EXPLORING THE DIVERSE FUNCTIONS OF PEPTIDOGLYCAN HYDROLASES IN
THE PLANT PATHOGEN, *AGROBACTERIUM TUMEFACIENS*

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EXPLORING THE DIVERSE FUNCTIONS OF PEPTIDOGLYCAN HYDROLASES IN
THE PLANT PATHOGEN, *AGROBACTERIUM TUMEFACIENS*

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EXPLORING THE DIVERSE FUNCTIONS OF PEPTIDOGLYCAN HYDROLASES IN THE PLANT PATHOGEN, *AGROBACTERIUM TUMEFACIENS*

Wanda Figueroa-Cuilan

Dr. Pamela Brown, Dissertation Advisor

Abstract

The plant pathogen *Agrobacterium tumefaciens* displays an atypical form of unipolar elongation, followed by incipient pole synthesis during cell division and cell separation. Currently, how polar growing bacteria modulate cell wall hydrolysis during growth and division remains largely unknown. This work includes the comprehensive analysis and characterization of the role of cell wall hydrolases involved in bacterial growth, division, recycling and beta-lactam resistance in *A. tumefaciens*. First, we performed bioinformatic analyses and used reverse genetics to better understand the role cell wall hydrolases in *A. tumefaciens*. Inactivation of most cell wall hydrolases, led to no phenotypic defects suggesting a high degree of redundancy. However, inactivation of the amidase, AmiD, and the lytic transglycosylase Atu3779, revealed significant changes in beta-lactam resistance suggesting that these proteins are involved in the activation beta-lactamases and outer-membrane integrity. Next, we developed a tool (Figueroa-Cuilan *et al.*, 2016) to dissect the role of essential genes, which enabled characterization of the essential regulator of cell division, DipM, a LytM-containing

factor. Absence of DipM causes severe cell division defects, including increased cell length, mid-cell width and lysis. A cell wall composition analysis of cells devoid of DipM shows an increase in the activity of the PG hydrolases, lytic transglycosylases, suggesting that DipM may inhibit the activity of these enzymes. Lastly, we find that deletion of individual lytic transglycosylases (LTs) from the DipM depletion strain delays the onset of the DipM depletion phenotype. Overall, this research provides mechanistic insights about the roles of peptidoglycan hydrolases and their regulators in cell growth and division. Understanding how bacterial cell wall hydrolysis is spatiotemporally regulated and coordinated with cell wall synthesis and cell division (Figueroa-Cuilan and Brown, 2018), will be applicable to other closely related polar-growing bacteria.

CHAPTER 1

Cell Wall Biogenesis During Elongation and Division in the Plant Pathogen

Agrobacterium tumefaciens

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ABSTRACT

A great diversity of bacterial cell shapes can be found in nature, suggesting that cell wall biogenesis is regulated both spatially and temporally. Although *Agrobacterium tumefaciens* has a rod-shaped morphology, the mechanisms underlying cell growth are strikingly different than other well studied rod-shaped bacteria including *Escherichia coli*. Technological advances such as the ability to deplete essential genes and the development of fluorescent D-amino acids have enabled recent advances in our understanding of cell wall biogenesis during cell elongation and division of *Agrobacterium tumefaciens*. In this review, we address how the field has evolved over the years by providing a historical overview of cell elongation and division in rod-shaped bacteria. Next, we summarize the current understanding of cell growth and cell division processes in *A. tumefaciens*. Finally, we highlight the need for further research to answer key questions related to the regulation of cell wall biogenesis in *A. tumefaciens*.

