All of these gene products contribute to the synthesis and export of the *C. crescentus* holdfast. Gene names for the major *A. tumefaciens* unipolar polysaccharide biosynthesis cluster (*uppA-F*) are listed above each arrow. Conserved coding indicates the predicted function in polysaccharide biosynthesis as described for holdfast. In the Rhizobiales, *uppA* (yellow arrows) is predicted to encode an acetyltransferase, *uppB* (shaded with

both light blue and grey) is a fusion of *hfsA* and *hfsB*, and flippase genes are typically found at another genomic loci. Several members of the Rhizobiales, including *P. hirschii*, have a *wzy*-type polysaccharide gene cluster that is similar in cluster structure to *A. tumefaciens*. Black arrows represent hypothetical proteins with no significant homology to known polysaccharide biosynthesis genes. Scale bar is 1Kb.

Figure 2.S2 *Phylogeny places P. hirschii within the Rhizobiales.* Maximum likelihood phylogeny inferred from *gyrA* sequences. Node values indicate clade frequency among 100 nonparametric bootstrapped datasets. Black nodes represent bootstrap values above 90.

Figure 2.S3 *P. hirschii forms microcolonies of a single cell type.* Differential interference contrast (DIC) microscopy image of *P. hirschii* microcolonies comprised of tightly packed, non-motile long-stalked cells (left) and motile short-stalked cells (right)

Figure 2.S4 *Rapid attachment of motile P. hirschii cells.* Quantification of the proportion of swimming and attached cells following cell synchrony.

Synchronized cells were collected in the analysis channel and videos were taken within 5 minutes of the synchrony and again 10 minutes later. Each field represents data from an independent synchrony. A total of 40 – 65 cells were classified as swimming or attached in each field at the indicated times. The proportion of attached cells increases from ~30% to 55% within 10 min, in two independent experiments

Figure 2.S5 *P. hirschii* cells are synchronized in the microfluidic device.

Representative white light images of cells attached to the analysis channel 15 and 200 min post synchronization are shown. Quantitation of multiple fields reveals that 15 min post synchrony 8.33% of cells (n=108) have pre-divisional septa, whereas 200 min post synchrony 70.86% of cells (n=127) have pre-divisional septa. Scale bar is 5 μ m.

Figure 2.S6 *CtrA* may function as a global regulator in *P. hirschii*. A. Alignment of the CtrA protein from *P. hirschii* to orthologs from other Alphaproteobacteria reveals a high degree of protein identity. *P. hirschii* CtrA is 94.2% identical to CtrA from *A. tumefaciens* (Atu_2334). Protein alignment was generated with the Clustal Omega program. B. Multiple putative CtrA binding sites are found in gene clusters encoding flagellum genes. The flagellin gene cluster (top panel) includes regulators of flagellin biosynthesis (blue arrows) and the flagellins (red arrows). The gene encoding FlgI, a protein found in the P ring, is just downstream and has a CtrA binding site in the intergenic region. A flagellum biosynthesis and export cluster (bottom panel) contains multiple CtrA binding sites. This region of genome encodes proteins found in the L-ring (FlgH; orange arrow), P-ring (FlgA; green arrow), rod (FlgG, FlgF, FliL, FlgB, FlgC, and FliE; pink arrows), export apparatus (FliP, FliQ, FliR, FlhB; yellow arrows), and C-ring (FliM; grey arrow). CtrA binding sites are indicated by closed black circles (exact match to

consensus) and open black circles (one substitution). Purple arrows indicate genes encoding chemotaxis proteins whereas black arrows indicate genes encoding hypothetical proteins. C. Multiple putative CtrA binding sites are observed in the unipolar polysaccharide biosynthesis gene cluster. Colored arrows indicate genes with homology to the upp biosynthesis cluster in *A. tumefaciens* as described in the legend for Figure 2.S1. Black genes indicate hypothetical proteins. Putative CtrA binding sites with one substitution are indicated by open black circles.

SUPPLEMENTARY TABLES

Table 2.S1 Orthologs of *A. tumefaciens* *UPP* biosynthesis cluster in *P. hirschii*.

<i>P. hirschii</i> NCBI locus	Ortholog in <i>P. hirschii</i>	Ortholog in <i>A. tumefaciens</i> (% identity)*	General description of function#
KPL55196 [‡]	C1_5522 [‡]	UppH Atu_0482 (29%) [^]	Deacetylase
KPL55195	C1_5521	UppA Atu_1240 (31.3%)	Acetyltransferase
KPL55194	C1_5520	UppB Atu_1239 (35.5%)	MPA-1
KPL55193	C1_5519	UppC Atu_1238 (63.4%)	Secretin
KPL55192	C1_5516	UppD Atu_1237 (44.4%)	Glycosyltransferase
KPL55191	C1_5515	UppE Atu_1236 (54.2%)	Glycosyltransferase
KPL55190	C1_5514	UppF Atu_1235 (44.6%)	Polymerase
KPL52370	C1_1957	Atu_0480 (80%)	Flippase
Not present	Not present	Atu_0481	Polymerase

* % identity of bidirectional best hits to *P. hirschii* orthologs were determined using Clustal Omega except where indicated

general descriptions of function are provided as a reference

[‡] Non-syntenic *P. hirschii* locus that has higher sequence homology to *A. tumefaciens* *uppH*. NCBI locus (KPL54727), Ortholog in *P. hirschii* (C1_4935) is the bidirectional best hit.

[^] Lower confidence hits due to low sequence similarity with less than 12% overall coverage

Table 2.S2 Orthologs of cell cycle regulators in *P. hirschii*

Regulatory Cell Cycle Protein	<i>P. hirschii</i> NCBI locus	Ortholog in <i>P. hirschii</i>	Ortholog in <i>A. tumefaciens</i> (% identity)*	Ortholog in <i>C. crescentus</i> (% identity)*	General description of function#
DivJ	KPL52058	C1_1571	Atu_0921 (46.6%)	CC_1063 (49.2%)	Histidine kinase responsible for DivK phosphorylation
DivK	KPL51044	C1_0307	Atu_1296 (80.5%)	CC_2463 (80.0%)	Response regulator; phosphorylation state controls cell fate

PleC	KPL54541	C1_4701	Atu_0982 (44.9%)	CC_2482 (39.8%)	Histidine kinase dephosphorylates DivK
PdhS1	KPL51802	C1_1248	Atu_0614 (51.5%)	Not present	Histidine kinase involved in polar differentiation
PdhS2	KPL51665	C1_1072	Atu_1888 (48.5%)	Not present	Histidine kinase involved in polar differentiation
CckA	KPL51705	C1_1122	Atu_1362 (53.5%)	CC_1078 (49.4%)	Hybrid histidine kinase; along with ChpT regulates phosphorylation state of CtrA
ChpT	KPL54390	C1_4527	Atu_2438 (52.0%)	CC_3470 (36.0%)	Hpt phosphotransfer ase; along with CckA regulates phosphorylation state of CtrA
CtrA	KPL54404	C1_4543	Atu_2434 (94.2%)	CC_3035 (82.3%)	Master regulator of cell cycle progression
SciP	KPL54413	C1_4554	Atu_2430 (84.6%)	CC_0903 (82.7%)	Accessory protein functioning in regulation of CtrA-dependent promoters
MucR2/ ros ⁺	KPL52063	C1_1579	Atu_0916 (62.3%)	CC_0949 (66.9%)	Repressor of CtrA-activated genes
CpdR	KPL55751	C1_1840	Atu_3883 (86.3%)	CC_0744 (63.0%)	Response regulator involved in ClpXP localization
RcdA	KPL52102	C1_1635	Atu_4742 (55.8%)	CC_3295 (53.3%)	Degradation factor required for ClpXP mediated proteolysis of CtrA
ClpP	KPL55257	C1_5596	Atu_1258 (69.0%)	CC_1963 (67.0%)	Essential ATP- dependent ClpXP protease component; responsible for CtrA proteolysis
ClpX	KPL55258	C1_5597	Atu_1259 (81.8%)	CC_1961 (81.3%)	Essential ATP- dependent ClpXP protease component;

					responsible for CtrA proteolysis
CcrM	KPL52394	C1_1988	Atu_0794 (68.7%)	CC_0378 (65.7%)	DNA methyltransferase; methylation state impacts cell cycle regulation
GcrA	KPL55789	C1_2139	PC-6 ⁺ (47.2%)	CC_2245 (42.9%)	Transcriptional regulator involved in late cell cycle progression
DnaA	KPL52602	C1_2255	Atu_0324 (52.0%)	CC_0008 (37.0%)	Initiates DNA replication and transcriptional activator during early cell cycle progression
DivL	KPL56005	C1_4100	Atu_0027 (43.2%)	CC_3484 (35.7%)	Tyrosine kinase required for cell division and growth
PodJ	KPL52461	C1_2083	Atu_0499 (40.0%)	CC_2045 (33.8%)	Polar development protein required for localization of several polar organelles

* % identity of bidirectional best hits to *P. hirschii* CDS were determined using Clustal Omega

general descriptions of function are provided as a reference

^ An additional MucR paralog (C1_2945) is present in the genome

+ GcrA was not annotated in the original *A. tumefaciens* genome sequence but was later identified during re-annotation.

CHAPTER 3

Cell Wall Synthesis and Remodeling Enzymes Required for Polar Elongation in the Rhizobiales

Author Contributions

Williams M, Aliashkevich A, and Kroll E, conducted the experiments. Williams M
Cava F, Becker A, and Brown PB designed the experiments. Williams M,
Aliashkevich A and Brown PB analyzed data and Williams M, and Brown PB
wrote and edited the paper.

ABSTRACT

Many well-studied rod-shaped bacteria insert new peptidoglycan (PG) by incorporation of PG precursors along the lateral sidewalls of the cell body using a well-defined elongasome complex, which consists of MreBCD, RodA, and PBP2. In contrast, bacteria in the Rhizobiales exhibit polar elongation, whereby new PG is inserted at a single pole, and the canonical cell elongation machinery for lateral insertion of PG is universally absent. Here we show that an essential bifunctional Penicillin-binding protein (PBP1a) plays a key role in polar elongation, while the monofunctional PBP3a and PBP3b are a synthetic lethal pair required for cell division. Cells depleted of PBP1a get shorter due to a loss of polar growth. By comparing the labeling pattern between two cell wall probes, fluorescent dipeptides (FDAADs) and fluorescent D-amino acids (FDAAs) we show that PBP1a is the major synthase required for polar PG insertion. In addition, depletion of PBP1a causes changes in PG composition indicative of altered LD-transpeptidase (LDT) activity. LDTs synthesize 3-3 cell wall crosslinks, which make up a high proportion (~30%) of the crosslinks in *A. tumefaciens* and are a prominent feature in the cell walls of other polar-growing bacteria. The genome of *A. tumefaciens* encodes 14 LDTs, and localization studies reveal that these LDTs are found in 4 distinct patterns: dispersed, mid-cell, polar, and sub-polar. Investigation of the two sub-polar LDTs shows that they contribute to remodeling of the cell wall distinctly in the old and new cell compartments. We have purified one of these LDTs and identified faropenem as an antibiotic inhibitor of this enzyme. Sub-lethal treatment with faropenem causes swelling of the growth pole,

leading to lollipop-shaped cells. This indicates that sub-polar remodeling by LDTs plays an important role during polar growth in *A. tumefaciens*. Using cell wall enzyme targeting antibiotics and deletion mutants we show that similar features of the cell wall machinery such as an essential PBP1a and sub-polar LDTs underlie the unique mechanism of polar growth in the agriculturally and medically important Rhizobiales, *Sinorhizobium* and *Brucella*.

INTRODUCTION

The Alphaproteobacterial order Rhizobiales is a diverse clade of bacteria with respect to their phylogeny, species morphology, and environmental niches [1, 2]. This includes many species of medical and agricultural significance such as the facultative intracellular pathogens *Bartonella* and *Brucella*, and the nitrogen-fixing plant symbiont *Sinorhizobium*. Also included in this order, *Agrobacterium tumefaciens* is the causative agent of crown gall disease and is considered one of the leading plant pathogens of economic importance [3, 4]. The Rhizobiales exhibit polar elongation, whereby new cell wall is inserted at a single pole. This growth mode is uniquely different from other model bacteria such as *E. coli* and *B. subtilis*, which incorporate new cell wall along the lateral sidewalls of the cell body. Lateral cell wall synthesis utilized by model bacteria has become the archetypal example for studies of bacterial growth [5, 6]. Thus, expanding studies of bacterial growth modes beyond the typical model systems will enhance our understanding of fundamental questions like how and why bacteria have evolved different growth strategies. Detailed investigations of the mechanism of growth

utilized by species in the Rhizobiales may also contribute to the discovery of antibiotics and novel therapeutics that target the bacterial cell wall of these polar-growing bacteria [7, 8].

Bacteria grow and divide, faithfully reproducing their characteristic cell shape, a process that requires enzymes to synthesize, hydrolyze and remodel peptidoglycan (PG) in a temporal and site-specific manner [9]. Since the PG is a closed polymer comprised of covalently linked sugars and peptides, synthesis of new PG cannot progress without first hydrolyzing the existing structure. There are several classes of enzymes that cleave PG including the DD-endopeptidases, which hydrolyze 4-4-crosslinked PG to make space for the insertion of new PG [10]. Following hydrolysis, class a and b penicillin-binding proteins (PBPs) can synthesize new cell wall. aPBPs are bifunctional enzymes that catalyze β -1,4 linkages between the N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugars in a process called transglycosylation, and also synthesize crosslinks between 4-3 and 4-4 peptides, known as transpeptidation [11]. The bPBPs are monofunctional enzymes that only have transpeptidase activity and often work as dimers [12]. Another class of enzymes known as LD-transpeptidases (LDTs) catalyze 3-4 and 3-3 crosslinks [13]. The cell wall of *A. tumefaciens* and other polar-growing bacteria have been shown to contain a high proportion of 3-3 and 3-4 crosslinks, indicating that LDT enzymes may play an important role during polar growth [14, 15].

Peptidoglycan labeling experiments in *Agrobacterium tumefaciens*, *Brucella abortus*, *Sinorhizobium meliloti*, and *Ochrobactrum anthropi* have confirmed unipolar growth is the main mode of elongation in these rod-shaped species [14], whereby new PG is inserted at a single pole; and the canonical cell elongation machinery for dispersed insertion of PG is universally absent, including homologs to the major cell wall scaffolding and PG synthesis enzymes MreBCD, RodA, and PBP2 [16]. Interestingly, the Rhizobiales also lack homologs to the known scaffolding proteins required for elongation (DivIVA, Scy, and FilP) in another polar-growing clade, the Actinobacteria [6, 17]. This indicates that species in the Rhizobiales have evolved a novel mechanism for elongation, but the proteins that drive polar peptidoglycan synthesis remain undefined.

Here we use microscopy, biochemical and genetic analyses to characterize all six high molecular weight PBPs and identify the major cell wall synthesis and remodeling enzymes required for polar growth of *A. tumefaciens*. We show that the essential PBP1a is a key enzyme required for polar PG expansion. Using dipeptide probes, we show that PBP1a is the major enzyme inserting nascent PG at the pole. Depletion of PBP1a results in a loss of rod-shape. Additionally, depletion of PBP1a modifies PG composition leading to an increase in LDT-linked PG. Of the 14 LDTs encoded in the *A. tumefaciens* genome, we have identified three that localize to the growth pole, one that localizes to the sub-pole of the growth pole compartment, and one LDT that localizes to the sub-pole of the old pole compartment. Together this suggests that the mechanism of polar

growth in the Rhizobiales has evolved through the diversification, expansion and regulation of known cell wall synthesis machinery. We confirmed the essentiality of PBP1a in the closely related bacterium *Sinorhizobium*, illustrating that the mechanisms that underly polar growth are well conserved in the Rhizobiales. Finally, we have identified faropenem as a specific inhibitor of polar growth in *Sinorhizobium* and *Brucella*, and these findings may inform antibiotic treatments of other pathogenic Rhizobiales such as *Bartonella*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. A list of all bacterial strains used in this study is provided in Table 3.S1. *Agrobacterium tumefaciens* C58 and derived strains were grown in ATGN minimal media [18] without exogenous iron at 28°C with shaking. When appropriate, kanamycin (KAN) was used at the working concentration of 300 µg/ml. When indicated, isopropyl β-D-1-thio-galactopyranoside (IPTG) was used as an inducer at a concentration of 1 mM, and cumate was used at a concentration of 0.2 µM. *Sinorhizobium meliloti* strains were grown in TY medium. When appropriate, KAN was used at the working concentration of 100 µg/ml, Gentamicin (GM) was used as 20 µg/ml, and IPTG was used at a concentration of 500 µg/ml. *Brucella abortus* strain S19 was grown in Brucella Broth. *E. coli* strains were grown in Luria-Bertani medium at 37°C. For *E. coli* DH5α and S17-1 λ *pir*, when appropriate 50 µg/ml and 30 µg/ml of KM, were added.

Construction of strains and plasmids. For amplification of target genes, primer names indicate the primer orientation and added restriction sites. To construct expression vectors containing *ldtA-N-sfgfp* the respective coding sequence was amplified from purified C58 genomic DNA. The amplicons were digested overnight and ligated into cut pSRKKM-P_{cym} using NEB T4 DNA ligase at 4 degrees Celsius overnight, to create an expression vector compatible with the depletion strains. All expression vectors were verified by sequencing. All vectors were introduced into *A. tumefaciens* strains utilizing standard electroporation protocols [19] with the addition of IPTG in the media when introducing plasmids into depletion backgrounds.

Construction of deletion/depletion plasmids and strains. Vectors for gene deletion by allelic exchange were constructed using recommended methods for *A. tumefaciens* [19]. Briefly, 500-bp fragments upstream and 500 bp downstream of the target gene were amplified using primer pairs P1/P2 and P3/4 respectively. Amplicons were spliced together by SOEing using primer pair P1/P4. The amplicon was digested and ligated into pNTPS139. The deletion plasmids were introduced into *A. tumefaciens* by mating using an *E. coli* S17 conjugation strain to create KM resistant, sucrose sensitive primary integrants. Primary integrants were grown overnight in media with no selection. Secondary recombinants were screened by patching for sucrose resistance and KM sensitivity. Colony PCR with primers P5/P6 for the respective gene target was

used to confirm deletion. PCR products from P5/P6 primer sets were sequenced to further confirm deletions.

For depletion strain construction, target genes (*pbp1a* or *pbp3a*) were amplified, digested and ligated into pUC18-mini-Tn7T-GM-P_{lac}. The mini-Tn7 vector, along with the pTNS3 helper plasmid, were introduced into C58Δ*tetRA*::*a-att*Tn7 as described previously [20]. Transformants were selected for GM resistance and insertion of the target gene into the *a-att* site was verified by colony PCR using the tet forward and Tn7R109 primer. PCR products were sequenced to confirm insertion of the correct gene. Next, the target gene was deleted from the native locus as described above in the presence of 1 mM IPTG to drive expression of the target gene from the engineered site.

Phase and Fluorescence microscopy. A small volume (~1 µl) of cells in exponential phase (OD₆₀₀ = 0.2 - 0.4) was applied to a 1% ATGN agarose pad as described previously [21]. DIC, Phase contrast and epifluorescence microscopy were performed with an inverted Nikon Eclipse TiE and a QImaging Rolera em-c2 123 1K EMCCD camera with Nikon Elements Imaging Software. For time-lapse microscopy, images were collected every ten minutes, unless otherwise stated. Expression of plasmid encoded LdtA-N-sfGFP was induced by the presence of 0.2 mM cumate for 1-2 hours (h) prior to imaging. Cells containing plasmids with fluorescent protein fusions were grown to exponential phase before imaging on agarose pads.

Quantification of cell length distributions. Cells were grown overnight in ATGN. Cells were diluted in ATGN to an $OD_{600} = 0.20$ and allowed to grow until reaching an $OD_{600} = 0.4 - 0.6$. Live cells were imaged using phase contrast microscopy and cell length distributions of at least 400 cells per strain were determined using the longest medial axis as measured by MicrobeJ software [22].

Quantification of cell morphologies , FDAA and FDAAD labeling patterns.

Cells were grown overnight in ATGN media and diluted in the same conditions to an $OD_{600} = 0.20$ and allowed to grow until reaching an $OD_{600} = 0.4 - 0.6$. At this point cells were labeled with the fluorescent-D-amino acid (FDAA) HADA or the fluorescent dipeptides (FDAADs) NADA-DA as previously described [23-25]. Immediately following labeling, cells were ethanol fixed to prevent further growth. Phase contrast and epifluorescence microscopy was performed on at least 600 cells. Demographs were constructed using MicrobeJ. For demographs, cells were arranged from top to bottom according to their cell lengths and each cell was oriented such that the new pole (defined as the cell pole with the higher *fluorescence* intensity as determined by HADA labeling or smaller pole diameter) was located on the left.

Peptidoglycan compositional analysis.

For PG analysis in Figure 3.4A, three cultures each of WT and + PBP1a depletion cells were grown overnight in 3 ml culture tubes of ATGN minimal media at 28°C with shaking; the + PBP1a strain was supplemented with 1mM IPTG. The 3 ml cultures were then added to 50 ml flasks of fresh ATGN and allowed to grow under the same conditions until reaching an exponential phase OD₆₀₀ of 0.5-0.6. Cells were then pelleted by centrifugation at 4000 x g for 10 minutes. Cell pellets were washed three times with ATGN by centrifugation and resuspension to remove IPTG. After the final wash the 3 cell pellets from the + PBP1a strain were split and resuspended in 50 ml ATGN with or without IPTG. Each culture was grown for 16 hours (hr). to an OD₆₀₀ of 0.6 After 146 h of growth, 50 ml of the exponential cultures were collected and pelleted by centrifugation at 4000 x g for 20 minutes. Cell pellets were resuspended in 3 mL of ATGN and 6 mL of 6% SDS and stirred with magnets while boiling for 4 h. After 4 h, samples were removed from heat but continued to stir overnight. Samples were then shipped to Dr. Felipe Cava's laboratory for purification and analysis. Upon arrival, cells were boiled and simultaneously stirred by magnets for 2 h. After 2 h, boiling was stopped, and samples were stirred overnight. Peptidoglycan was pelleted by centrifugation for 13 minutes (min) at 60,000 rpm (TLA100.3 Beckman rotor, Optima Max-TL ultracentrifuge; Beckman), and the pellets were washed 3 to 4 times by repeated cycles of centrifugation and resuspension in water. The pellet from the final wash was resuspended in 50 µl of 50 mM sodium phosphate buffer, pH 4.9, and digested overnight with 100 µg/ml of muramidase at 37°C. Muramidase digestion was stopped by boiling for 4

min. Coagulated protein was removed by centrifugation for 15 min at 15,000 rpm in a desktop microcentrifuge. The muropeptides were mixed with 15 μ l 0.5 M sodium borate and subjected to reduction of muramic acid residues into muramitol by sodium borohydride (10 mg/ml final concentration, 20 min at room temperature) treatment. Samples were adjusted to pH 3 to 4 with orthophosphoric acid and filtered (0.2- μ m filters). Analysis of muropeptides was performed on an ACQUITY Ultra Performance Liquid Chromatography (UPLC) BEH C18 column, 130Å, 1.7 μ m, 2.1 mm x 150 mm (Water, USA) and detected at Abs. 204 nm with ACQUITY UPLC UV-visible detector. Muropeptides were separated at 35°C using a linear gradient from buffer A (sodium phosphate buffer 50 mM pH 4.35) to buffer B (sodium phosphate buffer 50 mM pH 4.95 methanol 15% (v/v)) with a flow of 0.25 mL/min in a 20 minutes run.

Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards.

For PG analysis in Figure 3.5C, cell cultures were grown overnight in 3 ml culture tubes of ATGN minimal media at 28°C with shaking. The 3 ml cultures were then added to 50 ml flasks of fresh ATGN and allowed to grow under the same conditions until reaching an exponential phase OD₆₀₀ of 0.5-0.6. Cultures were then pelleted by centrifugation at 4,000 x g for 15 minutes. Pellets were resuspended in a solution of 1 ml ATGN and 2 ml of 6% SDS. Cells were boiled while simultaneously stirred by magnetic bars for 4 hours. After 4 hours, boiling was halted, but agitation was continued overnight. Peptidoglycan was pelleted by

centrifuging for 13 min at 60,000 rpm (TLA100.3 Beckman rotor; Optima Max-TL ultracentrifuge Beckman), and the pellets were washed 3-4 times by repeated cycles of centrifugation and resuspension in water. The pellet from the final wash was resuspended in 50 μ l of 50 mM sodium phosphate buffer pH 4.9 and subjected to overnight digestion with 100 μ g/ml of muramidase at 37°C. Muramidase digestion was stopped by boiling for 4 min. Coagulated protein was removed by centrifugation for 15 min, 15,000 rpm in a desk-top microcentrifuge. The muropeptides were mixed with 15 μ l 0.5M sodium borate and subjected to reduction of muramic acid residues into muramitol by sodium borohydride (10 mg/ml final concentration, 20 min at room temperature) treatment. Samples were adjusted to pH 3-4 with orthophosphoric acid and filtered (0.2 μ m filters). Analysis of muropeptides was performed on an ACQUITY Ultra Performance Liquid Chromatography (UPLC) BEH C18 column, 130Å, 1.7 μ m, 2.1 mm x 150 mm (Water, USA) and detected at Abs. 204 nm with ACQUITY UPLC UV-visible detector. Muropeptides were separated using a linear gradient from buffer A (sodium phosphate buffer 50 mM pH 4.35) to buffer B (sodium phosphate buffer 50 mM pH 4.95 methanol 15% (v/v)) with a flow of 0.25 mL/min in a 20 min run. Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards.

RESULTS

Cell wall synthesis enzymes play a key role in *A. tumefaciens* growth and division

To improve our understanding of polar PG biosynthesis and cell division in *A. tumefaciens* we began by characterizing the predicted PG synthase enzymes. Although *A. tumefaciens* lacks homologs to predicted cell elongation synthases RodA and PBP2, the genome of *A. tumefaciens* does encode four bifunctional aPBPs (PBP1a, PBP1b1, PBP1b2 and PBP1c), two monofunctional bPBPs (PBP3a and PBP3b), and one monofunctional glycosyltransferase (GTase), MtgA (Figure 3.1A). All of the predicted bifunctional and monofunctional PBPs are expressed during exponential growth of *A. tumefaciens* (Supplementary Figure 3.1A).

Fig. 3.1: Functional characterization of peptidoglycan synthases in *A.*

tumefaciens. A. Domain structure of the putative PG biosynthesis enzymes

showing the transmembrane (TM), Glycosyltransferase (GT, PF00912), Transpeptidase (TP, PF00905), OB-like (PF17092), PBP dimer domains (PF03717), and biPBP_C (PF06832) domains. The regions of intrinsic disorder (grey) as predicted by MoBiDB are also shown. The scale indicates the length in amino acids (aa). The corresponding ATU numbers are listed in parentheses beside each gene name. B. Phase microscopy images showing the phenotypes of PG synthase mutants. Each strain was grown to exponential phase, spotted on an ATGN agar pad, and imaged by phase microscopy. Scale bar: 2 μ M. C. Cell length distributions of PG synthase mutants. The indicated strains were grown as in B and subjected to cell lengths measurements using MicrobeJ. The data are shown as box plots. Distributions of cells significantly different from WT are indicated (***) One-Way ANOVA with Bonferroni correction, $p > 2 \times 10^{-16}$. $n = > 800$ cells per strain.

To explore the contribution of these enzymes to cell growth or division, we endeavored to make in-frame deletions of each of the respective PBPs. Single deletions of PBP1b1, PBP1b2, and PBP1c had no effect on cell length (Figure 3.1B, C) or cell viability (Supplementary Figure 3.1B). Surprisingly, a double mutant of PBP1b1 and PBP1b2 or a triple mutant of PBP1b1, PBP1b2 and PBP1c (referred to as $\Delta 3pbp$) also did not produce an obvious phenotype with respect to cell length (Figure 3.1B, C) or cell viability (Supplementary Figure 3.1B). Together, these enzymes may only minimally contribute to cell growth or division under the standard growth conditions tested here. Similarly, deletion of

MtgA produced cells of a normal cell length (Figure 3.1B,C). The lack of phenotype seen by deletion of MtgA is in line with finding from *E. coli* and *H. neptunium*, where deletion of MtgA also has no reported phenotypic effect [26-28].

Of the remaining genes, PBP1a was predicted to be essential by a transposon mutagenesis screen [29], and it was not possible to obtain a deletion mutant of PBP1a. As an alternative, we constructed a depletion strain of PBP1a by introducing a copy of the gene encoding *pbp1a* at a non-native site in the chromosome under an IPTG-inducible promoter and subsequently made an in-frame deletion of the native copy of *pbp1a* [20]. We confirmed depletion of PBP1a in the absence of IPTG (Supplementary Figure 3.1C). Strikingly, upon depletion of PBP1a for 16 hours cells lost their rod shape and became shorter (Figure 3.1B, C) and wider (Supplementary Figure 3.1D) and had a severe viability defect compared to the same strain when PBP1a is induced (Supplementary Figure 3.1B). These data suggest that the PBPs of *A. tumefaciens* do not have redundant functions, but instead have specialized roles during cell growth and division.

Saturating transposon mutagenesis indicated that PBP3a was likely essential [29]; however, it was possible to make a deletion of PBP3a in minimal medium. The saturating transposon mutagenesis screen was carried out in LB medium

and we find that PBP3a is not viable on LB. These observations suggest that the PBP3a deletion strain is conditionally essential. In minimal media, deletion of PBP3a caused a severe cell viability defect (Supplementary Figure 3.1C), and production of longer cells (Figure 3.1B, C), with several cells adopting a branched morphology, which is characteristic of a cell division defect in *A. tumefaciens* [30, 31]. In contrast, deletion of PBP3b, the other monofunctional bPBP, had no effect on cell viability (Supplementary Figure 3.1C) and cells were slightly shorter (Figure 3.1B, C). Together, these phenotypes indicate that PBP3a is the major bPBP contributing to cell division in *A. tumefaciens*.

Monofunctional PBP3a and PBP3b are required for cell division

While most bacteria possess a single, essential *ftsI* gene that encodes PBP3 and functions during cell division, some Rhizobiales, including *A. tumefaciens* encode two homologs of FtsI (PBP3a and PBP3b). In *A. tumefaciens* cells with a single deletion of PBP3a are still able to divide, thus we hypothesized that PBP3b may be able to partially compensate for the loss of PBP3a. To address this possibility, we first created a PBP3a depletion strain; this strain grown in the absence of IPTG for 24 hours phenocopies the cell viability and cell length defects of the PBP3a deletion mutant (Figure 3.2A). We then depleted PBP3a in the $\Delta pbp3b$ strain, and saw that cells fail to divide, and instead swell at the mid-cell (Figure 3.2A), indicating that PBP3a and PBP3b both contribute to septal PG biosynthesis during cell division in *A. tumefaciens*. In model bacteria, cell division begins by the recruitment of early divisome proteins such as the tubulin homolog,

FtsZ, to the mid-cell, which in turn recruit late divisome proteins like PBP3 and FtsW that synthesize PG at the mid-cell prior to cell division [32].

To determine if FtsZ rings form in the absence of PBP3a or PBP3b we localized FtsZ-sfGFP in the PBP3a depletion strain. Indeed, FtsZ rings do form in the PBP3a depletion strain, confirming that PBP3a is likely a late divisome protein that is recruited to the mid-cell after FtsZ. However, some FtsZ rings form asymmetrically (Figure 3.2 C yellow arrowhead), or FtsZ rings form near mid-cell and are unstable, leading to cell lysis (Figure 3.2 C white arrowhead). These observations suggest that proper PG biosynthesis affects the stability and placement of Z-rings. The partial loss of mid-cell PG biosynthesis may contribute to the slower growth rate and cell viability defects seen in the PBP3a depletion strain. We also monitored FtsZ ring placement in the $\Delta pbp3b$ strain depleted of PBP3b. These cells fail to divide, and instead begin to grow ectopic poles from the mid-cell. Despite the almost complete block in cell division, FtsZ rings still form under these conditions (Figure 3.2 C blue arrowheads). Later in the depletion, cells severely swell from the mid-cell and lose FtsZ ring foci, and instead FtsZ-sfGFP is dispersed mainly in the mid-cell buds (Figure 3.2 C pink arrows). Together, PG biosynthesis at the mid-cell by PBP3a and PBP3b is required for proper cell division and localization of FtsZ. The phenotype observed in the absence of both PBP3a and PBP3b is remarkably similar to the phenotype observed during depletion of FtsW in *A. tumefaciens* [30]. In model bacteria it has been shown that FtsW possesses GTase activity, and is a major synthase

required for cell division [33, 34]. In *A. tumefaciens*, we hypothesize that FtsW provides the GTase activity while PBP3a and PBP3b provide the DD-transpeptidase (TPase) activity necessary for proper septal PG biosynthesis during cell division.

Fig. 3.2: *Functional characterization of monofunctional synthases PBP3a and PBP3b* A. Phase microscopy images showing the phenotypes of PG synthase mutants. Each strain was grown to exponential phase, spotted on an ATGN agar pad, and imaged by phase microscopy. Scale bar: 2 μ M. B. Cell length distributions of PG synthase mutants. The indicated strains were grown to exponential phase, spotted on an agar pad, imaged by phase microscopy, and subjected to cell lengths measurements using MicrobeJ. The data are shown as box plots. Distributions of cells significantly different from WT are indicated (***) One-Way ANOVA with Bonferroni correction, $p < 2 \times 10^{-16}$. $n = > 500$ per strain. C. Time-lapse microscopy of the – PBP3a and $\Delta pbp3b$ – PBP3a depletion strains illustrates the cell division defects of the double mutant. Cells were grown overnight in the presence of 1mM IPTG to an OD₆₀₀ of 0.6, washed three times with ATGN, then spotted on an agarose pad and imaged every 10 minutes.

PBP1a is the major synthase incorporating PG at the pole

Given its predicted activity as a cell wall synthase, and the striking loss of rod shape upon depletion of PBP1a, we investigated the impact of the depletion of PBP1a on PG synthesis using fluorescent probes that incorporate into PG. Cells were labeled with either a fluorescent D-amino acid (FDAA) or fluorescent D-amino acid dipeptide (FDAAD) probe to observe sites of PG crosslinking in living cells. FDAADs are incorporated into the cell wall precursors by the cytoplasmic MurF ligase [24, 25, 35]. The resulting lipid II-linked fluorescent precursor is covalently crosslinked to an existing PG glycan strand by the activity of

bifunctional PBPs. Thus, tracking sites of FDAAD probe incorporation indicates the sites of new PG incorporation by bifunctional PBPs (Figure 3.3A). WT *A. tumefaciens* was labeled for ~10% of the cell cycle with the FDAAD probe NADA-DA. Cells exhibit labeling at the growth pole during elongation with a switch to labeling at mid-cell in cells undergoing cell division. This pole-to-mid-cell labeling pattern indicates that PBP-mediated insertion of new PG is consistent with other PG labeling methods including FDAAs [23] and D-cysteine labeling [14]. A similar polar-to-mid-cell labeling pattern was observed in the triple PBP mutant (Supplementary Figure 3.2A), further suggesting that these PBPs do not make major contributions to PG incorporation under the conditions tested.

Fig. 3.3: Comparison of FDAAD and FDAA labeling in *A. tumefaciens*. A.

Schematic of the incorporation pathway of FDAADs. FDAADs are incorporated into the mureopeptide precursor molecule in the cytoplasm by the MurF ligase. The mureopeptide precursor is flipped across the membrane and the activity of a bifunctional PBP crosslinks the new PG monomer into the existing PG sacculus. Demographs of WT and the PBP1a depletion strain grown in the presence or absence of IPTG labeled + PBP1a and - PBP1a, respectively are shown. Demographs depict incorporation of FDAAD at a population level. Median

profiles of the fluorescence channel of more than 1000 cells per strain are stacked and ordered by cell length. Merged phase and fluorescent channels of cells with representative polar and mid-cell labeling of dipeptides are shown below each demograph. Scale bar: 2 μ M. B. Schematic of the incorporation pathway of FDAA. FDAAs diffuse into the periplasm and are crosslinked into the existing PG through a transpeptidation reaction, which is typically carried out by an LDT. Demographs of WT and the PBP1a depletion strain grown in the presence or absence of IPTG labeled + PBP1a and - PBP1a, respectively are shown. Demographs depict incorporation of FDAA acids at a population level. Median profiles of the fluorescence channel of more than 1000 cells per strain are stacked and ordered by cell length. Merged phase and fluorescent channels of cells with representative polar and mid-cell labeling by FDAAs are shown below each demograph.

In contrast to WT or PBP1a replete cells, polar labeling of NADA-DA is almost completely absent from cells depleted of PBP1a for 16 hours (Figure 3.3A), indicating that PBP1a is the major cell wall synthase required for polar elongation. In addition, PBP1a depleted cells have more robust labeling at the mid-cell (Figure 3.3A), suggesting PBPs acting at the mid-cell may partially compensate for the loss of PBP1a. Alternatively, since PBP1a depletion cells divide much slower than WT (After 12-16 hours of depletion cells double once every ~4 hours compared to ~2 hours in WT cells), the increased intensity of

mid-cell labeling may be due to the continued insertion of PG at mid-cell while cell division is blocked.

In comparison to FDAAD incorporation, FDAAs are incorporated into the cell wall in the periplasm through transpeptidase reactions mediated by PBPs or LDTs (Figure 3.3B). WT and $\Delta 3pbp$ cells labeled with FDAAs show the characteristic polar to mid-cell labeling pattern that is typical of *A. tumefaciens* (Figure 3.3B, Supplementary Figure 3.2A). Notably, using the FDAA probe HADA, labeling is apparent in the sidewalls of the new pole compartment prior to cell division, while the old pole remains unlabeled (Figure 3.3B), suggesting that LDTs may preferentially incorporate or modify PG in the new pole compartment. The similarities and differences in labeling patterns between FDAAD and FDAA probe incorporation suggest that PBP-mediated incorporation overlaps with that of LDT-mediated incorporation or modification at the pole and mid-cell, but that LDTs do contribute to spatially distinct crosslinking of the cell wall, particularly in the new pole daughter cell.

In stark contrast to the absence of polar labeling that was seen following FDAAD labeling, cells depleted of PBP1a labeled robustly at the growth pole with HADA (Figure 3.3B). Continued HADA incorporation at the growth pole in the absence of PBP-mediated FDAAD incorporation suggests that the major enzymes incorporating HADA at the pole in *A. tumefaciens* are likely LDTs, consistent with

recent findings for incorporation of FDAAs in *E. coli* [36]. Furthermore, LDTs can crosslink PG at the pole in the absence of PBP1a, indicating that some LDT activity can occur independently of PBP1a. Comparison of the labeling patterns generated with FDAAs and FDAADs, in combination with characterization of genetic mutants, is an effective method to probe the spatial patterns of PG insertion or remodeling by different classes of cell wall enzymes. Here we find that both PBP1a and LDTs function during polar elongation of *A. tumefaciens*.

Depletion of PBP1a leads to increased levels of LD-crosslinks in PG

Since depletion of PBP1a led to less polar incorporation of FDAADs while maintaining polar FDAA incorporation we hypothesized that PG composition analysis may reveal chemical signatures of a shift in the activity of PBPs and LDTs. We grew the PBP1a depletion strain in the presence or absence of IPTG for 16 hours, collected the cell wall fraction, and analyzed muropeptides by ultra-performance liquid chromatography (UPLC). The major muropeptides found in WT *A. tumefaciens* PG and their quantification are shown in Figure 3.4B and include monomeric (M), dimeric (D) and trimeric (T) muropeptides. PG from PBP1a depleted cells has a significant reduction in the abundance of muropeptides with DD-crosslinks, evident in the ~3% reduction in the abundance of D44 (Figure 3.4A, B). As expected, this observation suggests that the activity of PBP1a-mediated DD-transpeptidases is decreased during PBP1a depletion. Depletion of PBP1a also caused an increase in muropeptides containing LD-crosslinks, as indicated by the ~4% increase in abundance of D34 (Figure 3.4A,

B). These data suggest that the activity of LD-transpeptidases is increased during PBP1a depletion. Since we only saw an increase in mucopeptide D34 but not D33 (Figure 3.4A), we hypothesize that only a subset of LDTs are activated during depletion of PBP1a, and another subset of LDTs may in fact function along with PBP1a during polar growth.

Fig. 3.4: *PG composition analysis of cell wall synthase mutants* A. Bar graphs showing the average abundance of muropeptides obtained by UPLC analysis from WT, and the PBP1a depletion strain grown in the presence or absence of IPTG for 16 hours. Data shown are averages taken from analysis of three

independent biological samples. B. Schematics of each of the mucopeptides from the bar graph are shown. The average percent abundance of each type of mucopeptide for each of the three strains is shown. A key labeling the components of each mucopeptide is provided. Monomer (M) Dimer (D), Trimer (T).

Carbapenem and penem antibiotics target LDTs

Compared to most other bacteria, *A. tumefaciens* possesses an expanded set of 14 LDT enzymes, half of which comprise a Rhizobiales-specific clade that has low homology to other known LDT enzymes [16]. Domain analysis shows that most of the Rhizobiales-specific LDTs are shorter in length, and do not contain PG binding domains, which seems to be a characteristic of most of the *E. coli* LDT homologs (Supplementary Figure 3.3A). To be relatively consistent with the *E. coli* and *C. crescentus* nomenclature, the LDTs of *A. tumefaciens* are now referred to as: LdtA-N (Supplementary Figure 3.3A).

β -lactam antibiotics are one of the most widely used classes of antibiotics that target cell wall synthesis enzymes, and primarily have been studied for their ability to target PBPs [37]. A subclass of β -lactam antibiotics known as the carbapenems, along with the penem antibiotic faropenem, are useful for probing the activity of LDTs [38, 39]. To characterize the roles of LDT activity during *A. tumefaciens* growth, we investigated the effect of five carbapenem antibiotics and

one penem antibiotic on cell growth and morphology. Sub-MIC treatments for 24 hours with any of five carbapenem antibiotics induces mid-cell swelling (Figure 3.5A). These data indicate that the carbapenem antibiotics may target an enzyme(s) with a specific role at the mid-cell during cell division. Interestingly, faropenem treated cells became wider and rounder (Figure 3.5A), which may indicate its cellular target is important for maintenance of rod shape during polar growth. In agreement, time-lapse microscopy of faropenem-treated cells reveals a loss of rod shape preferentially in the new daughter cell compartment, while the old compartment retains its rod shape (Figure 3.5B). This phenotype is distinct from the carbapenem antibiotics and indicates that the cellular target(s) of faropenem are important for proper polar PG synthesis.

Fig. 3.5: Phenotypic characterization of carbapenem and penem antibiotic

treatments A. Representative images of WT cells grown in the presence of 0.5 ug/mL of imipenem, 1.5 ug/mL doripenem, ertapenem, meropenem, faropenem, and 50 ug/mL tebipenem. Cells were incubated with antibiotics for 24 hours, then spotted on a 1% agarose pad and imaged using DIC microscopy. Scale bar: 2 μ M B. Time-lapse microscopy of WT cells spotted on a 1% ATGN agarose pad supplemented with 1.5 ug/mL of faropenem were imaged every 10 minutes. Indicated time in hours is shown. Scale bar: 2 μ M C. Abundance of total LD- and DD-crosslinkage in peptidoglycan isolated from WT cells grown in the presence of DMSO for 6 hours, WT cells grown in the presence of 1.5 ug/mL meropenem for 4 hours, and WT cells grown in the presence of 1.5 ug/mL of faropenem for 6

hours. Data shown are the abundance of each crosslinkage type from analysis of one representative independent biological sample. Schematics and percent abundance of the muropeptides containing LD- or DD-crosslinks for each condition are shown.

Although the phenotypes differed, carbapenem and penem antibiotics are both thought to target LDTs. Thus, we hypothesized that the PG derived from antibiotic-treated cells may show signatures of a loss of LDT activity. Because the antibiotics were dissolved in DMSO, WT cells grown in the presence of DMSO were used as a control. Treatment of WT cells with 1.5 $\mu\text{g/mL}$ meropenem or faropenem causes the abundance of muropeptides with LD-crosslinks to decrease (from ~33% in WT to ~20%), while the abundance DD-crosslinked muropeptides is minimally impacted (Figure 3.5C). Thus, both meropenem and faropenem are likely targeting LDT enzymes; however, the phenotypes of meropenem and faropenem-treated cells suggest that they may target different subsets of LDTs that act during different stages of growth or at distinct subcellular locations. Indeed, we find that the LdtM activity is blocked by faropenem but not meropenem *in vitro* (Supplementary Figure 3.2C).

LD-TPases have distinct localization patterns

To investigate if any of the LDTs may play a direct role in polar growth or division, we assessed their subcellular localization. Cells expressing LDT-sfGFP

fusions from a cumate-inducible promoter displayed four distinct localization patterns: dispersed, mid-cell, polar, and sub-polar (Supplementary Figure 3.4). Upon further investigation of the polarly-localized LDTs (LdtJ, LdtK and LdtL), each have a unique localization pattern. LdtJ, localized strictly to the growth pole (Figure 3.6A). Thus, LdtJ may play a role primarily at the growth pole during elongation. LdtK localized to the growth pole and to mid-cell in pre-divisional cells (Figure 3.6A). Finally, LdtL localized to the growth pole, then relocated to mid-cell prior to cell division, and after division, LdtL localized bipolarly in the new daughter cell and strictly to the growth pole of the old daughter cell (Figure 3.6A). Therefore, LdtL localized bipolarly in about half the population, and may play distinct roles at the new and old pole in *A. tumefaciens*. The unique localization patterns displayed by all three of the polarly localized LDTs indicate that they may similarly function in polar elongation, but that they likely each have distinct functions during the cell cycle.