INTRODUCTION

Hidden in the microscopic world, a great diversity of bacterial morphologies can be found. From simple rods and spheres, to very complex star- and helical-shaped bacteria, the mechanisms by which these fascinating organisms achieve, maintain, or evolve cell shapes are currently under investigation. The bacterial peptidoglycan (PG) cell wall is one of the fundamental determinants of cell shape and research has revealed that carefully regulated enzymatic processes are used to achieve and maintain a characteristic morphology. Thus, the constant

targeting of biosynthetic machineries and remodeling enzymes to specific subcellular locations ensure that a specific morphology can be maintained for many generations. The bacterial cell wall biosynthetic machinery is comprised of high molecular weight penicillin-binding proteins (HMW PBPs) (Typas et al. 2011) and glycosyltransferases such as shape, elongation, division, and sporulation proteins (SEDS) (Meeske et al. 2016). A second subset of enzymes including endopeptidases, carboxypeptidases, and L,D-transpeptidases (LDTs) are involved in remodeling of the PG (Typas et al. 2011). Finally, amidases and lytic transglycosylases (LTGs) are required for the hydrolysis of the PG mesh enabling cell separation (van Heijenoort 2011; Uehara and Bernhardt 2011). Accordingly, PBPs, SEDS proteins, remodeling enzymes and autolysins work in concert to continuously modify and expand the PG, allowing cell elongation and division to proceed. In recent years, technological advances such as the development of genetic tools (Morton and Fuqua 2012; Figueroa-Cuilan et al. 2016; Grangeon et al. 2017) and use of fluorescent D-amino acids (Kuru et al. 2012; Kuru et al. 2015; Siegrist et al. 2015; Howell et al. 2017b) have contributed to our understanding of cell growth patterning and the underlying mechanisms in diverse bacteria, including *Agrobacterium tumefaciens*.

A. tumefaciens is a rod-shaped, gram-negative soil bacterium, and the causative agent of crown gall disease in flowering plants (Escobar and Dandekar 2003; Nester 2014). Studies using amine-reactive dyes, non-canonical D-amino acids, and fluorescent D-amino acids have demonstrated that *A. tumefaciens* and

other members of the order Rhizobiales exhibit unipolar elongation (Brown et al. 2012; Kuru et al. 2012). In contrast, in *E. coli*, a classical model for studies of bacterial cell growth and division, PG insertion is dispersed along the existing lateral cell wall during elongation (Cava et al. 2013).

CELL GROWTH AND DIVISION OF ROD-SHAPED BACTERIA: A HISTORICAL PERSPECTIVE

Here, we provide a historical perspective of the advances in our understanding of cell growth and division of rod-shaped bacteria including *E. coli*, *A. tumefaciens*, and *Rhizobium meliloti*. These early studies laid the groundwork for more recent studies which highlight key mechanistic differences in cell elongation among rod-shaped bacteria.

In *E. coli*, disruption of chromosome replication or cell division causes cells to elongate dramatically. Gamma irradiation (Lea et al. 1937), ultraviolet irradiation (Barner and Cohen 1956) and treatments with antibiotics that cause DNA damage or target components of DNA replication machinery such as mitomycin C and nalidixic acid (Latch and Margolin 1997; Kilgore and Greenberg 1961; Helmstetter and Pierucci 1968; Goss et al. 1964) cause *E. coli* cells to form long, smooth filaments (Figure 1-1A:1-2). Similarly, treatment with antibiotics, including cephalixin and carbenicillin, that inhibit mid-cell PG biogenesis also causes *E. coli* cells to become filamentous (Figure 1-1A:3-4)

Figure 1-1. Morphological changes induced by inhibition of cell division.

Treatments include DNA damaging agents (1-1.1, 1-1.2) and beta-lactam antibiotics (1-1.3, 1-1.4). Cell division is also blocked in temperature sensitive (TS) mutants (1-1.5) when incubated at high temperatures. (A) In *E. coli*, when cell division is inhibited, a filamentous phenotype is consistently observed. (B) In the Rhizobiales, phenotypes induced by cell division blocks are more variable and induced branching, bulging, elongated and spherical cells. Schematics depicted in this figure show the impact of these treatments as observed in the

indicated references (Latch and Margolin 1997; Zupan et al. 2013; Fujiwara and Fukui 1972; Fujiwara and Fukui 1974a; Fujiwara and Fukui 1974b; Van De Putte et al. 1964, Kilgore and Greenberg 1961; Helmstetter and Pierucci 1968, Goss et al 1964).

(Latch and Margolin 1997; Rolinson 1980; Zupan et al. 2013). In addition, numerous temperature-sensitive (TS) with filamentous growth at elevated temperature were isolated (Figure 1-1A:5) (Van De Putte et al. 1964). Mapping of genes with mutations responsible for cell filamentation led to the identification of the *fts* (filamentous growth is thermosensitive) genes (Ricard and Hirota 1973; Lutkenhaus and Donachie 1979; Lutkenhaus et al. 1980). *fts* genes encode proteins belonging to a core complex of highly conserved proteins, termed the divisome, which functions in cell division (See section 1-4).

The first indication that *A. tumefaciens* may have a different growth pattern than *E. coli* emerged when isolation of TS mutants with a block in cell division failed to produce filamentous cells (Figure 1-1B:5)(Fujiwara and Fukui 1972). Among 17 temperature-sensitive mutants which do not divide at elevated temperatures, three exhibited an atypical branched morphology, while the remaining mutants exhibited a spherical morphology (Figure 1-1B:5). In the branching mutants, cell growth occurred exclusively at one pole of a cell and branches were formed from splitting of the growth-active poles (Fujiwara and Fukui 1974b). While the growth mode of individual wild-type cells could not be deduced, the pattern of microcolony formation led to the hypothesis that *A. tumefaciens* growth occurs at a single pole (Fujiwara and Fukui 1974b). These results were supported by the observation that the mitomycin C and nalidixic acid block cell division and cause branching of *R. meliloti* and *A. tumefaciens* cells (Figure 1-1B:1-2) (Latch and Margolin 1997; Fujiwara and Fukui 1974a).

Furthermore, treating *R. meliloti* and *A. tumefaciens* with cephalixin or carbenicillin also causes a branched morphology to emerge (Figure 1B:3-4) (Latch and Margolin 1997; Zupan et al. 2013). Despite the compelling evidence that blocking cell division causes branching, consistent with a model of polar elongation, these observations remained largely unrecognized. Indeed, Bergey's Manual of Systematic Bacteriology indicates that *Agrobacterium* does not reproduce by budding at one pole of the cell (Kuykendall 2005). More recently, the growth pattern of bacteria belonging to the order Rhizobiales, including *A. tumefaciens*, has been described as unipolar elongation (Figure 1-2) (Brown et al. 2012; Kuru et al. 2012).

CELL ELONGATION IN A. TUMEFACIENS

In rod-shaped bacteria, cell growth is defined as the insertion of peptidoglycan (PG) resulting in the elongation of the cell. In Gram-negative bacteria, the PG cell wall is located in the periplasmic space between the outer and inner membranes (Fig 1-3a). The PG cell wall is a net-like structure that aids bacteria to withstand osmotic pressure conferred by the environment and is comprised of a polysaccharide containing alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) sugars. The NAM sugar is decorated with a pentapeptide stem, composed of the following D-amino acids: L-Ala, D-Glu, D-DAP-L, D-Ala, D-Ala (Figure 1-3). The pentapeptide stem enables NAG-NAM disaccharides to be cross-linked to one another through peptide bridges (Figure 1-3). Although *A. tumefaciens* PG resembles the canonical structure of PG for a

Figure 1-2 Schematic and fluorescence images illustrating the *A. tumefaciens* growth pattern.

The growth pattern of *A. tumefaciens* includes three stages: 1) polar growth during cell elongation, 2) a transition when polar growth terminates and mid-cell PG synthesis is initiated, and 3) mid-cell PG synthesis during cell division. A) The schematic indicates areas of active PG synthesis (showed in cyan) throughout the cell cycle. B) Fluorescence images of individual *A. tumefaciens* cells following a short pulse label with a fluorescent D-amino acid (HADA) reveals sites of PG synthesis through the cell cycle. These images highlight the three different stages of PG synthesis.