Fig. 3.6: Polar and sub-polar localization of LDTs A. Representative images of the localization patterns for LdtJ-sfGFP, LdtK-sfGFP and LdtL-sfGFP expressed from a cumate-inducible promoter in wildtype cells. Exponential phase cells were grown in the presence of cumate for 2 hours prior to spotting on a 1% ATGN agarose pad and imaged. Shown are merged images of phase and fluorescent channels. White arrowheads indicate mid-cell localization. Yellow asterisks represent cells with bipolar localization. Scale bar: 2 μM B. Representative images of the localization pattern of LdtM-sfGFP expressed from a cumate-inducible promoter in wildtype cells. Cells were grown and imaged as described in A. Demograph depicts localization of LdtM-sfGFP at a population level. Median

profiles of the fluorescence channel of more than 1000 cells per strain are stacked and ordered by cell length. Cells were oriented with the new pole on the left using the HADA as a marker for the new pole. Scale bar: 2 μ M

Two of the LDTs (LdtM and LdtN) displayed sub-polar localization (Supplementary Figure 3.4A). Therefore, strains expressing either LdtM-sfGFP or LdtN-sfGFP were labeled with HADA as a marker for the new pole. In shorter cells, LdtN-sfGFP localized opposite the HADA, in the sub-polar region of the old pole compartment. As the new pole grows out LdtN seems to remain fixed to the sub-polar region of the old pole compartment. Finally, before cell division, a population of LdtN appears to be inherited to the sub-polar region of the new daughter cell (Supplementary Figure 3.4B). Based on this localization pattern, we infer that LdtN may function in remodeling of the PG specifically along the sidewalls of the old cell compartment.

In contrast, newborn cells expressing LdtM-sfGFP colocalized with HADA at the growth pole. As the growth pole extends, LdtM remains fixed at the sub-pole of the new cell compartment, and after cell division LdtM is inherited solely by the new daughter cell (Figure 3.6B). This localization pattern suggests that LdtM may play a role in crosslinking or modifying PG along the sidewalls of the new pole compartment. The observation that LdtN and LdtM localize to the old and new cell compartments, respectively, indicates that different LDTs are active along the

lateral sidewalls, particularly in sub-polar regions. Thus, the old and new cell compartments may have a distinct PG composition that confers polar identity between the daughter cells. This is an exciting finding that expands our understanding of PG synthesis and regulation in *A. tumefaciens* beyond simply, polar and mid-cell PG incorporation, and allows us to entertain the idea that sub-polar elongation or remodeling may partially contribute to the unique growth mode of *A. tumefaciens* and other Rhizobiales.

PG enzymes involved in polar growth have a conserved function in the Rhizobiales

In this work we have explored the essential role of PBP1a in polar PG insertion in *A. tumefaciens*. Unipolar cell wall elongation is a shared feature of growth within the Rhizobiales, but the mechanisms that drive polar PG insertion remain unexplored in other Rhizobiales species. The closely related plant symbiont *S. meliloti* has been shown to elongate at the pole, and the molecular mechanisms of polar growth are likely conserved between *S. meliloti* and *A. tumefaciens*. *S. meliloti* encodes six bifunctional PBP homologs, including one PBP1a homolog, four PBP1b homologs, and one PBP1c homolog. A $\Delta 5pbp$ mutant, which includes deletion of the four PBP1b homologs and deletion of the PBP1c homolog had a median cell length similar to that of WT *S. meliloti*, but a slightly broader distribution of cell lengths, with ~88% of cells falling between 1.5 and 4 μM compared to ~97% of WT cells falling between these cell lengths. Remarkably, the $\Delta 5pbp$ mutant retains rod shape, suggesting that these

enzymes contribute minimally to sustaining proper rod shape in standard growth conditions. Since we were unable to make a deletion of PBP1a, we constructed a PBP1a depletion strain. Depletion of PBP1a causes a severe viability defect (Supplementary Figure 3.5A) and cells become shorter (Figure 3.7A) and wider (Supplementary Figure 3.5B). Thus, PBP1a is essential for polar growth and maintenance of proper rod shape in both *S. meliloti* and *A. tumefaciens*. In addition, a transposon mutagenesis screen of *B. abortus* predicted that out of the bifunctional PBPs, only PBP1a may be essential for growth [40]. In addition, Bandara and colleagues were unable to obtain a PBP1a mutant in *Brucella melitensis*, indicating that PBP1a is likely essential for viability [41]. Together, these observations support the notion that an essential PBP1a is an important and conserved feature of polar growth in Rhizobiales.

Fig. 3.7: Mechanisms of polar growth are conserved in the Rhizobiales *Brucella* and *Sinorhizobium*. A. Phase microscopy images showing the phenotypes *S. meliloti* WT and PBP mutants. Each strain was grown to exponential phase, spotted on a 1% TY agarose pad, and imaged by phase microscopy. Cell length

distributions of PG synthase mutants. The indicated strains were grown to exponential phase, spotted on an agarose pad, imaged by phase microscopy, and subjected to cell lengths measurements using MicrobeJ (cite). The data are shown as box plots. Distributions of cells significantly different from WT are indicated (***; One-Way ANOVA with Bonferroni correction, $p < 2 \times 10^{-16}$). $n = >400$ per strain B. Representative phase microscopy images of the indicated species are shown. Cells were grown overnight to an OD_{600} of 0.6 and spotted on a 1% ATGN, TY or Brucella Broth agarose pad for *A. tumefaciens*, *S. meliloti*, or *B. abortus*, respectively, with or without 1.5 $\mu\text{g/mL}$ faropenem. Cells were imaged after 16 hours of growth.

The expansion of genes encoding LDTs is another shared feature of the genomes of most species in the Rhizobiales. *A. tumefaciens* encodes 14 LDTs, *S. meliloti* encodes 13 and *B. abortus* encodes 11. Remarkably, we saw swelling of the growth pole in *S. meliloti* and *B. abortus* cells treated with sub-lethal concentrations of faropenem (Figure 3.7B). These data suggest that LDTs have a conserved role in polar growth not only in *A. tumefaciens*, but other agriculturally and medically important Rhizobiales.

DISCUSSION

Bacteria employ diverse growth strategies, and unipolar growth, or incorporation of new cell wall at a single pole, is the primary mode of elongation for species in

the Rhizobiales. Here, we identified PBP1a as an essential protein synthesizing PG at the growth pole in *A. tumefaciens*. By comparing the different incorporation patterns of two fluorescent cell wall probes we were able to distinguish between LDT crosslinked and PBP1a crosslinked PG. This method identified PBP1a as the major PG synthase during polar elongation. One other report of an essential PBP1a enzyme has been described in *Mycobacterium smegmatis* [42], an Actinobacteria that grows by bipolar elongation. In addition, PBP1a has been shown to localize to growth poles and be important for maintenance of rod shape in other Actinobacteria [43, 44]. Therefore, the reliance on PBP1a for synthesis of PG at the pole may be a key feature of polar-growing bacteria.

The Actinobacteria comprise a clade of polarly-growing bacteria distantly related to the Rhizobiales, but species in these clades do share some features, which may be important for a polar-growing lifestyle. Notably, the cell wall of polar-growing bacteria in both clades contain a high proportion (~30-80%) of LDT-crosslinked PG [14, 45, 46]. This is distinct from that of laterally growing bacteria, where only ~1-5% of PG is LDT-crosslinked [47]. In this work we identified 3 LDTs that localize to the growth pole of *A. tumefaciens* and uncovered LDTs that localize dynamically to the sidewalls of the old and new pole compartments. This points to a role for LDTs not only in polar growth, but also in sidewall remodeling or expansion of the old and new daughter cells. In agreement, deletion of LDTs causes a loss of rod-shape in *Mycobacterium* [48], and LDTs were found to contribute to active PG synthesis of the sidewalls of *Mycobacterium* [35].

Whether the LDTs of *A. tumefaciens* contribute to remodeling, polar PG synthesis, or both will require future studies of LDT mutants. Additionally, retaining the ability to edit the sidewalls of the cell may be an important supplement in addition to the primary site of PG insertion at the growth pole, or allow for repair of damaged PG.

Since LDTs are resistant to most classes of beta-lactam antibiotics [48], crosslinking the cell wall via LDTs may be a contributing factor for the high antibiotic resistance to beta-lactam antibiotics, which is characteristic of bacteria in the Actinobacteria and Rhizobiales. Continuing evidence suggests that the carbapenem class of beta-lactam antibiotics is in fact able to target LDT enzymes, and carbapenem antibiotics are routinely used to treat *M. tuberculosis* infections [15]. Here we show that meropenem and faropenem inhibit LD-transpeptidation in *A. tumefaciens*, indicating that they are targeting LDTs. In particular, faropenem causes swelling of the growth pole in *A. tumefaciens*, *S. meliloti* and *B. abortus*. This suggests that an important role for remodeling by LDTs is a conserved feature of polar growth in the Rhizobiales. Expanding our understanding of the mechanism of polar growth in the Rhizobiales will help us discover ways we can interfere with this process for antibiotic development. For example, transpeptidation inhibitors, such as faropenem and the carbapenem class of antibiotics could be developed into novel therapeutics against polar-growing pathogens.

Several of the proteins required for the recruitment, scaffolding and regulation of PG biosynthesis enzymes have been identified in the Actinobacteria [17]. However, *A. tumefaciens* not only lacks homologs to the well-known rod system, but also to any of these known proteins required for polar growth in the Actinobacteria. Thus, how PBP1a is recruited to the pole and how its activity is regulated remains unexplored in the Rhizobiales. Recently, GPR (for Growth Pole Ring), a large (~6 Kb) apolipoprotein with homology to the polar organizing protein TipN, was shown to form a ring at the growth pole in *A. tumefaciens*, and depletion of this protein led to rounded cells [49]. This phenotype implicates GPR as a likely candidate to scaffold PG enzymes during elongation. In addition, PG synthesis by PBP1a also requires hydrolysis of the existing sacculus to allow for insertion of new muropeptides. A DD-endopeptidase (RgsM) that is predicted to have hydrolysis activity was recently shown to be essential for polar growth in *S. meliloti* [50]. Depletion of the RgsM homolog in *A. tumefaciens* revealed that this enzyme functions at the growth pole and is required for maintenance of rod shape (Chapter 4). Based on this, RgsM is likely the major enzyme required for polar PG hydrolysis, and it will be of interest to investigate if RgsM interacts with PBP1a at the growth pole. In recent years a few proteins essential for polar elongation in the Rhizobiales have been identified, and our characterization of PBP1a adds to our understanding of the polar elongation complex. In the future it will be interesting to explore how PBPs, LDTs, EPases, and scaffolding proteins interact to promote polar PG biosynthesis.

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CHAPTER 3: SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURES

Supplementary Figure 3.S1: Characterization of PBP mutants. A. Bocillin gel analysis of WT cell membrane prep. Each PBP is labeled according to its predicted molecular weight. B. Spot assays for cell viability of PBP deletion and

depletion strains. C. Bocillin gel analysis showing the successful depletion of PBP1a proteins levels after cells were grown in the absence of IPTG for 16 hours. D. Cell width distributions of WT cells compared to the PBP1a depletion strain grown with or without IPTG for 16 hours. The indicated strains were grown as in Figure 3.1B and subjected to cell width measurements using MicrobeJ. The data are shown as box plots.

Supplementary Figure 3.S2: *Comparison of FDAAD and FDAA labeling in the $\Delta 3pbp$ mutant.* A. Demographs depict incorporation of either FDAADs or FDAAs at a population level. Median profiles of the fluorescence channel of more than 1000 cells per strain are stacked and ordered by cell length. Merged phase and fluorescent channels of cells with representative polar and mid-cell labeling of dipeptides are shown below each demograph. Scale bar: 2 μ M.

Supplementary Figure 3.S3: Domain structure of the putative LDT enzymes. A. The conserved YkuD domain (orange). The regions of intrinsic disorder (grey) as predicted by MoBiDB are also shown. Green represents PG-Binding domain 1, and the blue color represents different domains for each protein that are labeled on the schematic. The schematics are drawn to scale, and the protein length in amino acids (aa) is indicated. The corresponding ATU numbers are listed for each gene, and the new names are in parentheses beside each gene name.

Supplementary Figure 3.S4: Characterization of LDT Enzymes A. Phylogenetic tree comparing the 14 *A. tumefaciens* LDTs to those of *C. crescentus*, *E. coli* and *M. tuberculosis*. Show are merged phase and fluorescence images of the localization pattern of all 14 LDTs from *A. tumefaciens*. B. Demograph of the

localization of LdtN-sfgfp. C. Nitrocefin Hydrolysis assay on purified LdtM incubated with the indicated antibiotics.

Supplementary Figure 3.S5: Characterization of *S. meliloti* PBP strains. A. Spot assays for cell viability of PBP deletion and depletion strains of *S. meliloti*. B. Cell width distributions of all strains from figure 3.7A. The indicated strains subjected to cell width measurements using MicrobeJ. The data are shown as box plots.

SUPPLEMENTARY METHODS

Spotting Assays For cell viability spot assays, exponentially growing cultures were diluted to $\text{OD}_{600} = 0.05$ and serially diluted in ATGN. (this is the undiluted cells – U). 4 μl of each dilution was spotted onto an ATGN plate with or without IPTG and incubated at 28°C for 2 days before imaging.

Bocillin-FL labeling of Membrane Extract Cells were grown from a single colony in a 3 mL of ATGN media overnight, then the 3 mL was transferred to a 50 mL flask grown overnight, and sub-cultured in 1L of ATGN media the next morning and grown to an OD₆₀₀ of 1.0. To extract the cell membrane, cells were pelleted at 4,400 x g for 10 minutes at 4 °C, resuspended in buffer (10 mM potassium phosphate + 140 mM NaCl, pH 7.0), and then sonicated. After sonication cells were passed through an 20G needle 10 times to further lyse the cells. Centrifugation cell lysate at 12,000 x g for 10 min at 4 °C. Centrifuge the supernatant fractions at 150,000 x g for 40 min at 38,000 rpm. Resuspend pellets in 1 mL buffer (10 mM potassium phosphate + 140 mM NaCl, pH 7.0). Total protein concentration was measured by Bradford assay and 0.7 µg/mL of the preparation was incubated in the presence of buffer (20 mM potassium phosphate + 140 mM NaCl) and 0.1 mM Bocillin-FL (Thermo Fisher Scientific, Waltham, MA) for 5 minutes at 37 °C in the dark, and then the reaction was quenched upon addition of Laemmli buffer to 1x final concentration. Samples were boiled for 5 minutes, then 30 µL was separated on a 12% SDS-PAGE gradient gel. Gels were imaged on BioRad gel image (Alexa488 setting).

Phylogenetic Tree Construction Homologs of the *E. coli*, *A. tumefaciens*, *C. crescentus*, and *M. tuberculosis* LDTs were identified by BLASTp. A ClustalW nucleotide alignment and a neighborjoining tree was created in Geneious using the Jukes-Cantor genetic distance model. These trees were imported and annotated in iTOL.

LdtM Expression and Purification The gene encoding the Atu2336 (LdtM) protein was cloned into the EcoRI and NheI sites of the pSV281 expression plasmid. The expressed protein has an N-terminal His6 tag that is cleavable with tobacco etch virus protease and is inducible under the control of the lac operon. Atu2336 was expressed in BL21 (DE3) cells. Starter cultures of 10mL were grown in LB in the presence of 50 ug/mL KAN. The starter cultures were used to inoculate 2 L of cells in Terrific broth. After the cultures reached an OD_{600} of 1.0-1.5 Atu2336 expression was induced with 0.5 mM IPTG. Protein expression was carried out overnight at 18 °C. The cells were harvested by centrifugation at 4000 x g. Cells were lysed by sonication in a buffer containing 5 % (w/v) glycerol, 300 mM NaCl, 1 % (v/v) Tween-20, 50mM Tris (pH 7.5), 0.5 mM TCEP, 10 mM imidazole. Soluble protein was separated from insoluble cell debris by centrifugation at 16,500 rpm in an SS34 rotor for 1 h at 4 °C. His-tagged Atu2336 was isolated by purification over a Ni-NTA column charged with $NiSO_4$ and equilibrated in 50 mM Tris (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 5 % (w/v) glycerol. Atu2336 was eluted from the resin in a similar buffer containing 500 mM imidazole. The N-terminal His-tag was removed by tobacco etch virus protease, at a ratio of 1 mg of protease/10 mg Atu2336, during an overnight dialysis into a buffer containing 5 % (w/v) glycerol, 300 mM NaCl, 1 % (v/v) Tween-20, 50mM Tris (pH 7.5), 0.5 mM TCEP, 10 mM imidazole, 0.5 mM EDTA. His-cleaved Atu2336 was re-run over the Ni-NTA resin to isolate the tag-free Atu2336, which was collected in the flow through.

Nitrocefin Hydrolysis Assay

Nitrocefin is a chromogenic β -lactam whose native and hydrolyzed forms can be monitored at 496 nm. LdtM at 10 μ M, was reacted with 100 μ M nitrocefin in a 1 mL reaction mixture volume at room temperature in a solution containing 25 mM HEPES-morpholineethanesulfonic acid (MES)-diethanolamine and 300 mM NaCl (pH 5.0). Nitrocefin hydrolysis was monitored by using spectrophotometer. To assess nitrocefin hydrolysis by LdtM in the presence of β -lactams, LdtM was pre-incubated with, 1.5 μ g/mL final concentration of antibiotics for 5 minutes, then added to a reaction mixture containing 100 μ M nitrocefin in a 1 mL total volume. The amount of hydrolyzed nitrocefin was then determined spectroscopically as described previously. Sample were normalized to Nitrocefin in buffer alone (without enzyme) to correct for the low level of non-enzymed driven hydrolysis of nitrocefin, and this data was then normalized to the enzyme alone control and plotted.

SUPPLEMENTARY TABLES

Table 3.S1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Genotype, Features or Characteristics	Source or Reference
Source Plasmids		
pNTPS139	Kmr ^r ; Suicide vector containing oriT and sacB	D. Alley
pUC18-mini-Tn7T-GM-Plac	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>lacI^q</i> and lac promoter	Figueroa-Cuilan et al 2016 AEM
pTNS3	Ap ^r ; helper plasmid encoding the site-specific TnsABCD Tn7 transposition pathway	Choi et al 2010 AEM

pSRKKm-Pcym	pSRKKm vector containing lac promoter flanked by <i>cuO</i> operator site along with the <i>cymR</i> repressor gene	Howell et al 2019 Mol Micro
Deletion Plasmids		
pNTPS139Δ <i>pbp1a</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1a</i>	This Study
pNTPS139Δ <i>pbp1b1</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1b1</i>	This Study
pNTPS139Δ <i>pbp1b2</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1b2</i>	This Study
pNTPS139Δ <i>pbp1c</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1c</i>	This Study
pNTPS139Δ <i>mtgA</i>	Km ^r Suc ^s ; deletion plasmid for <i>mtgA</i>	This Study
pNTPS139Δ <i>exoA</i>	Km ^r Suc ^s ; deletion plasmid for <i>exoA</i>	This Study
Depletion Plasmids		
pUC18-mini-Tn7T-GM-plac <i>pbp1a</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>pbp1a</i> under control of the lac promoter	This Study
pUC18-mini-Tn7T-GM-plac <i>pbp3a</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>pbp3a</i> under control of the lac promoter	This Study
Replicating Plasmids		
pSRKKm-Pcym- <i>Atu2336-sfgfp</i>	pSRKKm vector containing <i>Atu2336-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu5196-sfgfp</i>	pSRKKm vector containing <i>Atu5196-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu0048-sfgfp</i>	pSRKKm vector containing <i>Atu0048-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu0844-sfgfp</i>	pSRKKm vector containing <i>Atu0844-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu0845-sfgfp</i>	pSRKKm vector containing <i>Atu0845-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu3331-sfgfp</i>	pSRKKm vector containing <i>Atu3331-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu1164-sfgfp</i>	pSRKKm vector containing <i>Atu1164-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu1293-sfgfp</i>	pSRKKm vector containing <i>Atu1293-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu3631-sfgfp</i>	pSRKKm vector containing <i>Atu3631-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu1615-sfgfp</i>	pSRKKm vector containing <i>Atu1615-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pSRKKm-Pcym- <i>Atu2764-sfgfp</i>	pSRKKm vector containing <i>Atu2764-sfgfp</i> under control of the <i>cym</i> promoter	This Study
pTC130	pTC127 encoding <i>Atu3332-sfgfp</i> , with <i>atu3322</i> cloned with NdeI & SmaI sites Km ^r	Zambryski P. Cameron et al. 2014
pTC132	pTC127 encoding <i>Atu2133-sfgfp</i> , with <i>atu2133</i> cloned with NdeI & SmaI sites Km ^r	Zambryski P. Cameron et al. 2014
pTC140	pTC127 encoding <i>Atu0669-sfgfp</i> , with <i>atu0669</i> cloned with NdeI & SmaI sites Km ^r	Zambryski P. Cameron et al. 2014
pRV-ftsZ _{AT} -sfgfp	Constitutive expression of ftsZ _{AT} -sfgfp	Howell et al., 2017

Plasmids for protein production		
pSV281	pET15b derivative, Km ^r (IPTG)-inducible protein expression plasmid containing an N-terminal tobacco etch virus (TEV) protease-cleavable 6X histidine tag	Tanner W.
pSV281-atu2336-N-term	deletion of the N-terminal domain (amino acids 1-11) of Atu2336 for purification	This Study
<i>E. coli</i> strains		
DH5α	Cloning strain	Life Technologies
S17-1	Smr;RP4-2 TC::MU Km-Tn7; for plasmid mobilization	Simon et al 1983 Nat Biotech
BL21(DE3)	Strain for protein overproduction	Novagen
<i>A. tumefaciens</i> strains		
C58	Nopaline type strain; pTiC58; pAtC58	Lab Stock
C58ΔtetRA::a-attTn7	Replacement of the tetRA locus with an artificial attTn7 site	Figuerola-Cuilan et al 2016 AEM
C58ΔtetRA::a-attTn7 Δ <i>pbp3a</i>	Δ <i>pbp3a</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp3b</i>	Δ <i>pbp3b</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp1b1</i>	Δ <i>pbp1b1</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp1b2</i>	Δ <i>pbp1b2</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp1c</i>	Δ <i>pbp1c</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>mtgA</i>	Δ <i>mtgA</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp1b1</i> , Δ <i>pbp1b2</i>	Δ <i>pbp1b1</i> , Δ <i>pbp1b2</i>	This Study
C58ΔtetRA::a-attTn7 Δ <i>pbp1b1</i> , Δ <i>pbp1b2</i> , Δ <i>pbp1c</i>	Δ <i>pbp1b1</i> , Δ <i>pbp1b2</i> , Δ <i>pbp1c</i> (Δ3 <i>pbp</i>)	This Study
C58ΔtetRA::mini-Tn7-GM-Plac-pbp1a	Mini-Tn7T-GM-Plac-pbp1a inserted into a-attTn7 site	This Study
C58ΔtetRA::mini-Tn7-GM-Plac, Δ <i>pbp1a</i>	Chromosome-based complementation of Δ <i>pbp1a</i> with C58ΔtetRA::mini-Tn7-GM-Plac-pbp1a allowing depletion of PBP1a under control of the lac promoter	This Study
C58ΔtetRA::mini-Tn7-GM-Plac-pbp3a	Mini-Tn7T-GM-Plac-pbp3a inserted into a-attTn7 site	This Study
C58ΔtetRA::mini-Tn7-GM-Plac, Δ <i>pbp3a</i>	Chromosome-based complementation of Δ <i>pbp3a</i> with C58ΔtetRA::mini-Tn7-GM-Plac-pbp3a allowing depletion of PBP3a under control of the lac promoter	This Study
C58ΔtetRA::mini-Tn7-GM-Plac, Δ <i>pbp3a</i> , Δ <i>pbp3b</i>	Chromosome-based complementation of Δ <i>pbp3a</i> with C58ΔtetRA::mini-Tn7-GM-Plac-pbp3a allowing depletion of PBP3a under control of the lac promoter, Δ <i>pbp3b</i>	This Study
C58ΔtetRA::a-attTn7, <i>Atu2336-sfgfp</i>	Strain expressing <i>Atu2336-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu5196-sfgfp</i>	Strain expressing <i>Atu5196-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu0048-sfgfp</i>	Strain expressing <i>Atu0048-sfgfp</i> from a pSRK replicative plasmid	This Study