Figure 1-3 Crosslinking reactions catalyzed by D,D-transpeptidases and L,D-transpeptidases of *A. tumefaciens*.

Peptidoglycan (PG) crosslinking is necessary for the stability of the PG. (A) A representative D,D-transpeptidation reaction is illustrated. A monomeric donor with 5 peptides in the stem (M5) is crosslinked to a monomeric acceptor with 4

peptides in the stem (M4). This transpeptidation reaction produces a dimeric mucopeptide (D44). The catalysis of D,D-transpeptidation reactions is performed by bifunctional and monofunctional PBPs. (B) L,D-transpeptidation reactions involve the crosslinkage of an M4 donor and M4 acceptor producing a dimeric mucopeptide (D34). L,D-transpeptidation reactions are catalyzed by L,D-transpeptidases (LDTs). In *A. tumefaciens*, three different subfamilies of LDTs can be found: YcbB, YbiS and YafK. With the exception of Atu2764, the proteins belonging to the YbiS subfamily are specific to the Rhizobiales.

Gram-negative bacterium (Erbs et al. 2008), a number of distinctive features were revealed by compositional analysis of the PG (Brown et al. 2012). These features include an increased abundance of overall mucopeptide cross-linkage and an enrichment in L,D-Dap-Dap cross-links formed by LDTs. Interestingly, increased abundance of L,D- cross-linkages is a common feature among polar growing bacteria such as *Mycobacterium* (Lavollay et al. 2008; Lavollay et al. 2011), indicating that LDTs may play an important role during polar elongation. Another feature of *A. tumefaciens* PG is the absence of detectable anhydromuropeptides. Anhydromuropeptides occupy the terminal position in glycan strands of PG from Gram-negative bacteria, and thus the abundance of anhydromuropeptides provides an indirect measure of average glycan strand length. This inability to detect anhydromuropeptides in *A. tumefaciens* PG could be explained by: (1) modification of anhydromuropeptides masking their detection, (2) an unusually long glycan strand length, or (3) a novel mechanism for the glycan strand termination. Together, these observations suggest that the composition of PG in *A. tumefaciens* differs from that of other well-studied Gram-negative bacteria such as *E. coli*.

In this section, we first highlight the unipolar growth pattern during elongation of *A. tumefaciens*. Next, potential mechanisms for targeting PG biosynthesis to the growth pole and the role of enzymes in polar synthesis of PG are discussed. Finally, we consider how PG biosynthesis is terminated at the

growth pole, allowing establishment of a new growth zone near mid-cell for cell division.

A. tumefaciens Elongates Unipolarly

The growth of several rod-shaped bacteria is characterized by insertion of new PG alongside the lateral walls of the cell, a process mediated in part by the actin-like scaffolding protein MreB (for review, see (Errington 2015)). MreB interacts with multiple components of the cell wall machinery and functions as a regulator to maintain proper rod-cell shape during cell elongation. Remarkably, the genomes of representative members of the order Rhizobiales lack the core components of the elongasome, the protein complex responsible for insertion of new PG into the existing wall material during cell elongation, including MreB (Errington 2015; Margolin 2009). This observation, coupled with the branched morphologies resulting from treatment of *A. tumefaciens* with DNA damaging agents or antibiotics which inhibit mid-cell PG biosynthesis (Figure 1-1B), provides further evidence for an alternative mode of growth during cell elongation. To compare the growth pattern of *A. tumefaciens* and *E. coli*, cells were treated with the amine-reactive dye Texas Red-X succinimidyl ester (TRSE) to label the outer membrane proteins. During dispersed growth of *E. coli* cells in the absence of TRSE, incorporation of new unlabeled material along the lateral walls of the cell results in dilution of the TRSE signal. In contrast, the TRSE signal remains fixed in the cell body and old pole compartment of elongating *A. tumefaciens* because growth occurs at the new cell pole (Brown et al. 2012).

Similar growth patterns were observed using methods that directly label the peptidoglycan, including pulse-chase labeling of PG with D-cysteine (Brown et al. 2012) and short pulse labeling of PG with fluorescent D-amino acids (FDAAs) (Figure 1-2) (Kuru et al. 2012). Together, these experiments led to the elucidation of the growth patterning of *A. tumefaciens*, which consists of three main growth stages: (1) polar growth during cell elongation, (2) termination of polar growth and initiation of mid-cell PG synthesis, and (3) PG insertion at mid-cell to promote cell division (Figure 1-2) (Cameron et al. 2015; Howell and Brown 2016; Kysela et al. 2013; Yang et al. 2016). During elongation, the PG biosynthesis machinery is strictly targeted to the new cell pole. The cells continue to grow unipolarly for about a third of a cell cycle by addition of new PG at the cell pole, displacing older PG. Next, termination of polar growth coincides with recruitment of FtsZ to mid-cell and subsequent PG insertion at mid-cell. Insertion of PG at mid-cell continues until the septum is formed and the daughter cells separate. Whereas the growth pattern of *A. tumefaciens* has been clearly established we are just beginning to understand the mechanisms underlying unipolar elongation.

How Is the PG Synthesis Machinery Targeted to a Cell Pole During Elongation?

In *A. tumefaciens*, the strict polar targeting of PG biosynthesis machinery during cell elongation suggests that an underlying mechanism restricts growth to one pole. How can protein complexes be targeted to polar locations within bacterial cells? Several polar targeting principles have been described, including the ability of proteins to recognize negative membrane curvature and the accumulation of

polymer-forming proteins in DNA-free regions such as poles (Laloux and Jacobs-Wagner 2014). Polymer-forming landmark proteins function to recruit additional proteins to the pole, enabling polar protein complexes to form. For example, a polar hub established through interactions with the polar organizing protein PopZ enables proper chromosome segregation in *Caulobacter crescentus* (Bowman et al. 2008; Ebersbach et al. 2008; Bowman et al. 2010) and DivIVA is necessary for polar growth in *Actinobacteria* (Flärdh 2003; Kang et al. 2008; Letek et al. 2009; Fuchino et al. 2013; Hempel et al. 2008). Thus, it is likely that establishment of a polar protein complex to regulate polar growth in *A. tumefaciens* may also rely on a landmark protein. In *A. tumefaciens*, only two candidate polar landmark proteins, PopZ and PodJ have been characterized. Surprisingly, neither PopZ nor PodJ are strictly required for polar growth although both proteins contribute to the transition from polar growth to mid-cell growth (see section 3.4) (Grangeon et al. 2015; Howell et al. 2017a; Ehrle et al. 2017; Grangeon et al. 2017; Anderson-Furgeson et al. 2016). Thus, dissection of the molecular composition of *A. tumefaciens* growth poles will be necessary to determine whether novel polar organizing proteins are present and able to target the PG biosynthetic machinery to the new pole.

An alternative mechanism of polar targeting that may contribute to regulation of PG biosynthesis during elongation in *A. tumefaciens* is inheritance from the division site (Laloux and Jacobs-Wagner 2014). When rod-shaped bacteria undergo cell division, each newborn cell inherits a new pole formed

during cell division. Thus, proteins localizing to mid-cell during cell division will be present at the new pole of the new daughter cells. The absence of the canonical rod-shaped cell elongation machinery in *A. tumefaciens* (Brown et al. 2012; Cameron et al. 2014) indicates that the conserved cell division machinery may influence where new growth resumes after cell division. In *A. tumefaciens*, the cell division proteins FtsZ and FtsA display polar localization during elongation and then localize to mid-cell before cell division (Brown et al. 2012; Zupan et al. 2013; Grangeon et al. 2015; Howell et al. 2017a). The importance of the FtsZ and FtsA foci at the new cell pole during cell elongation is still largely unknown; however, the absence of FtsZ causes tip splitting to occur at growth poles, suggesting that FtsZ may be required for proper termination of polar growth (Howell and Brown 2016). It remains to be determined whether any of the divisome proteins that are retained at the new poles following cell division function in polar growth.