C58ΔtetRA::a-attTn7, <i>Atu0844-sfgfp</i>	Strain expressing <i>Atu0844-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu0845-sfgfp</i>	Strain expressing <i>Atu0845-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu3331-sfgfp</i>	Strain expressing <i>Atu3331-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu1164-sfgfp</i>	Strain expressing <i>Atu1164-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu1293-sfgfp</i>	Strain expressing <i>Atu1293-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu1615-sfgfp</i>	Strain expressing <i>Atu1615-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu2764-sfgfp</i>	Strain expressing <i>Atu2764-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, <i>Atu3631-sfgfp</i>	Strain expressing <i>Atu3631-sfgfp</i> from a pSRK replicative plasmid	This Study
C58ΔtetRA::a-attTn7, pTC130	Strain expressing <i>Atu3332-sfgfp</i> from a pSRK derived replicative plasmid	This Study
C58ΔtetRA::a-attTn7, pTC132	Strain expressing <i>Atu2133-sfgfp</i> from a pSRK derived replicative plasmid	This Study
C58ΔtetRA::a-attTn7, pTC140	Strain expressing <i>Atu0669-sfgfp</i> from a pSRK derived replicative plasmid	This Study
C58ΔtetRA::mini-Tn7-GM-Plac, Δ <i>pbp3a</i> , pRV-ftsZAT-sfgfp	Depletion strain of PBP3a under control of the lac promoter with constitutive expression of ftsZAT-sfgfp	This Study
C58ΔtetRA::mini-Tn7-GM-Plac, Δ <i>pbp3a</i> , Δ <i>pbp3b</i> , pRV-ftsZAT-sfgfp	Depletion strain of PBP3a under control of the lac promoter in the background of Δ <i>pbp3b</i> with constitutive expression of ftsZAT-sfgfp	This Study
<i>S. meliloti</i> strains		
Rm2011	Wild type, Str ^r	Casse et al 1979
Rm2011 <i>rgsP-egfp</i>	Rm2011 expressing <i>egfp</i> -tagged <i>rgsP</i> , markerless insertion	Schäper et al 2018
Rm2011 <i>rgsP-egfp</i> , Δ <i>5pbp</i>	Rm2011 <i>rgsP-egfp</i> carrying markerless deletions of <i>mrcA2</i> , <i>mcrB</i> , <i>pbp</i> , <i>pbpC</i> and SMC02856	This Study
Rm2011 <i>rgsP-egfp mrcA1dpl</i>	Rm2011 <i>rgsP-egfp</i> carrying markerless deletion of <i>mrcA1</i> , curable complementation plasmid pGCH14- <i>mrcA1</i> , and pSRKKm as a source of <i>lacI</i> to cure pGCH14- <i>mrcA1</i> , Gm ^r Km ^r	This Study
<i>B. abortus</i> strain		
S19	Wild type	J. Skyberg

CHAPTER 4

Decreased Peptidoglycan Synthesis Induces the RSI Invasion Switch in *Agrobacterium tumefaciens*

Author Contributions

Williams M and Bouchier J, conducted the experiments. Williams M and Brown PB designed the experiments. Williams M, and Brown PB analyzed data and Williams M and Brown PB wrote and edited the paper.

ABSTRACT

The absence of the essential, bifunctional penicillin-binding protein PBP1a in *Agrobacterium tumefaciens* results in shorter, rounder cells suggesting PBP1a is the major synthase for polar elongation. Remarkably, depletion of PBP1a also prevents microcolony formation. To explore this unexpected phenotype, we monitored gene expression levels during PBP1a depletion using RNA-seq. During PBP1a depletion, genes for motility are downregulated and genes for virulence, Type VI secretion, and exopolysaccharide (EPS) production are upregulated. Using a Calcofluor White binding assay we confirm that the exopolysaccharide succinoglycan is overproduced when PBP1a is depleted. Furthermore, deletion of a gene required for succinoglycan production in the PBP1a depletion strain restores microcolony formation. WT cells treated with flavomycin mimics the depletion of PBP1a, suggesting that loss of glycosyltransferase activity may trigger these transcriptional changes. Remarkably, the transcriptional changes induced by loss of glycosyltransferase activity are strikingly similar to those described during host invasion. The RSI (ExoR-ExoS-ChvI) invasion switch, originally found in *S. meliloti*, is a characteristic of the Rhizobiales, and is a key regulatory aspect in the switch from free-living to host-associated lifestyle. Together, these findings suggest that *A. tumefaciens* may use changes in peptidoglycan composition or structure specific to the loss of glycosyltransferase activity as a signal to modulate multiple behaviors during conditions such as antibiotic stress or virulence.

INTRODUCTION

The soil environment is constantly in flux and can undergo rapid changes in hydration, nutrient availability, temperature, acidity levels and many other abiotic and biotic factors [1]. To survive in these conditions, soil-dwelling bacteria must be able to monitor and respond to the changes around them. One of the main mechanisms bacteria employ to monitor changes in their environment are two-component systems (TCS) that induce changes in gene expression in response to environmental triggers [2]. In turn transcriptional changes can modify bacterial behavior. In *A. tumefaciens*, the VirA/VirG histidine kinase/response-regulator pair responds to plant phenolic compounds such as acetosyringone and induces expression of the *vir* regulon, which encodes genes that are required for pathogenicity and plant transformation [3],

Despite being a plant pathogen, *A. tumefaciens* is closely related to *S. meliloti*, and other plant symbionts with the ability to colonize plant roots [4]. In the case of either pathogenesis or symbiosis, activation of conserved pathways is required for invasion or colonization of host cells [5, 6]. The RSI invasion switch is employed by bacteria in the Rhizobiales to induce lifestyle-associated changes. The RSI signaling pathway is comprised of ExoR, ExoS and ChvI, and is required for the transition from a free-living bacterium to a host-associated lifestyle [7].

ExoR is a periplasmic regulatory protein, while ExoS (also known as ChvG in *A. tumefaciens*) and ChvI are a typical sensor kinase/response regulator pair. Under neutral conditions, ExoR binds to and represses ChvG; however, when cells are exposed to acidic conditions, ExoR is proteolyzed, which allows for activation of the ChvG/ChvI TCS [8, 9]. ChvI induces transcriptional changes in multiple genes across several major pathways, including suppression of genes for motility and chemotaxis, upregulation of genes for exopolysaccharide production, and in *A. tumefaciens* induction of the Type VI Secretion System (T6SS) [10]. While TCS are a widespread mechanism for transcriptional change in bacteria, conservation of the ExoR-ExoS-ChvI signaling pathway is unique to species in the Rhizobiales [6], and likely contributes to the lifestyles and diversity of niches occupied by bacteria in this order.

Despite the cell wall being an essential feature of bacteria that protects them from environmental stressors, relatively little is known about how bacteria sense and respond to changes in the composition of their cell wall. Remarkably, cells depleted of PBP1a no longer insert PG at the growth pole, leading to shorter cells that have compositional changes in PG (refer to chapter 3). In addition to its role in polar PG insertion, we observed that during PBP1a depletion, cells spread apart rather than form microcolonies. To better understand this phenotype, we used RNA-seq to get transcriptional profiles of cells depleted of PBP1a after 6 hours, corresponding to the onset of the spreading phenotype, and after 16 hours. Our data revealed 90 genes that were differentially expressed at the 6-

hour timepoint, and 309 genes that were differentially expressed at the 16-hour timepoint. Surprisingly, the transcriptome changes closely mimicked the transcriptome changes seen when the RSI invasion switch is activated in *A. tumefaciens*, including downregulation of genes for motility and chemotaxis and upregulation of genes for exopolysaccharide biosynthesis and T6SS. We developed a set of microscopy-based assays to experimentally validate the RNA-seq results, and to confirm the impacts of the PBP1a depletion on the physiology and behavior of *A. tumefaciens*. In this study we identified a myriad of genes that are regulated in response to compositional changes in the cell wall, and together our observations begin to shed light on the connection between changes in PG composition and complex behaviors in the Rhizobiales.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. A list of all bacterial strains and plasmids used in this study is provided in Table 4.S1. *Agrobacterium tumefaciens* C58 and derived strains were grown in ATGN minimal media [11] without exogenous iron at 28°C with shaking. When appropriate, kanamycin (KAN) was used at the working concentration of 300 µg/ml. When indicated, isopropyl β-D-1-thio-galactopyranoside (IPTG) was used as an inducer at a concentration of 1 mM, and cumate was used at a concentration of 0.2µM. *Sinorhizobium meliloti* stains were grown in TY medium. When appropriate, KAN was used at the working concentration of 200 µg/ml, gentamycin (GM) was used at 20 µg/ml, and IPTG was used at a concentration of 500 µg/ml. For *E. coli*

DH5 α and S17-1 λ *pir*, when appropriate 50 μ g/ml and 30 μ g/ml of KM, were added.

Construction of strains and plasmids. To construct expression vectors containing FlaA_{cys} and *impB-sfgfp* the respective coding sequence was amplified from purified C58 genomic. The amplicons were digested overnight and ligated into cut pSRKKM-P_{cym} using NEB T4 DNA ligase at 4° C overnight, to create an expression vector compatible with depletion strains. All expression vectors were verified by sequencing. All vectors were introduced into *A. tumefaciens* strains utilizing standard electroporation protocols [12].

Construction of deletion/depletion plasmids and strains. Vectors for gene deletion by allelic exchange were constructed using recommended methods for *A. tumefaciens* [12]. Briefly, 500-bp fragments upstream and 500 bp downstream of the target gene were amplified using primer pairs P1/P2 and P3/4 respectively. Amplicons were spliced together by SOEing using primer pair P1/P4. The amplicon was digested and ligated into pNTPS139. The deletion plasmids were introduced into *A. tumefaciens* by mating using an *E. coli* S17 conjugation strain to create KM resistant, sucrose sensitive primary integrants. Primary integrants were grown overnight in media with no selection. Secondary recombinants were screened by patching for sucrose resistance and KM sensitivity. Colony PCR with primers P5/P6 for the respective gene target was

used to confirm deletion. PCR products from P5/P6 primer sets were sequenced to further confirm deletions.

For depletion strain, target gene (*rgsM*) was amplified, digested and ligated into pUC18-mini-Tn7T-GM-P_{lac}. The mini-Tn7 vector, along with the pTNS3 helper plasmid, were introduced into C58Δ*tetRA*::a-*att*Tn7 as described previously [13]. Transformants were selected for GM resistance and insertion of the target gene into the a-*att* site was verified by colony PCR using the tet forward and Tn7R109 primer. PCR products were sequenced to confirm insertion of the correct gene. Next, the target gene was deleted from the native locus as described above in the presence of 1 mM IPTG to drive expression of the target gene from the ectopic site.

Phase and Fluorescence microscopy. A small volume (~1 µl) of cells in exponential phase (OD₆₀₀ = 0.2 - 0.4) was applied to a 1% ATGN agarose pad as described previously [14]. DIC, Phase contrast and epifluorescence microscopy were performed with an inverted Nikon Eclipse TiE and a QImaging Rolera em-c2 123 1K EMCCD camera with Nikon Elements Imaging Software. For time-lapse microscopy, images were collected every ten minutes, unless otherwise stated. Expression of plasmid encoded FlaA_{cys}, *ldtD-sfgfp*, or *impB-sfgfp* was induced by the presence of 0.2 mM cumate for 1 hour (h) prior to imaging. Cells containing plasmids with fluorescent protein fusions were grown to exponential phase before imaging on agarose pads. For calcofluor agar pad assays, calcofluor was added to agarose pads at a concentration of 25 µg/mL and

exposed to DAPI filter for 50ms. When appropriate agar pads were supplemented with 1mM IPTG.

Visualization of flagellar filaments with Alexa Fluor 488 maleimide stain. *A. tumefaciens* C58 or PBP1a depletion cells carrying the pSRK-cym plasmid encoding FlaAT213C were grown overnight and then subcultured until they reached exponential phase. Alexa Fluor 488 maleimide dye was diluted to 25 g/ml in ATGN minimal medium. Diluted dye was mixed with an equal volume of cells. The mixture was then added on a slide and incubated for 5 to 10 min with a coverslip on it. The samples were then observed using DIC and epifluorescence microscopy as described above.

Microfluidics The CellASIC™ ONIX Microfluidic Platform was used with CellASIC ONIX plate for bacteria cells (4 chamber) Cat number: B04A-03-5PK. The device was set up according to the CellASIC® ONIX B04A-02 Microfluidic Bacteria Plates User Guide in conjunction with an inverted Nikon Eclipse TiE and a QImaging Rolera em-c2 123 1K EMCCD camera with Nikon Elements Imaging Software. Images were taken with high-numerical aperture (NA) objectives in the analysis channel at 60x magnification every 10 minutes. The microfluidic device was inoculated by pumping +PBP1a cells from an OD₆₀₀ 0.1 culture in the loading chamber according the user manual. ATGN media + IPTG was flowed over cells at a low pounds per square inch (psi) for 1 hour. During this time, permanent

adhesion of the bacteria to the microchannel wall was observed. Device seeding was terminated after 1 h, and ATGN medium was pumped through the incubation region at a higher psi for 5 minutes to flush away any unadhered cells. The cells in the incubation region were grown for 4 hours with ATGN + IPTG media, then ATGN media without IPTG was flowed in at a higher psi for 5 minutes to quickly wash away the ITPG. The psi was returned to the normal rate for the remaining 20 hours.

RNA Isolation, Sequencing and Analysis. Four cultures each of WT, WT + IPTG and 12 cultures of + PBP1a depletion cells were grown overnight in 2 ml of ATGN minimal media at 28°C with shaking; the + PBP1a strains and WT + IPTG strains were supplemented with 1mM IPTG. Cells were then pelleted by centrifugation at 7000 x *g* for 5 minutes. Cell pellets were washed three times with ATGN by centrifugation and resuspension to remove IPTG. After the final wash the cell pellets from WT, WT + IPTG, and four of the 12 + PBP1a strains were resuspended to an OD₆₀₀ of 0.05 in 6mL ATGN, or ATGN with 1mM IPTG. The other eight + PBP1a strains were resuspended to an OD₆₀₀ of 0.05 in 6 ml ATGN without IPTG. This resulted in 4 replicate cultures each of WT, WT +IPTG, + PBP1a, - PBP1a_6hr and - PBP1a_16hr. Growth of the cultures was monitored and supplemented with fresh medium as needed so that the OD₆₀₀ never went over 0.3. RNA was isolated from the -PBP1a_6hr strains after 6 hours of growth, and RNA was isolated from all other strains after 16 hours of growth. To prepare samples, a culture volume equivalent to 6 ml at an optical density at 600 nm

(OD₆₀₀) of 0.2-0.3 was pelleted by centrifugation at 7000 x *g* for 5 minutes and pellets were resuspended in 1mL of ATGN media and incubated with 2 mL of RNAProtect reagent (QIAGEN) for 15 min at room temperature. Cells were lysed with 10 mg lysozyme, and RNA was extracted using the QIAGEN RNEasy kit.

DNA libraries for sequencing were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq mRNA stranded sample preparation kit. The sample concentration was determined by Qubit fluorometer (Invitrogen) using the Qubit HS RNA assay kit, and the RNA integrity was checked using the Fragment Analyzer automated electrophoresis system. Briefly, the poly-A containing mRNA is purified from total RNA, RNA is fragmented, double-stranded cDNA is generated from fragmented RNA, and the index containing adapters are ligated to the ends. The amplified cDNA constructs were purified by addition of Axyprep Mag PCR Clean-up beads. The final construct of each purified library was evaluated using the Fragment Analyzer automated electrophoresis system, quantified with the Qubit fluorometer using the Qubit HS dsDNA assay kit, and diluted according to Illumina's standard sequencing protocol for sequencing on the NextSeq 500.

For all samples, when adapter sequence was detected it was removed using cutadapt (0.16) [15]. All samples were purged of reads that mapped to transcripts for rRNA genes using bowtie2 (2.3.4.3) [16]. The reads were then mapped to the *A. fabrum* genome using STAR (version 2.5.4b) [17], which also produces the

number of read counts per gene. The index files used by STAR were derived from the files *Agrobacterium_fabrum_str_c58.ASM9202v1.dna.toplevel.fa* and *Agrobacterium_fabrum_str_c58.ASM9202v1.40.gtf*, both of which are part of Ensembl release 40 (<http://bacteria.ensembl.org/index.html>). Pairwise comparisons were performed to test for differential expression of genes using the Bioconductor package DESeq2 [18]. Gene annotations were collected from the annotations included with the file of cDNAs also at Ensembl *Agrobacterium_fabrum_str_c58.ASM9202v1.cdna.all.fa.gz*.

RESULTS AND DISCUSSION

Temporal loss of polar growth and microcolony formation during PBP1a depletion

PBP1a was previously characterized for its essential role in polar PG biosynthesis (Chapter 3), and we observed that cells depleted of PBP1a became shorter and wider after 16 hours. To better understand how cell elongation is impacted by loss of PBP1a activity, the depletion strain was grown using a microfluidic system. In the microfluidic device cells are grown under a constant flow of nutrients, and different media conditions can easily be exchanged; therefore, we were able to wash the cells of IPTG in a more controlled manner. Furthermore, in the device, new daughter cells are washed away to prevent overcrowding, so we can follow the same cells over multiple generations. At the onset, the PBP1a depletion strain was induced for four hours by flowing in media with IPTG. After 4 hours, media without IPTG was flowed in for 10 minutes at a

high psi to quickly wash away the IPTG, before returning the flow rate back to its original state for the remainder of the experiment. Three, two-hour windows of time were chosen to measure cell length, first during the 2-4 hours window of pre-depletion, then 4-6 hours post-depletion and 10-12 hours post-depletion, which should represent the onset and late stages of the PBP1a depletion phenotype. A timeline of the microfluidic experiment is illustrated in Figure 4.1A. The average cell length was measured one frame before the cells divided (M, for mother cell), and the cell length of the 2 daughter cells was measured the frame after the division event occurred (D1 and D2 for daughter cell 1 and daughter cell 2). D1 was always designated as the cell that remained attached to the microfluidic device, or the old pole daughter cell. If D2, the new pole daughter cell, washed away before it could be measured, the cell length was calculated by subtracting the length of D1 from M.

A **B** **C**

Figure 4.1 *Temporal analysis of PBP1a depletion.* A. Shown are cell length distributions of the PBP1a depletion strain grown in a microfluidic device. A timeline of the experiment is provided. Cells were left induced for 4 hours before washing out the IPTG to deplete cells of PBP1a for the remainder of the experiment. Mother cell (M) Daughter cell 1 (D1) Daughter cell 2 (D2). Median cell lengths are indicated above each boxplot. B. DIC microscopy images showing the microcolony phenotypes of PG synthase mutants. Each strain was grown to exponential phase, spotted on an ATGN agar pad, allowed to grow for 16 hours, and imaged by DIC microscopy. Scale bar: 2 μ M. C. Time-lapse microscopy of the PBP1a depletion grown on an agar pad with or without 1mM IPTG. DIC images were acquired every 10 minutes. Time is shown in hours (h). For the - PBP1a strain, cells were washed 3X with ATGN media and grown at 28 C with shaking for 4 hours before cells were spotted on an agar pad for imaging.

During the pre-depletion window, the median mother cell length was 3.3 μ M. After cell division, the median cell length of D2 was ~13% shorter than D1, which is consistent with WT *A. tumefaciens* and other Alphaproteobacteria that have an asymmetric cell cycle (Figure 4.1A, green) [19, 20]. Tracking the time between divisions during the window gave an average generation time of ~2 hours, which is consistent with the generation time of WT *A. tumefaciens*. Overall, the PBP1a depletion strain induced with IPTG is remarkably similar to the WT with regards to its cell cycle and cell growth. Interestingly, during the first post-depletion window the median cell length of D1 remained the same as during the pre-

depletion period, and only the cell length of D2 became shorter, while the average generation time increased to 2.6 hours (Figure 4.1A, dark pink). The reduction in cell length of the new pole daughter cell likely results from the loss of polar PG insertion by PBP1a, and the site of cell division remaining a fixed distance from the old pole. Thus, cells divide producing a D1 that is ~18% shorter than D2. During the second post-depletion window, the cell length of the mother cell, and both daughter cells are notably shorter, and the average generation time has increased to ~5 hours (Figure 4.1A, light pink). Thus, there is a mechanism to delay cell division when polar cell wall biosynthesis is limited and to reposition the division site closer to the old pole. Remarkably, D2 still remains ~13% shorter than D1, which suggests that there is a robust mechanism for maintaining an asymmetric cell cycle that is independent of PBP1a.

Early during the depletion of PBP1a, time-lapse microscopy on agarose pads reveals an additional phenotype, the inability to form microcolonies (Figure 4.1B). Instead of forming microcolonies where cells grow directly touching an adjacent cell, PBP1a depleted cells began to move apart from each other on an agar pad (Figure 4.1B). This change took place between ~4-7 hours after depletion of PBP1a, and by 9 hours the spreading became more pronounced. Additionally, we show that the cell separation phenotype is conserved in the closely related bacterium *Sinorhizobium meliloti* (Supplementary Figure 4.S1A), and that the onset of the spreading began between ~6 and 10 hours post-depletion. This phenotype is specific to the loss of PBP1a and did not occur upon deletion of any

other high molecular weight penicillin-binding proteins or the monofunctional glycosyltransferase (GTase) MtgA (Figure 4.1C). Based on these observations, some species in the Rhizobiales may have a common physiological response that is induced by loss of the cell wall synthase PBP1a.

Global transcriptome changes after depletion of PBP1a for 6 or 16 hours

The spreading phenotype that was seen during depletion of PBP1a is suggestive of a behavioral change in the cells that likely affects pathways beyond that of cell wall biosynthesis. To identify all genes affected by the depletion of PBP1a we compared the transcriptomes of cells with or without PBP1a for 6 or 16 hours.

This analysis identified 90 and 309 genes that were differentially expressed in the + PBP1a strain and the 6- or 16-hour depletion of PBP1a, respectively, using a p-value of less than 0.05 and minimum log₂ fold change (FC) of 2.0 (for complete list of differentially expressed genes from both depletion timepoints see

Supplementary Tables 4.S4 and 4.S5). The addition of IPTG, which was used as an inducer for the + PBP1a strain, did not alter gene expression profiles of WT cells compared to WT + IPTG samples (Supplementary Figure 4.2A).

Additionally, the transcriptome of the PBP1a depletion strain is comparable to the transcriptome of WT when both strains are grown in the presence of IPTG (Supplementary Figure 4.2B).

We plotted the RPKM (Reads Per Kilobase Per Million) of the PBP1a depletion for 6- and 16-hour samples against the same strain with PBP1a induced for 16-hours (Figures 4.2A, B). The larger dots correspond to transcripts that meet the criteria for differentially expressed genes. Two major functional categories of genes were upregulated when PBP1a is depleted; succinoglycan biosynthesis gene clusters (blue dots) and genes for Type VI secretion (pink dots). Notably, genes for flagella-mediated motility are downregulated (red dots). Within these categories, the genes became more significantly differentially expressed at the 16-hour timepoint, indicating a continued activation of genes for exopolysaccharide production, and Type VI secretion, and a continued repression of motility genes. In contrast, ~200 additional genes (dark grey dots) are differentially expressed only after 16 hours of PBP1a depletion. In total, approximately 6% of the genome is impacted by depletion of PBP1a after 16 hours.

Figure 4.2 *Analysis of the PBP1a transcriptomes by RNA-seq.* A. Plots comparing the RMKM values of the + PBP1a transcriptome to that of the - PBP1a 6-hour transcriptome. Both axes are scaled to log10. Smaller grey dots represent no change in the DE of those genes. Larger dots represent genes with a Log2 fold change of 2.0 and a p-value of less than 0.05. Colored dots represent groups of genes with a similar function. Type VI Secretion (blue), Succinoglycan biosynthesis (Pink) and motility (red). In total, 90 genes were DE in the - PBP1a 6-hour compared to +PBP1a. B. Plots comparing the RMKM values of the + PBP1a transcriptome to that of the - PBP1a 16-hour transcriptome. Plot and gene cut-off criterion is the same as in A. 309 genes were DE in the PBP1a 6-hour compared to + PBP1a.

A. tumefaciens has a circular chromosome, which houses roughly half (51.7%) of the protein-coding genes, a linear chromosome (34.7%) and two mega plasmids, the At plasmid (10%) and Ti plasmid (3.6%). Most of the genes differentially expressed during PBP1a depletion are encoded on the linear and circular chromosomes (Figure 4.3A). After 6 hours of PBP1a depletion, 96% of the differentially expressed genes are located on the circular and linear chromosome, while this number drops slightly to 91% after 16 hours of PBP1a depletion. The response to the depletion of PBP1a seems to be primarily mediated by chromosomally encoded rather than plasmid encoded genes. After 16-hours depletion, genes on the At and Ti plasmids are upregulated, indicating that induction of genes for virulence and plant colonization may be initiated.

To better understand the impacts of PBP1a depletion, we have grouped differentially expressed genes into 8 functional categories (Figure 4.3B). Remarkably, these same categories have been previously identified and experimentally validated as physiologically important for *A. tumefaciens* to survive during exposure to acidic conditions and loss of ExoR [9, 10]. This observation suggests that the loss of PBP1a activity may also provide a signal leading to a lifestyle switch via the RSI pathway. During PBP1a depletion, all 14 genes in the *imp* operon, which are located on the linear chromosome and encode the structural proteins of the T6SS, are upregulated during PBP1a depletion. Phosphorylated ChvI binds to the *imp* promoter region and is required for expression of the operon [21] suggesting that the transcriptional changes are likely due to activation of ChvI early during depletion of PBP1a. Further evidence for activation of the ChvI signaling pathway early during PBP1a depletion is the upregulation of the succinoglycan biosynthesis pathway and downregulation of flagella-mediated motility [22]. In contrast, the downregulation of chemotaxis proteins, which are important for directing bacteria motility, is only observed after 16 hours of PBP1a depletion. Similarly, a large number of ABC transporters and ABC transporter binding proteins are up and down regulated after 16 hours of PBP1a depletion. Together, these observations suggest that modulating directional movement, and amino acid or sugar transport in and out of the cell may be an attempted adaptation to the loss of PBP1a.

Figure 4.3 *Distribution of differentially expressed genes across replicons and functional groups.* A. Gene groups of interest that are DE in response to depletion of PBP1a. The annotated genes of interest represented are from the + PBP1a versus the - PBP1a 6-hour strain and the + PBP1a versus the - PBP1a 16-hour strains were assigned to functional groups based on previous . The

number of genes up or down regulated in each functional category are plotted and color coded according to the key. B. Comparison of how the DE genes from the 6 and 16-hour depletion are distributed across the genome of *A. tumefaciens*.

Although there is significant overlap between the acid-induced and ExoR regulons and the transcriptome changes that occur after depletion of PBP1a, not all of the changes observed in our data can be explained through the ExoR regulon. Degradation of ExoR allows downstream activation of ChvG and ChvI, leading to changes in gene expression characteristic of the RSI pathway. Surprisingly, we observe upregulation of ExoR and ChvI during PBP1a depletion. One possible explanation is that ExoR transcript levels are upregulated, but that the protein is proteolyzed, which may still induce the RSI signaling pathway. Alternatively, loss of PBP1a activity may activate ChvI through an alternative sensor kinase. Two additional predicted sensor kinases are upregulated during PBP1a depletion but neither has an established link to regulation of the RSI pathway. Further investigation is merited to characterize the function of these regulators during PBP1a depletion.

Overall, we have revealed a large number and variety of genes that are regulated in response to depletion of PBP1a. Although many of these changes in gene expression have been reported previously in response to low pH or deletion of the RSI regulator ExoR, these changes have never been associated with any cell

wall enzymes, and provide insight into the temporal aspect of the regulation of these genes in response to a stress condition. The early response begins with changes in gene expression mainly on the circular and linear chromosomes. This response includes succinoglycan production, which may function as an osmoprotectant to guard cells from the slightly acidic environment of the rhizosphere. Upregulation of T6SS may allow *A. tumefaciens* to compete with other bacteria in the rhizosphere. The late response consists of upregulation of genes on the Ti and At plasmids, including a few genes for virulence (*virG* and *virH*), several ABC transporters, and cell surface remodeling genes.

Expression of cell surface structures upon loss of PBP1a

To better understand the biological implications of upregulation of the T6SS during PBP1a depletion, we monitored T6SS assembly in *A. tumefaciens* using a sfGFP fusion to ImpB, a component of the contractile sheath. The T6SS is a needle complex used for interbacterial competition and allows predatory bacteria to inject effectors into the cytoplasm of a prey cell to kill or disrupt the function of the prey bacteria [23]. WT *A. tumefaciens*, primarily assembles T6SS at the three-quarter position of the cell closest to one pole, and extended sheaths appear as a line spanning the width of the cell, while contracted sheaths appear as a foci (Figure 4.4A). In contrast to WT cells, the T6SS sheaths primarily appear as foci at one pole of the cell during PBP1a depletion (Figure 4.4A). Rarely, T6SS are seen to assemble perpendicular to the pole instead of horizontal, which was never observed for WT cells. Interestingly, cells depleted of

PBP1a often assemble 2-3 secretion systems per cell (Figure 4.4A, white arrows), whereas WT cells almost always assemble a single complex. Overall, T6SS gene expression, localization and assembly dynamics are altered in the PBP1a depletion strain compared to WT. The ability to track ImpB-sfGFP will enable future studies to monitor the assembly and contraction dynamics of the T6SS, potentially shedding light on the biological implication of upregulation of the T6SS during PBP1a depletion.

Figure 4.4 *Microscopy-based tools to visualize extracellular structures of A.*

tumefaciens. A. ImpB, a component of the T6SS sheath was tagged with sfGFP on a cumate-inducible plasmid. + PBP1a and cells depleted of PBP1a for 16 hours were induced with 0.2 mM of cumate for one hour and imaged. B. Flagella are visualized with fluorescence microscopy of cells labeled with Alexa Fluor maleimide dye linkage to cysteine residues of FlaA_{cys} expressed from a cumate-inducible plasmid.

Downregulation of motility genes, including those for flagella basal body, filament, and motor assembly suggests that cells depleted of PBP1a do not build functional flagella, and therefore are non-motile. To determine if functional flagella are assembled during PBP1a depletion, we modified the flagella filament protein FlaA to express a surface exposed cysteine, enabling flagella to be labeled with maleimide, a thiol reactive dye. When PBP1a is expressed, FlaA_{cys} labels with maleimide and appears as foci near the sub-polar region, where flagella filaments are assembled [24] (Figure 4.4B). In contrast, cells depleted of PBP1a do not form visible foci (Figure 4.4B). In the absence of FlaA as a target, the maleimide binds to the cell surface, resulting in a dispersed localization pattern. These data are consistent with the downregulation of genes for motility and the notion that cells no longer assemble functional flagella following the depletion of PBP1a.

Succinoglycan overproduction is required for cell spreading

One of the most striking phenotypes of the PBP1a depletion is that cells spread apart instead of forming compact microcolonies. Since genes for motility and chemotaxis, are all downregulated (Figure 4.2B) and flagella do not appear to be assembled during PBP1a depletion (Figure 4.4B) and there is no evidence for twitching or swarming motility in *A. tumefaciens*, it is unlikely that the spreading is dependent on one of these forms of motility. Here we find that overproduction of the exopolysaccharide succinoglycan is required to allow the cells to spread apart. Succinoglycan is a β -1,4-linked sugar made of glucose and galactose, and the most abundant exopolysaccharide produced by *A. tumefaciens* [25]. Utilizing the knowledge that calcofluor white can bind β -1,4-linked glycans and fluoresces under UV light, we developed a microscopy-based assay to observe succinoglycan production in *A. tumefaciens*. Calcofluor white was added to agarose pads, and either WT cells or different mutants were spotted on the pads and grown overnight, then imaged using the DAPI filter to detect succinoglycan production (Figure 4.5A). WT *A. tumefaciens* and the PBP1a depletion strain under conditions where PBP1a is present both synthesize and secrete succinoglycan that is contained within the boundary of each microcolony, with enrichment of succinoglycan near the center of the microcolony (Figure 4.5A). In comparison, depletion of PBP1a triggers secretion of a large quantity of succinoglycan that defines the boundary of where the cells spread. The excess amount of succinoglycan secreted by the PBP1a depletion, is consistent with the upregulation of succinoglycan biosynthesis genes during PBP1a depletion (Supplementary Table 4.S2). To test the hypothesis that the secretion of

succinoglycan contributes to the spreading phenotype of the PBP1a depletion, we generated an in-frame deletion of *exoA*, a glycosyltransferase required for succinoglycan production in *A. tumefaciens* [26]. Indeed, secretion of succinoglycan was almost undetectable in the *exoA* deletion strain (Figure 4.5A). Succinoglycan production was also nearly absent during PBP1a depletion in an *exoA* deletion strain and the cells no longer spread apart on the agarose pad (Figure 4.5A). While the *exoA* deletion abolishes excess succinoglycan production in the during PBP1a depletion, it does not impact production of short, round cells. During PBP1a depletion, Δ *exoA* cells have a median cell length of 1.42 μ M after 16 hours of depletion, which is similar to that of the PBP1a depletion strain (Chapter 3). This assay provides strong evidence that the overproduction of succinoglycan during PBP1a depletion contributes to the spreading phenotype.

Figure 4.5 *Calcofluor white binding assay to visualize succinoglycan production.*

1% ATGN agarose pads were supplemented with 25 μ g/mL calcofluor white and

1mM IPTG when appropriate. Mid log phase cells were spotted onto the pads, incubated at 28° C for 16 hours, and imaged.

To determine if the loss of PBP1a activity is directly triggering the overproduction of succinoglycan, we took advantage of the fact that flavomycin and moenomycin antibiotics are well-known to target the GTase activity of PBP1a enzymes [27].

WT *A. tumefaciens* cells treated with sub-MIC concentrations of flavomycin phenocopied both the cell spreading and reduction in cell length phenotypes of the PBP1a depletion strain (Supplementary Figure 4.1F). Furthermore, flavomycin-treated cells overproduce succinoglycan in our agar pad assay (Figure 4.5B). These data indicate that loss of the GTase activity of PBP1a is sufficient to mimic the physiological and behavioral changes of the PBP1a depletion. Together, these findings suggest that inefficient cell wall biogenesis may lead to accumulation of a signal that triggers changes in gene expression leading to striking changes in bacterial physiology reminiscent of lifestyle changes. Possible signals may include the accumulation of cytoplasmic peptidoglycan precursors such as lipid II or increased levels of naked glycan strands in the cell wall or the production of peptidoglycan hydrolysis [28]. Alternatively, the loss of cell wall biosynthesis may disrupt cell wall integrity leading to a more conventional stress responses due to osmotic sensitivity. It will be of interest to explore each of these possibilities to determine how the absence of PBP1a activity triggers the RSI pathway.

Regulation of Cell Wall Synthase and Hydrolase Genes in response to PBP1a depletion

In addition to the surprising finding that the depletion of PBP1a triggers an RSI-like invasion response, our transcriptome profiling reveals expected changes in the expression of proteins involved in cell surface remodeling, including cell wall synthesis and hydrolysis enzymes (Figure 4.3B). Consistent with the observation that PBP1a depletion causes increases in LD-crosslinkage in the peptidoglycan (Chapter 3), here we observe three LDTs that are upregulated after 16-hours of PBP1a depletion (LdtB, LdtD, and LdtF) (Table 4.1). LdtB-sfGFP and LdtF-sfGFP localize to mid-cell in WT cells (Chapter 3) suggesting that enzymes which normally contribute to mid-cell PG biosynthesis machinery may be upregulated to compensate for the loss of polar PG biosynthesis. LdtD was also upregulated during PBP1a depletion. Although LdtD is typically localized throughout the cell (Chapter 3), overexpression of LdtD-sfGFP leads to the accumulation of this protein at the growth pole (Supplementary Figure 4.1B), making it a candidate to crosslink PG at the pole in the absence of PBP1a. Future studies exploring the phenotypes of LDT mutants may shed light on their functional roles in crosslinking PG during cell division and polar growth. One LDT in particular, LdtK, was strongly downregulated after 16 hours of PBP1a depletion (Table 4.1). LdtK-sfGFP localized to the pole and mid-cell (Chapter 3), reminiscent of the localization pattern of PBP1a [29]. The observation that LdtK is downregulated during PBP1a depletion may suggest that LdtK functions alongside PBP1a at the pole to crosslink PG during elongation. LdtM was also downregulated during

PBP1a depletion (Table 4.1) and LdtM-sfGFP localized to the sub-polar region of the new cell compartment (Chapter 3). In addition to its role at the growth pole, PBP1a may either directly or indirectly be required for proper remodeling of newly synthesized PG in the sub-polar region. Together, the localization of LDTs observed in chapter 3, combined with the RNA-seq analysis during PBP1a depletion (Table 4.1) suggest that one subset of LDTs may function with PBP1a at the growth pole while another subset of LDTs may compensate for the loss of PBP1a.

Gene ID	Name	Log2 FC	p-value
Atu1615	LdtA	0.469	7.755E-11
Atu2133	LdtB	1.839	5.469E-85
Atu1293	LdtC	0.883	1.65E-14
Atu1164	LdtD	1.718	1.16E-64
Atu3631	LdtE	0.936	4.75E-29
Atu3332	LdtF	1.906	2.29E-114
Atu2764	LdtG	-0.140	0.2005777
Atu0669	LdtH	0.681	0.0002757
Atu3332	LdtI	1.594	3.56E-27
Atu0048	LdtJ	-0.712	1.737E-06
Atu0844	LdtK	-4.950	4.505E-90
Atu0845	LdtL	0.181	0.0976398
Atu2336	LdtM	-1.993	5.573E-51
Atu5196	LdtN	-0.125	0.2736784

Table 4.1 List of fold change (FC) of LDTs from the 16-hour depletion compared to + PBP1a.

In addition to the differential expression of LDTs, depletion of PBP1a for 16 hours also impacted the regulation of DD-endopeptidases (DD-EPases). DD-EPase class proteins cleave peptide bonds between the fourth D-ala and mDAP residues and

are likely candidates to cleave existing PG to allow for PBP-mediated synthesis of D44 crosslinks [30]. *A. tumefaciens* encodes three enzymes with potential DD-EPase activity, and two of these fell just below the log₂ FC cut-off for being considered differentially expressed (Figure 4.6A). Of the three predicted DD-EPases, only DD-EPase4178, now called RgsM (Figure 4.6B), was predicted to be essential [31]. We failed to make a deletion mutant of RgsM, suggesting that it is indeed essential. We constructed a depletion strain as described previously [13] by placing RgsM under an IPTG inducible promoter. Cells grown in the absence of IPTG had a severe cell viability defect compared to cells grown in the presence of IPTG (Supplementary Figure 4.1C). Depletion of RgsM for 16 hours led to the production of shorter cells (Figure 4.6C, D), reminiscent of the phenotype of cells depleted of PBP1a (Chapter 3 Figure 3.1 C). Together these data indicate that RgsM is a likely candidate to function as a spacing-making enzyme by hydrolyzing PG at the growth pole to enable insertion of new PG by PBP1a in *A. tumefaciens*. Furthermore, an RgsM homolog was found to be essential in *S. meliloti* suggesting that this protein likely has a conserved function in the maintenance of rod-shape in polar-growing bacteria in the Rhizobiales [32]. In the future, investigating if hydrolysis of PG by RgsM is required for PBP1a to insert new PG could be explored by observing the dipeptide labeling patterns of the EPase depletion.

Figure 4.6 Functional characterization of the essential DD-endopeptidase RgsM.

A. Schematic of the domain structure of RgsM. TM, transmembrane domain; M23, peptidase M23 B. Representative phase images of each condition. Scale bar: 2 μ M. C. Cell length analysis. The indicated strains were grown to exponential phase, spotted on an agar pad, imaged by phase microscopy, and subjected to cell lengths measurements using MicrobeJ. The data are shown as box plots. Distributions of cells significantly different from WT are indicated (***)

One-Way ANOVA with Bonferroni correction, $p < 2 \times 10^{-16}$). $n = >400$ cells per strain.

Since the DD-EPase RgsM is essential for polar growth and had a strikingly similar reduction in cell length to the PBP1a depletion, we tested whether RgsM overproduces succinoglycan. Surprisingly, RgsM depleted cells form hyper-compact microcolonies (Figure 4.6D) that produced visibly less succinoglycan than WT or RgsM replete cells (Figure 4.6E). Although PBP1a and RgsM are both essential for polar growth, and proper rod shape it seems that loss of RgsM is not sufficient to induce the behavioral changes that are seen in the PBP1a depletion. If RgsM is required for insertion of new PG by PBP1a, then the activities of both RgsM and PBP1a may be comprised during RgsM depletion. In contrast, during PBP1a depletion, the enzymatic activity of RgsM likely continues. Thus, during PBP1a depletion, an imbalance of PG hydrolysis and synthesis may occur. Perhaps a hydrolyzed PG fragment accumulates and serves as a signal to activate the RSI pathway. These hypotheses require further investigation but provide future directions to explore how bacteria monitor changes in their cell wall composition or integrity, resulting in transcriptional changes that impact physiology and behavior.

Concluding Remarks

In this work we make the surprising finding that the absence of PBP1a activity at the growth pole during elongation triggers an RSI-like response including the activation of T6SS and succinoglycan production and the repression of flagella-mediated motility. The bacterial cell surface is the conduit through which *A. tumefaciens* cells must sense and respond to a variety of environmental conditions in both the soil and the rhizosphere. The RSI pathway is known to be triggered by environmental changes associated with conditions favorable for plant association. Perhaps during plant colonization, *A. tumefaciens* decreases cell wall biogenesis to form cells which are relatively persistent and can survive the harsh *in plantae* environment? Alternatively, maybe the integrity and composition of the cell wall is routinely monitored and used as a signal for the activation of pathways associated with lifestyle choice. The overproduction of succinoglycan provides cells with passive protection against a number of stresses including detergents, salt, acidity, heat, antimicrobial peptides, and reactive oxygen species [33-36]. The decreased activity of PBP1a may mimic one or more of these conditions leading to the activation of the RSI pathway. Remarkably, our results indicate that depletion of PBP1a (Figure 4.1B), treatment with flavomycin (Figure 4.5B), and exposure to acid conditions (Supplementary Figure 4.1E) are all sufficient to trigger ExoA-dependent overproduction of succinoglycan. These observations suggest that compromised PG arising from an imbalance of PG synthesis and hydrolysis are sufficient to trigger the lifestyle switch in *A. tumefaciens*. Future investigations will be necessary to determine if

the RSI circuit directly responds to PG stress or if other regulators which presumably interact with the RSI circuit are involved in this response.

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CHAPTER 4: SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURES

Supplementary Figure 4.S1 *Additional characterization of PBP1a and RgsM depletion strains and WT cells* A. Time-lapse microscopy of *S. meliloti* PBP1a depletion strain spotted on a 1% TY agarose pad supplemented with 100 µg/mL KAN, 20 µg/mL GM and 500 µg/mL IPTG or without IPTG in the pad to induce depletion conditions. Cells were imaged every 10 minutes. B. WT cells carrying a plasmid with *IdtD-sfgfp* were grown to mid log phase and induced with 0.2 mM cumate for 1 hour, or 8 hours for the overexpression strain, then spotted on a 1% ATGN agarose pad and imaged. Shown are merged images of phase and fluorescent channels C. For cell viability spot assays, exponentially growing cultures were diluted to OD₆₀₀ = 0.05 and serially diluted in ATGN. 4 µl of each dilution was spotted onto ATGN agar plates and incubated at 28°C for 3 days before imaging. For the + RgsM strain ATGN plates contained IPTG 1mM. D. WT or WT grown with 24 µg/mL Flavomycin (FLA) for 24 hours was grown to exponential phase, spotted on an ATGN agar pad, and imaged by phase microscopy. Cell length distribution measurements were analyzed using MicrobeJ. The data are shown as box plots. E. Phase microscopy images showing the microcolony phenotypes of WT and $\Delta exoA$. Each strain was grown to exponential phase, spotted on an ATGN agar pad buffered with MES to pH 7 or 5.5 and allowed to grow for 16 hours and imaged.

A **B**

Supplementary Figure 4.S2 *RPKM plots of control strains* A. Plots comparing the RMKM values of the WT transcriptome to that of the WT + IPTG transcriptome. Data was transformed with log10. No genes were considered differentially expressed (DE) between the two conditions using the criteria Log2 fold change of 2.0 and a p-value of less than 0.05. B. Plots comparing the RMKM values of the WT + IPTG to the + PBP1a transcriptome. One gene belonging to the TetR locus was considered DE according to the criteria in A. We do not consider this change biologically relevant because it is due to the deletion of the TetR locus in the + PBP1a strain, which was engineered to allow us to make chromosomal deletions.