PG Biosynthesis at a Pole: Candidate Enzymes Contributing to Cell Wall Expansion in A. tumefaciens

The enzymes contributing to PG biosynthesis are well-conserved across bacteria and are likely to contribute to polar PG biosynthesis in *A. tumefaciens* (Cameron et al. 2014; Howell and Brown 2016). The first group of enzymes consists of the high molecular weight penicillin binding proteins (HMW-PBPs) (Figure 1-3A)(Scheffers and Pinho 2005; Egan et al. 2015; Sauvage et al. 2008; Typas et al. 2011). HMW-PBPs are subdivided into two groups: bifunctional PBPs, which

possess transglycosylase and DD-transpeptidase activities (PBP1), and monofunctional PBPs, which have DD-transpeptidase activity only (PBP2, PBP3). The transglycosylase activity of PBPs is responsible for the addition of the nascent NAG-NAM disaccharide to the existing glycan strand while the DD-transpeptidase links adjacent peptide stems. A representative peptide bond formed by DD-transpeptidase activity occurs between the D-Ala at the fourth position of the peptide stem (M5 donor) and the D-stereogenic center of DAP at the third position of the peptide stem (M4 acceptor) in two adjacent monomeric muropeptides to form a dimeric muropeptide (Figure 1-3A). In addition to PBPs, another enzyme that may be contributing to the PG biosynthesis in *A. tumefaciens* is the monofunctional peptidoglycan glycosyltransferase, MtgA, which is responsible for elongation of glycan strands during cell division in *E. coli* (Derouaux et al. 2008).

The genome of *A. tumefaciens* contains genes predicted to encode six HMW-PBPs; four bifunctional (PBP1a, PBP1b1, PBP1b2, and PBP1c), and two monofunctional (PBP3a and PBP3b) (Figure 1-3A) (Cameron et al. 2014). *A. tumefaciens* PBP1a localizes to the growth pole during most of the cell cycle (Cameron et al. 2014) and is essential for cell survival (Curtis and Brun 2014), suggesting that PBP1a may have an important function during polar elongation. In contrast, PBP3a and PBP3b localize at mid-cell during cell division (Cameron et al. 2014). PBP3a is essential for viability (Curtis and Brun 2014) whereas no changes in growth rates or phenotype were observed in the absence of PBP3b

(Cameron et al. 2014). These results suggest that PBP3a may be a major contributor to PG biosynthesis during cell division; however, other PBPs may also function during cell division because labeling PBPs with a fluorescent penicillin derivative, Bocillin-FL (Zhao et al. 1999), results in a strong mid-cell signal (Cameron et al. 2014). Furthermore, treatment of WT cells with carbenicillin, a penicillin derivative that blocks the DD-transpeptidase activity of PBPs, leads to mid-cell bulges with no obvious effects on the cell poles (Figure 1-1B:4)(Zupan et al. 2013). Together, these results indicate that PBP1a may contribute to polar growth, whereas PBP3a and possibly other PBPs are involved in septal PG synthesis during cell division. There are many open questions regarding the activities of HMW-PBPs in *A. tumefaciens*. If PBP1a functions in polar elongation, how its activity is restricted to the new pole during elongation and how its activity is terminated prior to cell division? In *E. coli*, the activity of PBP1A and PBP1B is dependent on cognate lipoproteins LpoA and LpoB, respectively (Paradis-Bleau et al. 2010; Typas et al. 2010). Although Lpo-like proteins are not present in the genome of *A. tumefaciens*, the presence of a domain of unknown function between the enzymatic domains of PBP1a suggests that its activity may be regulated. Are both PBP3a and PBP3b core components of the division machinery? What is the function of the remaining HMW-PBPs? As answers to these questions emerge, we expect to gain insights into the molecular mechanism underlying the cell growth pattern.

The next set of enzymes hypothesized to contribute to the polar elongation of *A. tumefaciens* are the L,D-Transpeptidases (LDTs) (Cameron et al. 2014; Howell and Brown 2016; Brown et al. 2012). LDTs are penicillin-insensitive enzymes involved in the catalysis of direct cross-links between two D-DAP-L peptides at the third position of adjacent mucopeptide stems (Figure 1-3B). Mucopeptide composition analyses indicate that *A. tumefaciens* PG is enriched in mucopeptides containing LD-cross-links (Brown et al. 2012). PG from other polar growing bacteria such as *Mycobacterium* also contain mucopeptides with a high percentage (60-80%) of LD-cross-links, suggesting an important role of LDTs in polar growth (Lavollay et al. 2008; Lavollay et al. 2011). Moreover, a loss of several LDTs in *Mycobacterium tuberculosis* leads to defects in cell morphology, such as cell rounding (Sanders et al. 2014), and β -lactam antibiotic sensitivity (Gupta et al. 2010). Consistent with the high abundance of LD-cross-links in the PG, the genome of *A. tumefaciens* reveals an enrichment in genes encoding LDT enzymes (Cameron et al. 2014). Genes encoding a total of 14 candidate LDTs have been identified in the *A. tumefaciens* genome. Interestingly, the YbiS subfamily of LDTs consists of seven Rhizobiales-specific LDTs and one commonly found in other bacterial classes. The remaining six LDTs belong to two subfamilies called YcbB and YafK (Figure 1-3B). Two of the Rhizobiales-specific LDTs (ATU0845, ATU0669) localize to the growth pole during most of the cell cycle (Cameron et al. 2014). These results indicate that LDTs may play a crucial, and perhaps essential, role during polar growth. More studies will be required to dissect the precise role and functional redundancy of LDTs in *A. tumefaciens*.

RELEASE FROM THE POLE: TRANSITION FROM POLAR GROWTH TO MID-CELL GROWTH

Before termination of polar elongation, a transition of growth from the new cell pole to mid-cell occurs (Figure 1-2). During this transition, the septal PG biosynthetic machinery is recruited to mid-cell to form the septum of the incipient daughter cells. In *A. tumefaciens*, so far three proteins have been found to contribute to this transition. First, the polar organizing protein (PopZ) is a polymer-forming landmark protein that serves as a hub for polar proteins. In *C. crescentus*, PopZ is required to tether the chromosomal origin to the cell pole and for assembly of proteins required for stalk development (Bowman et al. 2008; Ebersbach et al. 2008; Bowman et al. 2010; Bowman et al. 2013; Laloux and Jacobs-Wagner 2013; Holmes et al. 2016). In *A. tumefaciens*, PopZ strictly localizes to the new cell pole during cell elongation and then to mid-cell during late stages of cell division (Grangeon et al. 2015). The absence of PopZ results in cell division defects such as ectopic pole formation, tip splitting, bulging, and formation of small cells that lack DNA (Howell et al. 2017a; Grangeon et al. 2017). The chromosome partitioning protein, ParB, and cell division proteins, FtsZ and FtsA, are mislocalized in cells lacking PopZ (Howell et al. 2017a; Ehrle et al. 2017). Together, these observations suggest that PopZ participates in multiple processes including chromosome segregation and the transition from polar growth to mid-cell PG biosynthesis during cell division. It remains to be determined whether PopZ has a direct role in regulation of PG biosynthesis at

the growth pole or during cell division. Alternatively, the observed defects in cell division could be secondary to the defect in chromosome segregation caused by the absence of PopZ.