SUPPLEMENTARY TABLES

Table 4.S1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Genotype, Features or Characteristics	Source or Reference
Source Plasmids		
pNTPS139	Km ^r ; Suicide vector containing oriT and sacB	D. Alley
pUC18-mini-Tn7T-GM-Plac	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>lacI^q</i> and lac promoter	Figuerola-Cuilan et al 2016 AEM
pTNS3	Ap ^r ; helper plasmid encoding the site-specific TnsABCD Tn7 transposition pathway	Choi et al 2010 AEM
pSRKKm-Pcym	pSRKKm vector containing lac promoter flanked by <i>cuO</i> operator site along with the <i>cymR</i> repressor gene	Howell et al 2019 Mol Micro
Deletion Plasmids		
pNTPS139Δ <i>pbp1a</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1a</i>	This Study
pNTPS139Δ <i>pbp1b1</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1b1</i>	This Study
pNTPS139Δ <i>pbp1b2</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1b2</i>	This Study
pNTPS139Δ <i>pbp1c</i>	Km ^r Suc ^s ; deletion plasmid for <i>pbp1c</i>	This Study
pNTPS139Δ <i>mtgA</i>	Km ^r Suc ^s ; deletion plasmid for <i>mtgA</i>	This Study

pNTPS139 Δ exoA	Km ^r Suc ^S ; deletion plasmid for <i>exoA</i>	This Study
Depletion Plasmids		
pUC18-mini-Tn7T-GM-plac <i>pbp1a</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>pbp1a</i> under control of the lac promoter	This Study
pUC18-mini-Tn7T-GM-plac <i>rgsM</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>rgsM</i> under control of the lac promoter	This Study
Replicating Plasmids		
pSRKKm-Pcym- <i>impB-sfgfp</i>	pSRKKm vector containing <i>impB-sfgfp</i> under control of the cym promoter	This Study
pSRKKm-Pcym-FlaAcys	pSRKKm vector containing FlaAcys single amino acid change T213C	This Study
E. coli strains		
DH5 α	Cloning strain	Life Technologies
S17-1	Smr;RP4-2 TC::MU Km-Tn7; for plasmid mobilization	Simon et al 1983 Nat Biotech
A. tumefaciens strains		
C58	Nopaline type strain; pTiC58; pAtC58	Lab Stock
C58 Δ tetRA::a-attTn7	Replacement of the tetRA locus with an artificial attTn7 site	Figueroa-Cuilan et al 2016 AEM
C58 Δ tetRA::a-attTn7 Δ <i>pbp1b1</i>	Δ <i>pbp1b1</i>	This Study
C58 Δ tetRA::a-attTn7 Δ <i>pbp1b2</i>	Δ <i>pbp1b2</i>	This Study
C58 Δ tetRA::a-attTn7 Δ <i>pbp1c</i>	Δ <i>pbp1c</i>	This Study
C58 Δ tetRA::a-attTn7 Δ <i>mtgA</i>	Δ <i>mtgA</i>	This Study
C58 Δ tetRA::mini-Tn7-GM-Plac- <i>pbp1a</i>	Mini-Tn7T-GM-Plac- <i>pbp1a</i> inserted into a-attTn7 site	This Study
C58 Δ tetRA::mini-Tn7-GM-Plac, Δ <i>pbp1a</i>	Chromosome-based complementation of Δ <i>pbp1a</i> with C58 Δ tetRA::mini-Tn7-GM-Plac- <i>pbp1a</i> allowing depletion of PBP1a under control of the lac promoter	This Study
C58 Δ tetRA::mini-Tn7-GM-Plac- <i>rgsM</i>	Mini-Tn7T-GM-Plac- <i>rgsM</i> inserted into a-attTn7 site	This Study
C58 Δ tetRA::mini-Tn7-GM-Plac, Δ <i>rgsM</i>	Chromosome-based complementation of Δ <i>rgsM</i> with C58 Δ tetRA::mini-Tn7-GM-Plac- <i>pbp3a</i> allowing depletion of RgsM under control of the lac promoter	This Study
C58 Δ tetRA::mini-Tn7-GM-Plac, Δ <i>pbp1a</i> , Δ exoA	Chromosome-based complementation of Δ <i>pbp1a</i> with C58 Δ tetRA::mini-Tn7-GM-Plac- <i>pbp1a</i> allowing depletion of PBP1a under control of the lac promoter in the background of <i>exoA</i> deletion	This Study
C58 Δ tetRA::a-attTn7, <i>impB-sfgfp</i>	Strain expressing <i>impB-sfgfp</i> from a pSRK replicative plasmid	This Study

C58 Δ tetRA::a-attTn7, FlaAcys	Strain expressing FlaAcys from a pSRK replicative plasmid	This Study
<i>S. meliloti</i> strains		
Rm2011 rgsP-egfp mrcA1dpl	Rm2011 rgsP-egfp carrying markerless deletion of mrcA1, curable complementation plasmid pGCH14-mrcA1, and pSRKKm as a source of lacI to cure pGCH14-mrcA1, Gm ^r Km ^r	This Study

Table 4.S2. List of Succinoglycan genes regulated in the 16 hr depletion

Gene ID	Name	Description	log ₂ FC	p-value
Atu3325	exoQ	exopolysaccharide production protein	4.165	0
Atu3327	exoY	succinoglycan exopolysaccharide synthesis protein	4.262	0
Atu4049	exoP	exopolysaccharide polymerization/transport protein	3.695	0
Atu4050	exoN	UTP-glucose-1-phosphate uridylyltransferase	4.399	0
Atu4051	exoO	succinoglycan biosynthesis glycosyltransferase	3.886	0
Atu4052	exoM	UDP-hexose transferase	3.848	0
Atu4053	exoA	succinoglycan biosynthesis protein	3.470	0
Atu4059	exoV	succinoglycan biosynthesis protein	3.410	0
Atu4054	exoL	succinoglycan biosynthesis protein	3.571	5.2E-277
Atu4060	exoU	succinoglycan biosynthesis glycosyltransferase	2.941	1.3E-267
Atu4055	exoK	endo-1,3-1,4-beta-glycanase	3.771	4.4E-244
Atu4166	galE/exoB	UDP-glucose 4-epimerase	2.489	6.7E-244
Atu4056	exoH	succinoglycan biosynthesis protein	3.688	2.6E-242
Atu3326	exoF	exopolysaccharide production protein	4.150	4.2E-203
Atu4058	exoW	succinoglycan biosynthesis protein	2.753	2E-124
Atu4057	exoT	succinoglycan biosynthesis transport protein	2.519	1.28E-85
Atu1715	exoR	exopolysaccharide production negative regulator	2.204	1.64E-37

Table 4.S3. List of T6SS genes regulated in the 16 hr depletion

Gene ID	Name	Description	log ₂ FC	p-value
Atu4337	impG	conserved hypothetical protein	2.813	3.7E-212
Atu4340	impD	conserved hypothetical protein	2.736	1.7E-211
Atu4333	impK	OmpA-like porin	2.717	1.2E-177
Atu4341	impC	conserved hypothetical protein	2.854	3E-157
Atu4332	impL	lcmF family protein	2.597	3.8E-145
Atu4342	impB	conserved hypothetical protein	2.827	3E-126
Atu4334	impJ	conserved hypothetical protein	2.832	1.6E-118

Atu4339	impE	hypothetical protein	2.704	5.75E-98
Atu4343	impA	conserved hypothetical protein	2.627	3.66E-93
Atu4331	impM	serine/threonine phosphoprotein phosphatase	2.454	2.6E-74
Atu4335	impl	putative forkhead-type phosphopeptide-binding protein	2.620	2.4E-69
Atu4338	impF	conserved hypothetical protein	2.737	3.5E-67
Atu4330	impN	serine/threonine protein kinase	2.431	2.44E-60
Atu4336	impH	conserved hypothetical protein	2.610	6.28E-57

Table 4.S4. List of Differentially Expressed Genes in the 6 hr depletion

Gene ID	Name	Description	log ₂ Fold Change	p-value
Atu1341	mrcA	penicillin-binding protein 1a	-7.189	0
Atu4337	impG	conserved hypothetical protein	2.813	3.74E-212
Atu4340	impD	conserved hypothetical protein	2.736	1.69E-211
Atu4333	impK	OmpA-like porin	2.717	1.25E-177
Atu4341	impC	conserved hypothetical protein	2.854	3.04E-157
Atu4332	impL	lcmF family protein	2.597	3.78E-145
Atu4342	impB	conserved hypothetical protein	2.827	3.04E-126
Atu4334	impJ	conserved hypothetical protein	2.832	1.61E-118
Atu4339	impE	hypothetical protein	2.704	5.754E-98
Atu4343	impA	conserved hypothetical protein	2.627	3.662E-93
Atu4331	impM	serine/threonine phosphoprotein phosphatase	2.454	2.599E-74
Atu4335	impl	putative forkhead-type phosphopeptide-binding protein	2.620	2.405E-69
Atu4338	impF	conserved hypothetical protein	2.737	3.497E-67
Atu0875	-	hypothetical protein	3.311	5.706E-61
Atu4330	impN	serine/threonine protein kinase	2.431	2.444E-60
Atu3738	-	Na ⁺ /H ⁺ antiporter	3.393	1.315E-58
Atu8089	-	conserved hypothetical protein	3.049	5.249E-58
Atu4336	impH	conserved hypothetical protein	2.610	6.28E-57
Atu0944	cscA	sucrose hydrolase	3.426	3.466E-53
Atu4347	-	hypothetical protein	2.069	2.103E-52
Atu4345	-	conserved hypothetical protein	2.169	5.186E-52
Atu3325	exoQ	exopolysaccharide production protein	2.507	1.746E-51
Atu2162	pp26	periplasmic immunogenic protein	2.777	2.91E-51
Atu2444	-	conserved hypothetical protein	3.360	4.445E-50
Atu1150	-	conserved hypothetical protein	2.174	4.894E-46
Atu4026	-	conserved hypothetical protein	2.419	1.177E-45
Atu2112	slt	soluble lytic transglycosylase	4.298	1.385E-45

Atu4498	-	conserved hypothetical protein	2.077	2.5E-45
Atu6178	virG	two component response regulator	2.607	5.853E-45
Atu1762	-	conserved hypothetical protein	2.381	7.92E-43
Atu0898	-	conserved hypothetical protein	2.682	2.993E-42
Atu1001	-	conserved hypothetical protein	2.243	2.666E-41
Atu1131	ropB	outer membrane protein	3.416	3.192E-34
Atu0034	chvI	two component response regulator	2.181	4.892E-34
Atu3326	exoF	exopolysaccharide production protein	2.599	1.281E-31
Atu4049	exoP	exopolysaccharide polymerization/transport protein	2.072	2.968E-31
Atu4051	exoO	succinoglycan biosynthesis glycosyltransferase	2.211	2.577E-30
Atu4052	exoM	UDP-hexose transferase	2.215	3.241E-30
Atu8017	-	hypothetical protein	2.080	8.201E-30
Atu4050	exoN	UTP-glucose-1-phosphate uridylyltransferase	2.604	1.221E-29
Atu4323	rdh	ribitol 2-dehydrogenase	2.011	1.491E-28
Atu8139	-	hypothetical protein	2.439	6.094E-28
Atu0381	-	hypothetical protein	2.949	5.23E-27
Atu4401	-	conserved hypothetical protein	-2.293	2.733E-26
Atu4640	-	hypothetical protein	2.488	3.824E-26
Atu3327	exoY	succinoglycan exopolysaccharide synthesis protein	2.296	6.043E-26
Atu6162	-	conserved hypothetical protein	2.152	7.655E-25
Atu3975	glf	UDP-galactopyranose mutase	-2.165	1.874E-23
Atu3540	-	hypothetical protein	2.467	2.358E-23
Atu5146	attU	conserved hypothetical protein	2.753	1.497E-22
Atu1155	-	conserved hypothetical protein	2.488	1.615E-22
Atu2303	-	conserved hypothetical protein	2.515	5.129E-22
Atu2198	-	hypothetical protein	3.087	1.506E-18
Atu4638	-	two component response regulator	2.410	2.682E-18
Atu0552	flgG	flagellar basal-body rod protein	-2.460	3.831E-17
Atu3976	-	conserved hypothetical protein	-2.572	2.063E-16
Atu0548	flgH	flagellar L-ring protein precursor	-2.156	3.841E-16
Atu0563	fliG	flagellar motor switch protein FliG	-2.311	6.466E-15
Atu0547	fliL	flagellar protein	-2.202	1.026E-14
Atu8132	-	conserved hypothetical protein	-2.556	1.606E-14
Atu0555	flgB	flagellar basal-body rod protein FlgB	-2.172	1.634E-14
Atu1534	fixP	cytochrome-c oxidase, FixP chain	-2.119	1.642E-14
Atu1233	-	hypothetical protein	-2.168	1.903E-14
Atu3974	ina	ice nucleation-like protein	-2.422	3.042E-14
Atu0585	-	conserved hypothetical protein	-2.306	3.08E-14
Atu0562	fliN	flagellar motor switch protein FliN	-2.368	5.63E-14
Atu0523	fliF	flagellar M-ring protein	-2.257	1.389E-13

Atu0561	fliM	flagellar motor switch protein	-2.361	1.409E-13
Atu0570	MotC	Chemotaxis protein	-2.180	2.257E-13
Atu0584	-	conserved hypothetical protein	-2.452	2.332E-13
Atu0560	motA	flagellar motor protein	-2.276	3.502E-13
Atu0569	motB	flagellar motor protein, PAL/OmpA family	-2.109	3.887E-13
Atu0558	flgF	flagellar basal body rod protein	-2.292	4.934E-13
Atu0568	-	conserved hypothetical protein	-2.231	6.939E-13
Atu1221	-	hypothetical protein	3.597	8.148E-13
Atu0581	flhA	flagellar biosynthesis protein	-2.136	1.452E-12
Atu0557	fliI	flagellum-specific ATP synthase	-2.313	3.453E-12
Atu0550	flgI	flagellar P-ring protein precursor	-2.221	4.332E-12
Atu0551	flgA	flagellar protein FLGA precursor	-2.279	8.234E-12
Atu0549	-	conserved hypothetical protein	-2.402	1.048E-11
Atu3293	dadA	D-amino acid dehydrogenase, small subunit	-2.346	1.365E-11
Atu0572	-	conserved hypothetical protein	-2.249	1.61E-11
Atu0554	flgC	flagellar basal-body rod protein	-2.476	2.808E-11
Atu0553	fliE	flagellar hook-basal body complex protein	-2.550	3.961E-11
Atu0844	-	conserved hypothetical protein	-2.297	2.24E-10
Atu4400	-	ferrienterobactin-like protein	-2.318	3.167E-09
Atu0559	-	conserved hypothetical protein	-2.280	3.576E-09
Atu4402	-	ABC transporter, membrane spanning protein (anion)	-2.217	1.458E-08
Atu4205	tetR	transcriptional regulator, TetR family	5.459	1.549E-07
Atu4080	-	glutamine amidotransferase	-2.210	0.0010223

Table 4.S5. List of Differentially Expressed Genes in the 16 hr depletion

Gene ID	Name	Description	log ₂ Fold Change	p-value
Atu0802	-	conserved hypothetical protein	2.929	0
Atu0875	-	hypothetical protein	4.429	0
Atu0944	cscA	sucrose hydrolase	5.030	0
Atu1155	-	conserved hypothetical protein	4.381	0
Atu1341	mrcA	penicillin-binding protein 1a	-7.204	0
Atu2162	pp26	periplasmic immunogenic protein	3.570	0
Atu3325	exoQ	exopolysaccharide production protein	4.165	0
Atu3327	exoY	succinoglycan exopolysaccharide synthesis protein	4.262	0
Atu3976	-	conserved hypothetical protein	-5.320	0
Atu4026	-	conserved hypothetical protein	3.107	0
Atu4049	exoP	exopolysaccharide polymerization/transport protein	3.695	0
Atu4050	exoN	UTP-glucose-1-phosphate uridylyltransferase	4.399	0

Atu4051	exoO	succinoglycan biosynthesis glycosyltransferase	3.886	0
Atu4052	exoM	UDP-hexose transferase	3.848	0
Atu4053	exoA	succinoglycan biosynthesis protein	3.470	0
Atu4059	exoV	succinoglycan biosynthesis protein	3.410	0
Atu4337	impG	conserved hypothetical protein	2.702	0
Atu4340	impD	conserved hypothetical protein	2.594	0
Atu4341	impC	conserved hypothetical protein	2.527	0
Atu4640	-	hypothetical protein	4.474	0
Atu6178	virG	two component response regulator	4.019	0
Atu3715	tolA	conserved hypothetical protein	2.062	2E-293
Atu4054	exoL	succinoglycan biosynthesis protein	3.571	5.2E-277
Atu0585	-	conserved hypothetical protein	-4.406	3.4E-276
Atu4060	exoU	succinoglycan biosynthesis glycosyltransferase	2.941	1.3E-267
Atu0841	-	conserved hypothetical protein	2.157	7.1E-260
Atu4498	-	conserved hypothetical protein	3.147	5.1E-251
Atu2253	moxR	MoxR family protein	2.577	4.2E-248
Atu4055	exoK	endo-1,3-1,4-beta-glycanase	3.771	4.4E-244
Atu4166	galE/exoB	UDP-glucose 4-epimerase	2.489	6.7E-244
Atu4056	exoH	succinoglycan biosynthesis protein	3.688	2.6E-242
Atu3752	-	conserved hypothetical protein	3.398	2.3E-237
Atu1150	-	conserved hypothetical protein	3.446	3.4E-230
Atu4499	-	conserved hypothetical protein	2.624	1.4E-227
Atu4333	impK	OmpA-like porin	2.593	8E-220
Atu2257	-	conserved hypothetical protein	2.812	1.3E-218
Atu2444	-	conserved hypothetical protein	5.092	2.3E-213
Atu0555	flgB	flagellar basal-body rod protein FlgB	-5.017	4.7E-212
Atu4334	impJ	conserved hypothetical protein	2.458	6.4E-208
Atu4342	impB	conserved hypothetical protein	2.371	6.2E-207
Atu3326	exoF	exopolysaccharide production protein	4.150	4.2E-203
Atu4332	impL	IcmF family protein	2.573	4.3E-199
Atu0553	fliE	flagellar hook-basal body complex protein	-5.715	3.8E-197
Atu2272	-	conserved hypothetical protein	2.576	3.8E-197
Atu0547	fliL	flagellar protein	-4.212	2.5E-196
Atu0578	flbT	FlbT post-transcriptional regulator of flagellin	-2.722	3.4E-195
Atu8089	-	conserved hypothetical protein	4.048	9.7E-194
Atu0551	flgA	flagellar protein FLGA precursor	-4.460	3.5E-191
Atu4541	pcaC	carboxymuconolactone decarboxylase	-3.422	1.5E-185
Atu0523	fliF	flagellar M-ring protein	-4.926	1.5E-185
Atu0548	flgH	flagellar L-ring protein precursor	-4.308	4.8E-182
Atu4532	aroE	shikimate 5-dehydrogenase	-2.462	2.1E-178

Atu1741	-	conserved hypothetical protein	2.737	1.2E-177
Atu1001	-	conserved hypothetical protein	3.668	2.3E-177
Atu0584	-	conserved hypothetical protein	-5.019	5.7E-168
Atu3974	ina	ice nucleation-like protein	-5.196	1.4E-167
Atu3975	glf	UDP-galactopyranose mutase	-3.725	3E-167
Atu4549	pcaF	beta-ketoadipyl CoA thiolase	-3.118	2.5E-164
Atu0515	-	conserved hypothetical protein	-3.336	1.9E-161
Atu8175	-	putative transmembrane protein, RNA-binding region RNP-1 (RNA recognition motif)	2.966	4.2E-161
Atu0522	-	conserved hypothetical protein	-3.070	7.4E-161
Atu3616	-	conserved hypothetical protein	2.081	2.2E-159
Atu1877	-	OmpA family protein	3.297	3.5E-159
Atu4339	impE	hypothetical protein	2.598	7.6E-158
Atu1559	-	conserved hypothetical protein	-2.757	7.3E-155
Atu1468	-	conserved hypothetical protein	-5.450	6.2E-154
Atu8139	-	hypothetical protein	3.584	5E-153
Atu6162	-	conserved hypothetical protein	3.126	5.2E-150
Atu1131	ropB	outer membrane protein	5.043	1.4E-149
Atu0554	flgC	flagellar basal-body rod protein	-5.399	3.1E-148
Atu3738	-	Na ⁺ /H ⁺ antiporter	4.656	1.3E-147
Atu3540	-	hypothetical protein	3.849	2.9E-147
Atu0998	bacA	undecaprenyl pyrophosphate phosphatase, possible bacitracin resistance protein	2.850	4.6E-146
Atu0550	flgI	flagellar P-ring protein precursor	-4.334	3.4E-145
Atu0571	motD	chemotaxis MotD protein	-4.624	1.2E-144
Atu0518	cheR	chemotaxis methyltransferase	-3.233	2.1E-144
Atu3716	tolR	tolR protein	2.060	4E-143
Atu6150	virH1	P-450 monooxygenase	2.178	1.3E-142
Atu6164	tzs	trans-zeatin secretion protein	2.050	6.5E-142
Atu8200	-	Conserved hypothetical protein	2.841	1.1E-139
Atu0549	-	conserved hypothetical protein	-4.583	1.6E-138
Atu0574	flgE	flagellar hook protein	-4.139	4.3E-138
Atu0581	flhA	flagellar biosynthesis protein	-4.411	1.8E-137
Atu8017	-	hypothetical protein	3.556	2.1E-137
Atu3741	-	MFS permease	2.162	7.9E-137
Atu8132	-	conserved hypothetical protein	-4.552	1.7E-134
Atu4518	livH	ABC transporter, membrane spanning protein (branched chain amino acid)	-2.431	8.8E-132
Atu0653	-	conserved hypothetical protein	-2.276	1E-130
Atu4726	sodBIII	superoxide dismutase	2.329	2E-127
Atu0575	flgK	flagellar hook associated protein	-3.161	9.2E-127

Atu0579	flgD	hook formation protein	-2.556	2.6E-126
Atu6151	virH2	P-450 monooxygenase	2.399	2E-125
Atu4322	rbsC	ABC transporter, membrane spanning protein (ribose)	2.292	2.2E-125
Atu0520	cheY	chemotaxis receiver protein	-3.026	2.5E-125
Atu0572	-	conserved hypothetical protein	-4.647	3.7E-125
Atu4058	exoW	succinoglycan biosynthesis protein	2.753	2E-124
Atu0700	-	two component sensor kinase	-2.277	5.1E-124
Atu2254	-	conserved hypothetical protein	2.586	1.7E-122
Atu0563	fliG	flagellar motor switch protein FliG	-4.702	1.1E-117
Atu4533	-	ABC transporter, nucleotide binding/ATPase protein (amino acid)	-2.388	1.1E-117
Atu3714	tolB	tolB protein	2.264	1.6E-116
Atu4539	pcaG	protocatechuate 3,4-dioxygenase alpha chain	-2.569	1E-115
Atu1146	-	acetyl transferase	2.962	2.4E-115
Atu0576	flgL	flagellar hook-associated protein	-2.845	4.7E-115
Atu0570	-	Chemotaxis protein	-4.725	6.3E-115
Atu4538	pcaB	3-carboxy-cis,cis-muconate cycloisomerase	-2.654	2.4E-113
Atu1467	-	hypothetical protein	-3.159	3.2E-113
Atu2822	garD	D-galactarate dehydratase	2.206	4.3E-113
Atu8052	-	conserved hypothetical protein	2.480	4.1E-112
Atu0514	-	methyl-accepting chemotaxis protein	-2.787	9.1E-112
Atu2760	-	conserved hypothetical protein	2.568	3.4E-108
Atu0521	cheD	methyl-accepting chemotaxis protein	-2.901	2.1E-107
Atu0517	cheA	Chemotaxis protein histidine kinase	-3.205	2.5E-107
Atu0892	-	hypothetical protein	-3.540	5.9E-107
Atu0569	motB	flagellar motor protein, PAL/OmpA family	-4.749	4.3E-106
Atu2112	slt	soluble lytic transglycosylase	5.165	1.1E-104
Atu4658	-	hypothetical protein	2.934	4E-104
Atu0560	motA	flagellar motor protein	-4.780	4.6E-101
Atu4639	-	two component sensor kinase	2.740	2.9E-99
Atu4343	impA	conserved hypothetical protein	2.591	1.29E-98
Atu0565	-	conserved hypothetical protein	-2.003	1.82E-98
Atu1633	kefA	potassium efflux system KEFA	2.330	9.04E-97
Atu0898	-	conserved hypothetical protein	3.430	1.16E-95
Atu4330	impN	serine/threonine protein kinase	2.313	1.81E-95
Atu4338	impF	conserved hypothetical protein	2.471	1.83E-95
Atu0552	flgG	flagellar basal-body rod protein	-5.024	2.45E-95
Atu0034	chvI	two component response regulator	2.274	3.6E-95
Atu4529	-	conserved hypothetical protein	-2.051	2.63E-92
Atu0556	-	conserved hypothetical protein	-3.978	3.28E-92

Atu0844	-	conserved hypothetical protein	-4.950	4.51E-90
Atu0568	-	conserved hypothetical protein	-5.078	1.09E-89
Atu0561	fliM	flagellar motor switch protein	-5.650	2.55E-89
Atu0573	-	transcriptional regulator	-2.580	4.53E-89
Atu0900	-	conserved hypothetical protein	2.066	3.78E-87
Atu3704	-	conserved hypothetical protein	2.030	1.35E-86
Atu1997	cysJ	sulfite reductase	-4.896	1.48E-86
Atu4057	exoT	succinoglycan biosynthesis transport protein	2.519	1.28E-85
Atu4517	livM	ABC transporter, membrane spanning protein (branched chain amino acid)	-2.103	1.41E-84
Atu0557	fliI	flagellum-specific ATP synthase	-5.318	1.61E-84
Atu8116	-	hypothetical protein	-2.757	1.79E-84
Atu2075	cheW	chemotaxis protein	-2.090	5.74E-84
Atu4540	pcaH	protocatechuate 3,4-dioxygenase beta chain	-2.525	2.85E-83
Atu0943	-	hypothetical protein	2.856	3.58E-82
Atu0562	fliN	flagellar motor switch protein FliN	-5.966	3.99E-82
Atu1745	-	conserved hypothetical protein	2.095	8.24E-81
Atu4331	impM	serine/threonine phosphoprotein phosphatase	2.364	1.34E-80
Atu2198	-	hypothetical protein	4.864	2.7E-80
Atu0543	flaB	flaB	-3.241	5.31E-80
Atu5020	-	conserved hypothetical protein	-2.098	7.54E-80
Atu1637	-	hypothetical protein	-2.364	2.13E-79
Atu4175	hemK	protoporphyrinogen oxidase	2.122	1.28E-78
Atu4736	-	methyl-accepting chemotaxis protein	-3.920	6.01E-78
Atu0681	-	hypothetical protein	-2.710	4.96E-76
Atu0309	-	conserved hypothetical protein	-3.190	6.33E-76
Atu4534	-	ABC transporter, substrate binding protein (amino acid)	-2.517	6.73E-76
Atu4335	impl	putative forkhead-type phosphopeptide-binding protein	2.540	6.92E-76
Atu3977	ina	ice nucleation-like protein	-4.158	2.25E-73
Atu4596	-	conserved hypothetical protein	-2.023	3.27E-73
Atu1852	-	hypothetical protein	2.013	7.74E-73
Atu0519	cheB	protein-glutamate methylesterase	-3.191	1.27E-72
Atu3080	-	conserved hypothetical protein	2.812	2E-72
Atu2064	-	conserved hypothetical protein	2.811	2.21E-71
Atu2160	-	hypothetical protein	3.112	6.5E-70
Atu3016	gpmB	phosphoglyceromutase	2.763	1.68E-69
Atu4638	-	two component response regulator	3.213	2.32E-68
Atu3081	-	conserved hypothetical protein	2.065	3.75E-68
Atu4400	-	ferrienterobactin-like protein	-3.483	4.23E-68

Atu1631	rrpX	two component response regulator	-2.155	6.89E-68
Atu4535	-	ABC transporter, substrate binding protein (amino acid)	-2.487	8.26E-68
Atu8153	-	conserved hypothetical protein	2.287	2.02E-66
Atu3420	-	monooxygenase	5.240	2.2E-66
Atu5526	-	monooxygenase	4.701	2.2E-66
Atu1969	-	conserved hypothetical protein	2.734	5.41E-66
Atu4323	rdh	ribitol 2-dehydrogenase	2.154	1.22E-65
Atu5516	-	hypothetical protein	2.143	9.94E-65
Atu2421	-	chitooligosaccharide deacetylase	2.634	3.09E-64
Atu5545	-	hypothetical protein	2.083	8.24E-64
Atu4548	pcaJ	3-oxoadipate CoA-transferase subunit B	-3.132	1.28E-63
Atu4336	impH	conserved hypothetical protein	2.504	1.87E-63
Atu0516	cheY	chemotaxis receiver protein	-3.296	4.51E-63
Atu3643	-	hypothetical protein	2.178	7.74E-63
Atu1233	-	hypothetical protein	-4.093	1.34E-62
Atu5022	-	conserved hypothetical protein	-2.185	2.75E-62
Atu4515	livF	ABC transporter, nucleotide binding/ATPase protein (branched chain amino acid)	-2.229	1.77E-61
Atu3435	-	ABC transporter, membrane spanning protein (oligopeptide)	5.915	6.86E-61
Atu1486	-	conserved hypothetical protein	2.274	8.88E-61
Atu0881	-	cytochrome C-556	2.008	1.81E-60
Atu3437	-	ABC transporter, nucleotide binding/ATPase protein (oligopeptide)	3.472	2.45E-60
Atu8018	-	hypothetical protein	3.031	5.57E-60
Atu4516	livG	ABC transporter, nucleotide binding/ATPase protein (branched chain amino acid)	-2.336	9.34E-60
Atu4666	-	HlyD family secretion protein	-2.529	4.08E-59
Atu5146	attU	conserved hypothetical protein	3.620	5.09E-59
Atu0577	flaF	FLAF protein	-2.685	5.75E-58
Atu3416	-	ABC transporter, substrate binding protein	4.802	5.19E-57
Atu2303	-	conserved hypothetical protein	3.212	1.14E-56
Atu1762	-	conserved hypothetical protein	2.703	2.25E-56
Atu3725	-	methyl-accepting chemotaxis protein	-2.215	5.57E-56
Atu5021	-	conserved hypothetical protein	-2.082	4.99E-55
Atu3439	acd	acyl-CoA dehydrogenase	4.287	5.06E-55
Atu4536	-	ABC transporter, membrane spanning protein (amino acid)	-2.468	1.76E-54
Atu0933	-	beta-lactamase class D	2.373	2.22E-54
Atu3418	-	acetyltransferase	4.717	1.18E-52
Atu4668	-	ABC transporter, membrane spanning protein	-2.383	1.76E-52

Atu3015	-	conserved hypothetical protein	2.524	5.69E-52
Atu0738	mcpG	chemotaxis methyl-accepting protein	-2.847	1.22E-51
Atu3440	-	transcriptional regulator, LysR family	4.063	3.32E-51
Atu2336	-	conserved hypothetical protein	-1.993	5.57E-51
Atu3438	-	putative oxidoreductase	4.237	2.99E-50
Atu4547	pcaI	3-oxoadipate CoA-transferase subunit A	-3.143	4.05E-50
Atu0373	-	methyl-accepting chemotaxis protein	-3.122	8.68E-50
Atu4667	-	ABC transporter, nucleotide binding/ATPase protein	-2.829	9.31E-50
Atu3415	-	ABC transporter, substrate binding protein	4.507	1.64E-49
Atu2173	mcpA	methyl-accepting chemotaxis protein	-2.165	7.13E-49
Atu3434	-	ABC transporter, membrane spanning protein (oligopeptide)	5.232	1.09E-48
Atu0381	-	hypothetical protein	3.580	2.81E-48
Atu3433	-	ABC transporter, substrate binding protein (oligopeptide)	4.324	1.56E-47
Atu0545	flaA	flagella associated protein	-2.057	7.23E-47
Atu3838	-	hypothetical protein	-3.586	1.09E-46
Atu3450	-	nitrilotriacetate monooxygenase	4.490	9.41E-46
Atu8182	-	conserved hypothetical protein	2.016	6.2E-45
Atu3436	-	ABC transporter, nucleotide binding/ATPase protein (oligopeptide)	3.803	2.55E-44
Atu3432	-	desulfurizing enzyme	4.321	3.95E-44
Atu0544	-	hypothetical protein	-2.216	7.13E-43
Atu2451	-	conserved hypothetical protein	2.047	3.72E-42
Atu6080	-	transcriptional regulator, LysR family	2.601	4.24E-42
Atu2223	mcpA	methyl-accepting chemotaxis protein	-2.028	2.38E-41
Atu5523	-	ABC transporter, membrane spanning protein (branched chain amino acid)	4.710	2.44E-41
Atu4537	-	ABC transporter, membrane spanning protein (amino acid)	-2.664	3.25E-41
Atu0755	-	conserved hypothetical protein	2.231	1.08E-40
Atu3441	-	nitrilotriacetate monooxygenase	3.397	1.04E-39
Atu5094	tnpA	ISL3 family transposase	2.074	1.45E-39
Atu4531	aroQ	3-dehydroquinate dehydratase	-2.257	1.62E-39
Atu3076	uraA	uracil transport protein	-2.031	3.03E-39
Atu3396	-	ABC transporter, substrate binding protein (iron)	-2.170	3.68E-39
Atu3417	-	ABC transporter, nucleotide binding/ATPase protein	4.076	2.54E-38
Atu1534	fixP	cytochrome-c oxidase, FixP chain	-2.363	2.95E-38
Atu0567	flaD	flagellin protein FlaD	-2.898	6.66E-38
Atu1715	exoR	exopolysaccharide production negative regulator	2.204	1.64E-37

Atu5525	-	ABC transporter, nucleotide binding/ATPase protein (branched chain amino acid)	3.510	1.41E-36
Atu4401	-	conserved hypothetical protein	-2.599	1.57E-36
Atu5524	-	ABC transporter, membrane spanning protein (branched chain amino acid)	4.102	2.4E-36
Atu1535	fixQ	cytochrome c oxidase, FixQ chain	-3.008	4.7E-36
Atu0646	mcpA	methyl-accepting chemotaxis protein A	-1.996	9.17E-36
Atu5521	-	ABC transporter, nucleotide binding/ATPase protein (branched chain amino acid)	4.310	1.18E-35
Atu5515	-	hypothetical protein	2.147	4.9E-34
Atu3837	-	hypothetical protein	-2.219	5.23E-34
Atu2283	-	pseudoazurin	-2.473	5.35E-33
Atu2611	-	conserved hypothetical protein	2.346	3.02E-32
Atu5520	acd	acyl-CoA dehydrogenase	3.246	3.36E-32
Atu3442	-	ABC transporter, substrate binding protein (oligopeptide)	3.312	1.28E-31
Atu1469	-	hypothetical protein	-1.956	1.87E-31
Atu3497	-	hypothetical protein	2.737	4.3E-31
Atu1485	prtI	ECF family sigma factor	2.013	1.4E-30
Atu4221	-	conserved hypothetical protein	2.351	4E-30
Atu3178	-	ABC transporter, substrate binding protein	-6.609	1.84E-29
Atu3431	acd	acyl-CoA dehydrogenase	3.190	2.26E-29
Atu1537	fixN	cytochrome-c oxidase, FixN chain	-2.553	2.39E-29
Atu3191	-	outer membrane protein	-2.549	6.29E-29
Atu3179	-	ABC transporter, membrane spanning protein	-7.330	8.52E-29
Atu0558	flgF	flagellar basal body rod protein	-6.112	1.55E-28
Atu3017	-	phosphoglyceromutase	2.006	1.48E-27
Atu3446	-	monooxygenase	3.526	4.11E-27
Atu4542	pcaD	beta-ketoadipate enol-lactone hydrolase	-3.205	6.74E-27
Atu3180	-	ABC transporter, nucleotide binding/ATPase protein	-7.443	8.93E-27
Atu2287	-	outer membrane heme receptor	-2.296	9.09E-27
Atu4729	-	ABC transporter, nucleotide binding/ATPase protein	2.973	1.35E-26
Atu3444	-	ABC transporter, substrate binding protein (oligopeptide)	3.111	3.45E-26
Atu4519	livJ	ABC transporter, substrate binding protein (branched amino acid)	-2.141	1.49E-25
Atu6067	-	conserved hypothetical protein	3.711	2.26E-25
Atu1528	fixI	nitrogen fixation protein FixI	-2.387	2.39E-25
Atu5522	-	conserved hypothetical protein	4.934	7.94E-25
Atu3395	-	ABC transporter, membrane spanning protein (iron (III))	-2.220	8.95E-25

Atu1601	hemN	oxygen-independent coproporphyrinogen III oxidase	-2.344	1.08E-24
Atu3419	-	hippurate hydrolase	5.478	2.71E-24
Atu0559	-	conserved hypothetical protein	-5.625	4.11E-22
Atu1536	fixO	cytochrome C oxidase, FixO chain	-2.914	6.32E-21
Atu1221	-	hypothetical protein	4.210	9.04E-21
Atu3421	-	conserved hypothetical protein	4.842	1.35E-20
Atu3447	-	conserved hypothetical protein	3.445	1.85E-20
Atu3425	-	conserved hypothetical protein	2.770	2.27E-20
Atu3181	-	conserved hypothetical protein	-8.493	2.35E-20
Atu3394	fecE	ABC transporter, nucleotide binding/ATPase protein (iron)	-2.286	5.34E-20
Atu3424	-	hypothetical protein	2.593	6.47E-20
Atu3393	-	pseudoazurin	-2.332	1.67E-19
Atu1049	-	conserved hypothetical protein	-7.786	2.02E-19
Atu4382	nirK	nitrite reductase, copper-containing	-2.467	1.19E-18
Atu1521	znuA	ABC transporter, substrate binding protein (zinc)	-3.929	1.12E-16
Atu4407	napD	periplasmic nitrate reductase, NapD protein	-2.001	5.81E-16
Atu1884	ssuA	ABC transporter, substrate binding protein (aliphatic sulfonate)	2.085	2.41E-15
Atu2157	-	tyrosine/serine protein phosphatase	2.112	8.99E-15
Atu3426	-	putative sulfonate monooxygenase	2.023	1.38E-14
Atu3184	-	conserved hypothetical protein	-6.377	3.4E-14
Atu4397	-	protease	-2.227	1.65E-13
Atu4526	-	ABC transporter, nucleotide binding/ATPase protein	-2.379	1.13E-12
Atu5527	-	NADH-dependent FMN reductase	4.035	1.6E-12
Atu1529	fixH	nitrogen fixation protein FixH	-2.150	2.76E-12
Atu3664	ephA	epoxide hydrolase	-2.434	2.95E-12
Atu6075	dfpC	ABC transporter, membrane spanning protein (peptide)	2.492	4.55E-12
Atu6057	ssuC	ABC transporter, membrane spanning protein (nitrate/sulfonates)	2.145	3.29E-11
Atu3445	-	hippurate hydrolase	3.680	4.31E-11
Atu4396	-	conserved hypothetical protein	-2.141	6.86E-11
Atu4898	-	hypothetical protein	2.804	2.14E-10
Atu3183	-	hypothetical protein	-5.066	1.74E-09
Atu3097	ugpA	ABC transporter, membrane spanning protein (sn-Glycerol-3-phosphate)	2.376	2.07E-09
Atu4399	-	conserved hypothetical protein	-2.243	5.03E-09
Atu1814	-	epoxide hydrolase	-2.621	4.91E-08
Atu3823	metE	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	-2.656	5.99E-07
Atu3824	-	hypothetical protein	-2.456	1.87E-06

Atu3182	-	conserved hypothetical protein	-2.422	7.14E-05
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CHAPTER 5

Conclusions and Future Directions

Advancing our understanding of the mechanisms that underlie polar growth in the Rhizobiales

Bacterial cell shape is determined by growth patterning, defined as when and where the synthesis of new peptidoglycan (PG) is directed. *E. coli* and *A. tumefaciens* are both rod-shaped bacteria, but despite this they utilize strikingly different growth patterns during elongation [1, 2]. *E. coli* inserts new PG along the lateral sidewalls of the cell body, while *A. tumefaciens* inserts new PG primarily in a narrow region at one pole of the cell. This illustrates that even though bacteria may share a common morphology, there are diverse mechanisms to arrive at a similar rod-shape. Yet, the mechanisms of bacterial growth have primarily been studied in canonical rod-shaped bacteria. In this work, we identified key proteins required for polar PG insertion and maintenance of rod shape in the Rhizobiales order, using *A. tumefaciens* as a model and representative member.

Mechanistic insights and remaining questions about polar growth of *A. tumefaciens*

Previously [1], and in this work the spatiotemporal insertion of PG in *A. tumefaciens* was determined using the fluorescent cell wall probe HADA, which labels sites of newly crosslinked PG [3]. Utilizing the method, we observed that newborn daughter cells primarily incorporated new PG at a single pole during elongation. Cell wall synthesis is then re-directed from the pole to the division plane [4]. At the division plane, the growth machinery incorporates and remodels PG that will become part of the new poles of the mother and daughter cells after division (Figure 5.1). Although the growth pattern of *A. tumefaciens* was established [1], the molecular mechanisms that drive polar PG biosynthesis during cell elongation and division remain unexplored.

Figure 5.1 *Model of the A. tumefaciens cell cycle.* Labeling of *A. tumefaciens* cells with FDAAs reveals the patterns of PG incorporation across the stages of the cell cycle. Here we present an updated model of the sites of PG incorporation based on the localization of cell wall enzymes. Blue represents PG incorporation at the growth pole, purple PG incorporation at the mid-cell, yellow at the sub pole of the new daughter cell, and orange at the sub pole of the old pole compartment.

Cell elongation in bacteria requires a multitude of essential and non-essential proteins to scaffold (MreB), synthesize (RodA, PBP2) and regulate (LpoA, LpoB) PG insertion during growth. In model rod-shaped bacteria like *E.coli* and *B. subtilis* these proteins constitute the “elongasome” or “rod” complex [5]. The rod complex functions to polymerize glycan strands and crosslink peptide stems. Of the various rod complex proteins, the bifunctional enzyme PBP1a was classically thought to be the major protein required to polymerize and crosslink PG during elongation. In recent years, single-molecule tracking studies and improved assays to biochemically measure PG crosslinking have demonstrated that the Glycosyltransferase (GTase) RodA forms a complex with the monofunctional Transpeptidase (TPase) PBP2 to direct PG biosynthesis by the Rod complex, without a requirement for PBP1a [6, 7]. Thus, the hypothesis that PBP1a activity is required for rod shape has given way to a newer hypothesis, where PBP1a instead synthesizes PG in a manner that is distinct from the rod complex [8].

In stark contrast to growth in well-studied model bacteria, species in the Rhizobiales lack homologs to the canonical, essential rod complex proteins. Interestingly, the Rhizobiales have retained a PBP1a homolog, and the gene for PBP1b has undergone an early duplication event (Chapter 1). In addition, there has been an obvious expansion of the number of genes encoding LD-transpeptidases (LDTs) [2]. Overall, gene loss and expansion of different subsets of cell wall synthase enzymes suggests that the Rhizobiales evolved a novel pathway for cell elongation. Indeed, in Chapter 3, we characterized PBP1a as an essential PG synthase required for polar growth and proper rod shape. We showed that fluorescent dipeptide cell wall probes (FDAADs) were primarily incorporated into the cell wall by the activity of PBP1a, and cells depleted of PBP1a were impaired for PG insertion at the growth pole. We also detected changes in the composition of PG in the absence of PBP1a, which suggested that LD-transpeptidase enzymes could partially compensate for the loss of PBP1a at the growth pole. Localization studies revealed that three LDTs localized to the growth pole during portions of the cell cycle, and two LDTs localized to the sub-polar region of the growth pole and old pole compartments (Chapter 3). Our findings open up a new line of questioning regarding what recruits and retains enzymes at the pole and sub-pole in the absence of known scaffolding proteins such as MreB. A recent study of *A. tumefaciens* identified GPR (Growth Polar Ring), as essential for rod-shape and polar growth, and homologs are found only within the Rhizobiales. GPR has two N-terminal transmembrane domains with a large, cytoplasmic apolipoprotein domain; the

Figure 5.2 Model of known proteins required for polar elongation. This study identified and characterized PBP1a and RgsM as essential proteins required for polar elongation in *A. tumefaciens*. There are 4 LDTs that may play an important role in polar growth based on their localization to the growth pole or sub pole of the new daughter cell. GPR was also recently characterized as essential for polar growth and may potentially be a scaffold to recruit and/or retain proteins at the pole. Arrows represent potential physical interactions that require future experimental validation.

topology of GPR is reminiscent of the polar landmark protein TipN from *Caulobacter crescentis*, and in *A. tumefaciens* GPR forms a ring-like structure at the growth pole [9, 10]. Future studies aimed at determining the role that GPR

plays in recruiting proteins to the growth pole may shed light on the mechanisms that the Rhizobiales use to scaffold proteins during polar elongation. Proteins required for polar growth in the Rhizobiales are just beginning to be identified and characterized. In this work we characterized two essential proteins, PBP1a and RgsM, required for polar PG synthesis and hydrolysis, respectively (Chapters 3, 4 and Figure 5.2). Recently, RgsM was shown to be essential for polar growth in *S. meliloti* [11], consistent with a conserved mechanism underlying polar growth in the Rhizobiales.

Another interesting avenue that remains to be explored is the diversification in function of PBP enzymes. Given that there are several classes of bifunctional PBPs, why has PBP1a specifically been co-opted to function during polar growth? Since we show that the other class aPBPs from *A. tumefaciens* and *S. meliloti* are dispensable for growth and proper rod-shape (Chapter 3), what role do these additional PBPs play in PG biosynthesis? Since many Rhizobiales can occupy more than one niche (Chapter 1), perhaps the class aPBPs function during growth in different environmental conditions. This is a likely hypothesis given that the *E. coli* aPBPs have been shown to have different pH optima to support bacteria growth in acidic or alkaline environments [12], and PBP2 of *C. crescentis* shifts its localization in response to changes in osmolality [13].

Exploring the connection between cell wall integrity, environmental stimuli, and bacterial behaviors

In gram-negative bacteria, the cell wall is sandwiched between an inner and outer membrane in a compartment called the periplasm. The bacterial cell wall provides a framework to support envelope-spanning structures such as pili, secretion systems and flagella, and protects bacteria from a myriad of environmental stressors including osmotic pressure and changes in pH [14].

Thus, bacterial cells need to be able to sense and respond to changes in their extracellular environment that may cause damage to the peptidoglycan.

However, relatively little is known about the signaling pathways that can sense the state of the bacterial cell wall. In Chapter 4, we use RNA-seq, genetic deletions and microscopy-based assays to show that the loss of GTase activity of PBP1a in *A. tumefaciens* triggers a temporal cascade of transcriptional changes that result in altered behavior. Specifically, it appears that depletion of PBP1a induces the RSI invasion switch, which is responsible for a lifestyle change from a free-living bacterium to plant-pathogen. The RSI switch is known to be triggered by exposure to acidic conditions in *A. tumefaciens*, and is characterized by down-regulation of motility, and up-regulation of T6SS and succinoglycan production (Figure 5.3). These findings provide a starting point for investigating the connection between changes in peptidoglycan composition and the signaling pathways that induce complex behaviors in the Rhizobiales. Many questions remain, including what is the signal that the bacterial cell senses upon loss of PBP1a? Could it be that loss of PBP1a GTase activity causes an imbalance of

PG synthesis and hydrolysis, thereby creating cell wall fragments that are detected by the cell? These and many other hypotheses will require future testing to fully elucidate the PG-sensing pathway of *A. tumefaciens* and how much this pathway may overlap with the existing RSI pathway.

Figure 5.3 *Model of behavioral changes that occur after exposure of A.*

tumefaciens to acidic conditions or upon depletion of PBP1a. Top represents free-living soil bacteria. Bottom represents the physiological and behavioral changes that occur when *A. tumefaciens* is exposed to acid conditions such as the mildly acidic environment of the plant rhizosphere.

Another interesting possibility is that *A. tumefaciens* promotes plant colonization by decreasing cell wall synthesis to halt growth and enter a form of dormancy. Indeed, in the plant symbiont *S. meliloti* growth and cell division are inhibited during differentiation into a plant-associated bacteroid [15]. The ability of *A. tumefaciens* and *S. meliloti* to adopt dramatically different lifestyles in response to environmental conditions by altering their morphology and behavior may be a common strategy in the Rhizobiales. Many Rhizobiales are known to have dimorphic lifestyles (Chapter 1). For example, in Chapter 2 we show that *P. hirschii* possess a short-stalked form and a long-stalked form. The long-stalked cells are a minority in the population and are non-motile. Could the long-stalked form of *P. hirschii* represent a persistent state that allows the bacteria to survive fluctuating environmental conditions? Uncovering the mechanisms that sense and respond to the cell wall to induce changes in behavior, lifestyle, or morphology changes is an interesting and currently unexplored avenue for future studies. Testing these hypotheses in *A. tumefaciens* will also shed light on similar strategies utilized by Rhizobiales with more complex lifestyles such as *P. hirschii* or human pathogens such as *Brucella* and *Bartonella*.

Mechanistic studies of cell growth in polar-growing bacteria are severely underrepresented in the field of bacterial cell biology compared to studies of lateral-growing bacteria. Especially considering that two phylogenetically distinct orders, the Rhizobiales and Actinobacteria, are known to grow by polar elongation, and representative members from these orders include important human pathogens like *Mycobacteria tuberculosis*, and important agents of biotechnology like *A. tumefaciens*. We expect that discoveries made in *A. tumefaciens* may reveal fundamental principles that underlie polar growth. In support of this idea, the cell wall of polar-growing bacteria from both the Rhizobiales and Actinobacteria have a high percentage of LDT-crosslinked PG compared to non-polar-growing species [1, 16]. The shared dependence on LDTs may represent a common strategy for polar growth, which could be exploited to develop novel antibiotics and therapeutics that act against polar-growing bacteria.

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APPENDIX A

Modeling the Emergence of Antibiotic Resistance in Bacterial Populations

Published as

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Abstract

Since antibiotics have become routinely used to treat infections, antibiotic resistance is now an emerging concern for public health. To understand how bacteria become resistant to antibiotics, many students draw from the common misconception that bacteria gain resistance upon antibiotic exposure. We have designed models and a corresponding lab that explores how a population of bacteria can evolve antibiotic resistance, with emphasis on dispelling common misconceptions surrounding the mechanism of antibiotic resistance. Using an antibiotic disk diffusion assay, students compare the antibiotic resistance level of a harmless *E. coli* strain of bacteria over time. Then, students compare their lab data to the models, which together illustrate the roles that initial genetic variation and random mutation play in the evolution of antibiotic resistance. In this guided investigation, basic microbiology concepts and techniques are made accessible to students in a high school classroom. The models developed here are in line with the practices of the *Next Generation Science Standards (NGSS)*. The models, together with the lab, are used to guide students through the process of argumentation using a claim, evidence, and reasoning (CER) format to explain the evolutionary mechanisms of antibiotic resistance.

Key Words: microbiology; evolution; modelling; antibiotic resistance; bacteria

Introduction

It is widely accepted that evolution is central to understanding all other aspects of biology [1]; however, teaching evolution can be conceptually challenging [2]. Thus, it is not surprising that a recent survey revealed that almost half of teachers are spending fewer than three class periods teaching key topics in evolutionary biology. Teachers cited a lack of good laboratory exercises and supplemental materials as a major obstacle to effective teaching of evolution [3-5]. Therefore, we have developed an investigation that illustrates the phenomenon of natural selection using the example of emergence of antibiotic resistance in a bacterial population. This investigation incorporates the *Next Generation Science Standards (NGSS)* high-leverage practices of scientific modeling and argumentation. Antibiotic resistance is often taught as a black box—students are taught that bacteria survive or die in the presence of an antibiotic; however, there is no discussion of the underlying mechanism(s) of antibiotic resistance. In this investigation, we highlight several mechanisms of antibiotic resistance that are easy for students to understand based on their prior knowledge of cell structure, function, and processes. We use this investigation to introduce a larger unit on natural selection taught in a tenth-grade honors biology course, and the students have already been introduced to the peppered moth example in middle school.

Bacteria are an attractive model organism for teaching evolution because natural selection can be observed on a short time scale, due to the rapid growth rate of bacteria. In addition, antibiotic resistance provides a contemporary example of

evolution that will promote student engagement. Despite being a hot topic in the media, a clear understanding of antibiotic resistance is severely lacking in the general population. A study conducted by the World Health Organization found that adults realize antibiotic resistance is a problem, but don't fully understand what causes it [6]. Although disc diffusion assays are widely used in lab exercises to classify antibiotic resistance, the bacteria are typically classified as sensitive or resistant. In this module, students observe antibiotic resistance as a trait that changes over time due to accumulation of random mutations. This analysis is coupled with introducing students to the mechanisms bacteria use to become antibiotic resistant. Completion of this lab module enables wide-ranging discussions of the socio-scientific issues surrounding antibiotic resistance including the overuse of antibiotics, agricultural practices, and the emergence of herbicide-resistant plants.

One challenge to teaching microbiology in a classroom setting is that microorganisms are invisible to the naked eye, which is a barrier to understanding bacteria-related concepts. For this reason, we have developed a set of two models that make bacterial replication visible to students. Students can use these models to help conceptualize microbiology and evolutionary concepts, and reason with the models to promote their understanding of the antibiotic resistance laboratory investigation. In using the scientific models to reason, students are engaged in the authentic practices of scientists. Evaluation of the models helps students rule out the needs-based model of evolution (Figure A.1,

Model 1), which is a common misconception and barrier to understanding evolution [7, 8]. As a final activity, students write an argument explaining which model is best supported by their data.

Figure A.1: Models of antibiotic resistance emerging in a population. In both models, the white circles depict a susceptible population of bacteria that is either grown overnight on a plate with antibiotics (top) or grown for several days in liquid and then grown overnight on a plate with antibiotics (bottom). Model 1

represents the needs-based acquisition of antibiotic resistance. Here, bacteria become antibiotic resistant (black circles) when they are exposed to the antibiotics. Model 2 illustrates the acquisition of antibiotic resistance through random mutation independent of antibiotic exposure. On day 1 the population will remain susceptible (white circles, top row) because mutations have not accumulated. If mutations leading to antibiotic resistance are acquired later during growth, the population may have an intermediate level of resistance (middle row). If antibiotic resistance is acquired early during growth (black circles, bottom row), the population will become resistant.

Here we present a laboratory module that provides teachers with additional tools to teach evolution, allows students to directly experience evolution in the context of microbiology, and actively engages students by drawing on current topics such as the emergence of superbugs.

Student Objectives

1. Identify prokaryotic cell structures and their functions.
2. Define antibiotics and selective toxicity.
3. Describe how antibiotics work to kill bacteria.
4. Explain mechanisms bacteria use to evade antibiotics.

5. Identify aspects of natural selection, specifically random mutation resulting in genetic variation, selective pressure, and differential survival and reproduction.
6. Evaluate the merits of two models to explain the results of the investigation.
7. Explain how and why bacteria become resistant to antibiotics.
8. Demonstrate the following microbiology laboratory skills: sterile technique, culturing bacteria, and antibiotic disk diffusion assay.

Alignment with Next Generation Science Standards (NGSS)

This lab module highlights two high-leverage NGSS science practices, scientific modeling and argumentation, and includes the practices of carrying out an investigation, and analyzing and interpreting data. The modeling activity aligns with the NGSS modeling learning progression for grades 9–12, which states that students should be able to “evaluate merits and limitations of two different models of the same proposed tool, process, mechanism or system to select or revise a model that best fits the evidence or design criteria” [9](NGSS Lead States, 2013, App. F, p. 6). In the past, concrete scale models such as DNA models and fluid mosaic membrane models [10] have been used to make molecular and cellular structures visible to students. In these examples, students do not view a model as a tool to generate new knowledge, but *as a means of showing others what the phenomenon looks like* [11]. Thus, we wanted to

expand our use of models from concrete scale models to “generative tools for explaining and prediction” [11]. The models developed here (Figure A.1) are used as tools to help students think and reason about evolutionary phenomena in the context of microbiology. At the end of the module, students write an argument, using a claim, evidence, and reasoning (CER) format [12], defending their choice of Model 1 or Model 2 as best explaining why bacteria acquire antibiotic resistance. The argumentation activity aligns with the NGSS argumentation learning progression for grades 9–12, which states students should be able to “construct, use, and/or present an oral and written argument or counter-argument based on data and evidence” (NGSS Lead States, 2013, App. F, p. 13).

Introducing the Investigation

Prior to the laboratory investigation, we show a 9-minute video clip from the Frontline video, “Hunting the Nightmare Bacteria,” which introduces the issue of methicillin-resistant *Staphylococcus aureus* (MRSA) through the story of an adolescent girl who has a MRSA infection [13]. We elicit students’ prior knowledge and experiences with MRSA and explain that we will be investigating antibiotic resistance using *Escherichia coli*, a harmless rod-shaped bacterium that colonizes the guts of living organisms, in place of the dangerous MRSA (PowerPoint slides 1–3). At the start of the module, students are introduced to several key concepts, beginning with a background on antibiotics, bacterial cell

structures, and the mode of action of nalidixic acid (Student Handout 1, PowerPoint slides 4–14). Students are then given instructions for the lab exercise (Student Handout 2), and the lab exercise is started. Prior to completing the lab exercise, collecting data, and analyzing results, students are introduced to mechanisms used by bacteria to gain antibiotic resistance (Student Handout 3, PowerPoint slides 15–29).

Details of the Lab Module

The module is carried out over five class periods; days 1, 3, and 5 are student workdays, and days 2 and 4 require minimal work from only the teacher (Table A.1, Figure A.2). Students work in small groups of 2 or 3 to complete the tasks involved in this lab module. During day 1, students resuspend a colony of bacteria in growth media. They use this cell suspension to swab an agar plate with a lawn of bacteria and start a liquid culture of bacteria. Finally, the students place two disks on the plate: a blank disk and an antibiotic disk. An overview of this procedure is illustrated in Figure A.2A, and the step-by-step procedure is provided in Student Handout 2, page 1. The plates are incubated at 37°C for one day, then moved to the refrigerator (4°C). The day 1 student lab portion can be completed in approximately 45 minutes. Next, students are introduced to how the antibiotic disk diffusion assay works and to key terms in antibiotic resistance such as “susceptible,” “intermediate,” and “resistant” (PowerPoint slides 16–22). Students make predictions for where bacterial growth will occur if a bacterium is

resistant or susceptible to the antibiotic, to illustrate their understanding of the disk diffusion assay and the expected results (Student Handout 2, p. 3). On day 2 of the lab, the teacher subcultures the student's test tube of bacteria by transferring 100 μ L of *E. coli* from the student's tube into a new test tube with fresh media and places the tube in the shaking incubator (Figure A.2B). Student plates are removed from the incubator and placed in the refrigerator (Table A.1). On day 3 the students swab a new plate using the bacterial culture that originated from their initial colony, and again place a blank and antibiotic disk on the plate (Figure A.2C). The plates are incubated at 37°C overnight and then placed in the refrigerator. Day 3 of the student lab portion can be completed in approximately 20–30 minutes. After completing the lab exercise, students are introduced to the mechanisms of antibiotic resistance (PowerPoint slides 24–29) and are challenged to draw models explaining how MRSA can become resistant to different classes of antibiotics (Student Handout 3). On the final day of the lab, students record and analyze their data. Students measure the zone of inhibition (ZOI) surrounding the antibiotic disk for the bacteria plated on day 1 and day 3. Students are provided with detailed instructions on how to measure the ZOI, and a table to record their data (Student Handout 2, p. 5, and PowerPoint slides 30–31). The entire class contributes their data to a classroom data set, which is used to interpret and evaluate the two models. The students will discuss the models in small groups (PowerPoint slides 30–33), and use information from the entire lab module to fill out the CER sheet to answer the question, “How and why do antibiotics become useless?” (PowerPoint slides 34–37, and Guide for

Instructors, p. 6). As described, this lab was completed for a class that met on a Monday-Wednesday-Friday schedule; however, this lab can readily accommodate other schedules (see Guide for Instructors). For example, the subculture step on day 2 can be continued over multiple days and the plates from days 2 and 4 can be stored in the cold for more than one day before data collection and analysis.

	In Class Exercise	Supplemental Material
Pre-Lab Day 1	<ul style="list-style-type: none"> Show MRSA video, introduction to antibiotics and prokaryotic cell structure. 	<ul style="list-style-type: none"> Student Handout 1 PowerPoint slides 1–14
Day 1 Students	<ul style="list-style-type: none"> Students carry out day 1 of the lab exercise. Discuss the lab setup and make predictions. 	<ul style="list-style-type: none"> Student Handout 2, pages 1 and 3 PowerPoint slides 15–22
Day 2 Teachers	<ul style="list-style-type: none"> Wrap student plates from day 1 and store in the fridge. Subculture students' <i>E. coli</i> cultures. 	<ul style="list-style-type: none"> Student Handout 2, page 2 Guide for Instructors, page 3
Day 3 Students	<ul style="list-style-type: none"> Students carry out day 3 of the lab exercise. Discuss bacterial resistance strategies. 	<ul style="list-style-type: none"> Student Handout 2, page 2 Student Handout 3 PowerPoint slides 23–29
Day 4 Teachers	<ul style="list-style-type: none"> Wrap student plates from day 3 and store in the fridge. 	<ul style="list-style-type: none"> Guide for Instructors, page 3
Day 5 Students	<ul style="list-style-type: none"> Data analysis: students observe and record data from their day 1 and day 3 plates. Compile all class data. Discuss models. Students work on their CER arguments supporting either Model 1 or 2. 	<ul style="list-style-type: none"> Student Handout 2, pages 4–7 PowerPoint slides 30–33 PowerPoint slides 34–37 Guide for Instructors, page 6

Table A.1 *Timeline of lab exercises and corresponding supplemental material*

Figure A.2: Overview of the lab module.

Discussing the Models

Our use of prepared models was inspired by the work by HmeloSilver, Duncan, and Chinn [14, 15], who scaffold students' scientific modeling by giving them a

set of models to reason with, some of which represent commonly held misconceptions. The two models created for this module are shown in Figure A.1. Both models illustrate a starting population of susceptible bacteria that gain antibiotic resistance. Model 1 illustrates needs-based acquisition of antibiotic resistance. Here, the bacteria rapidly gain antibiotic resistance only when exposed to the antibiotic. This model addresses a common misconception that bacteria gain resistance because of antibiotic exposure. Model 2 illustrates acquisition of antibiotic resistance through mutation. Here, the bacteria acquire mutations over time that increase their level of resistance to the antibiotic independent of antibiotic exposure. If antibiotic resistance is acquired early during growth, the population will become resistant. If antibiotic resistance is acquired later during growth, the population may have an intermediate level of resistance. Using their data, students are asked to consider the fit of each model with the class data. Based on the observation that the starting bacterial populations are susceptible to the antibiotic, despite the antibiotic exposure, students should recognize that Model 1 is inaccurate. Once students reject Model 1, they are challenged to use their data to support Model 2. Since the ZOI indicates that the bacterium is susceptible on day 1, but intermediate or resistant on day 3, students recognize that their data supports Model 2. We allotted time on the final lab day for students to compare individual data to class data and discuss the models in small groups. The time spent considering their data and the models allows students to recognize that not all the individuals in a population are the same and that a population can gain various random

mutations over time. Finally, students are asked to use the CER approach [12] to outline their argument about which model best explains how and why antibiotics become useless (Student Handout 2, pp. 6–7). Students are expected to draw evidence not only from the data acquired in the lab exercise, but also from the introductory material presented throughout the lab module, including that random mutagenesis is required for antibiotic resistance to emerge. Students should argue that both the type and timing of mutation can influence the level of antibiotic resistance. An example of evidence and argument based on student answers related to data from the lab component is provided in the Guide for Instructors (see supplemental materials). We recommend the teacher resource, *Scientific Argumentation in Biology: 30 Classroom Activities* [16] for additional support in teaching the NGSS practice of argumentation.

Assessment of the Laboratory Module

This laboratory experience was carried out with nearly 45 pairs of tenth-grade honors biology students across eight classes. Sample student plates are shown in Figure A.3. Students measured their ZOI and used that number to classify the bacteria as susceptible, intermediate, or resistant per Table A.2. The resistance classifications laid out in Table A.2 were empirically determined for the conditions tested in this lab exercise and don't necessarily reflect actual numbers used in a clinical setting. Varying the media, temperature, bacteria, or other conditions may change the ZOI classifications. We pooled the data taken from all classes and

calculated the average ZOI to be 31.5 mm on day 1 (Table A.2). This indicates that on average, the population of bacteria on day 1 were susceptible to nalidixic acid. Indeed, nearly 93 percent of student pairs had a ZOI that fell into the susceptible range on day 1 (Table A.2). Using the class data provides an opportunity to discuss the initial genetic variation within the starting bacterial population. For example, the ZOI ranged from 26 to 44 mm for the susceptible bacteria on day 1 (Table 2). The diameters of the ZOI observed on day 3 were smaller than those observed on day 1. On average, the diameter of the ZOI was reduced by 10.7 mm. All the student pairs (~93%) that began with susceptible bacteria observed a shift in the classification of their bacteria to either intermediate (~32%) or resistant (61%) (Table A.2). Overall, this lab module was easy to execute and provided valuable data to help students learn how antibiotic resistance emerges through mutagenesis.

	Day 1	Day 3
Susceptible: ≥ 26 mm	92.9%	7.1%
Intermediate: 25–23 mm	5.4%	32.1%
Resistant: ≤ 22 mm	1.8%	60.7%
Average ZOI	31.5 mm	20.8 mm

Table A.2 *Resistance classification (%) and average ZOI (mm) measurements of compiled student data*

Troubleshooting the Laboratory Module

In implementing this lab module in eight classes, we observed some common problems that were readily corrected with clearer instruction. In this lab, it is ideal

to create an even bacterial lawn and appropriately space the disks (see Figure A.3A for well-executed sample student plates). In some instances, we observed that the bacterial growth on the day 1 plates was spotted instead of even (Figure A.3B, Day 1); however, the edge of the zone was still visible, and students could easily measure the ZOI. In contrast, if the procedure for swabbing the plate was not followed correctly, then the bacterial growth occurred in streaks, which made it harder for proper ZOI measurement (Figure A.3C, Day 1). The best procedure for measuring the ZOI was to draw a line through the center of the disk, mark the edges of the zone, and then measure along that line (as illustrated in Figure A.3B). In some cases, students placed the disk too close to the edge of the plate (Figure A.3C, Day 3). In this case, students can measure the radius of the ZOI and multiply by 2 to get the diameter. Other issues that were infrequently observed included lack of a bacterial lawn, disks that fell off the plate, and contamination. To help prevent or address these issues, we have prepared a section devoted to troubleshooting in the Guide for Instructors.

Figure A.3: *Representative plates from day 1 and day 3 of the lab exercise.*

Supplemental Material

All supplemental materials in this text can be accessed here: <http://ri2.missouri.edu/content/Supplemental-Online-Materials>. Supplemental materials include student handouts 1–3, a bank of PowerPoint slides, and the Guide for Instructors as referenced in the text. The PowerPoint slide bank also contains

additional, optional materials for using a CER format to explain why antibiotics become useless (slides 34–37), for explaining how antibiotic resistance genes on plasmids make antibiotic resistance worse via lateral gene transfer (slides 38–42), and includes videos that demonstrate emergence of antibiotic resistance (slide 43) and summarize key issues regarding antibiotic resistance (slide 44). The Guide for Instructors contains four parts including: materials list, tips for running the laboratory exercise, troubleshooting the laboratory exercise, and an example of how to fill out the CER sheet.

Conclusion

Through this lab module, students practice basic microbiology lab techniques and learn key microbiology concepts such as bacterial cell structures and their functions and the mode of action of antibiotics. Students use *NGSS* scientific practices of carrying out an investigation and analyzing data. This laboratory investigation of antibiotic resistance provides an opportunity for teachers to incorporate the high-leverage *NGSS* practices of scientific modeling and argumentation into high school biology classes. A variety of factors can hamper student understanding of microbiology and evolutionary phenomenon, including: a lack of familiarity with the phenomenon, complicated lab procedures, an abstractness in which the students cannot see what's happening (in this case, individual bacteria are too small to be seen with the naked eye), and/or common misconceptions associated with the phenomenon. In developing this lab and the

corresponding models, we included a model that illustrated the common misconception that evolution occurs based on need. Once students considered the needs-based model, they quickly rejected it. By asking students to evaluate the alternative model, we expand their understanding of models, shifting from viewing models only as concrete representations to viewing models as tools for reasoning and predicting biological phenomena. Finally, this lab engages students by applying key concepts in evolutionary biology to a current socio-science topic, leading to discussion of the emergence of antibiotic-resistant bacteria and how we can combat these superbugs.

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VITA

I was born in Kansas City, Missouri to parents Mary and Wallace Williams and grew up with my sister Rachelle. I attended North Kansas City High School from which I graduated with an International Baccalaureate (IB) Diploma in the top 10% of my class. I attended college at Missouri State University (MSU) with a Governor's Scholarship for full tuition. During college, Microbiology was my favorite class, and after working in the Microbiology Prep room across the hall from the research labs for a semester I was sure that I wanted to pursue undergraduate research. I joined the lab of Dr. Kyoungtae Kim as an undergraduate researcher investigating the intracellular trafficking pathway of the dynamin-related protein Vps1. Following graduation from MSU Magna Cum Laude in the Honor's College, with a Bachelor of Science in Biology area of emphasis in Microbiology and Biotechnology and a minor in chemistry, I remained at MSU and decided to pursue an accelerated Master's degree in Dr. Kim's lab. Managing an undergraduate course load and research simultaneously was challenging but solidified my love for research and I applied to PhD programs. I was accepted into the PhD graduate program at Mizzou in the department of biological sciences with a Life Sciences Fellowship that has allowed me to be fully funded for four years of my program. I rotated and landed in the lab of Dr. Pam Brown. During my time at Mizzou, I have dedicated myself to service and outreach by serving as a Center for Inclusion, Diversity, and Equity (IDE) Ambassador, Science on Wheels Outreach Speaker, Alternative

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Additionally, I was a teaching assistant for Introduction to Biological Systems, and a graduate assistant for General Microbiology. Following completion of my dissertation, I have accepted a position as a post-doctoral researcher in the lab of Dr. Marie Elliot at McMaster University in Hamilton, Ontario where I will investigate the mechanisms of exploratory growth in *Streptomyces* and other Actinobacteria. After completion of my post-doc, I plan on pursuing a career in Academia.