Another candidate polymer-forming landmark protein is PodJ. In *C. crescentus*, PodJ is located at the old pole, recruits cell cycle regulators to the pole, and enables development of polar structures including the flagellum and pili (Hinz et al. 2003; Viollier et al. 2002). In *A. tumefaciens*, PodJ initially localizes to the old pole but slowly accumulates at the new pole indicating a possible role in the transition from a new, growth-active pole to an old, growth-inactive pole. (Grangeon et al. 2015). The absence of PodJ causes the formation of elongated cells with multiple constrictions and branching, suggesting that polar growth is not terminated efficiently leading to subsequent defects in cell division (Anderson-Furgeson et al. 2016). Remarkably, FtsZ and FtsA have atypical localization patterns and FtsZ-rings and FtsA-rings often form at failed sites of cell division in the absence of PodJ. These data suggest that PodJ contributes to the transitioning of PG biosynthesis machinery from the cell pole to mid-cell. One possibility is that the transition of the growth active pole to a growth-inactive pole mediated by PodJ is coordinated with onset of PG biosynthesis at mid-cell for cell division. Further mechanistic studies will be necessary to determine why the sites of cell division that are established in the absence of PodJ often fail to septate.

Because proper establishment of future sites of cell division appears to be important for the transition of polar growth to mid-cell PG biosynthesis, it is logical to consider the role of cell division proteins in these processes. Both FtsZ and FtsA remain at the pole for a portion of the cell cycle before localizing near mid-cell to mark the future site of cell division (Brown et al. 2012; Cameron et al. 2014; Zupan et al. 2013). FtsZ-depleted cells exhibit a complete block of cell division leading to cell branching, multipolar elongation, and tip splitting (Howell and Brown 2016). This observation suggests that FtsZ is required not only for the establishment of PG biosynthesis at mid-cell, but also for the termination of polar PG biosynthesis. While it is clear that the absence of FtsZ prevents the transition of the growth-active pole to a growth-inactive pole, additional studies are needed to determine the precise functions of FtsZ and the proteins it recruits to mid-cell during the inactivation of the growth pole.

CELL DIVISION IN A. TUMEFACIENS

The onset of cell division is characterized by a burst of septal PG synthesis at mid-cell (Figure 1-2). Remarkably, septal growth must be regulated and the PG must be remodeled to generate the incipient bacterial cell poles. Indeed, precise coordination of these processes depends on the divisome, a multiprotein complex dedicated to orchestrating cell division. The divisome consists of over 30 proteins, which are highly conserved among bacteria (Haeusser and Margolin 2016). Placement of FtsZ at mid-cell leads to the recruitment and assembly of the other cell division proteins to the site of cell division. In rod-shaped bacteria

such as *E. coli* and *Bacillus subtilis*, FtsZ and its membrane anchor FtsA move the septal PG biosynthesis machinery around the circumference of the site of cell division using a treadmilling motion (Bisson-Filho et al. 2017; Yang et al. 2017).

Here, we speculate on the mechanism of cell division in *A. tumefaciens* using the linear hierarchical model for cell division of *E. coli* (Du and Lutkenhaus 2017) as a reference. We also discuss of what little is known about the function of the proteins contributing to the cell division of *A. tumefaciens* and highlight questions for future work.

Establishment of the FtsZ-Ring

The typical assembly pathway for the divisome is comprised of two stages: establishment of the Z-ring and recruitment of late cell division proteins for PG biosynthesis (Du and Lutkenhaus 2017). During the first stage, GTP-dependent interactions between FtsZ monomers allow the formation of FtsZ protofilaments at mid-cell, where the concentration of proteins that inhibit FtsZ-polymer formation is the lowest (Oliva et al. 2004; Rowlett and Margolin 2013; Wu and Errington 2011). FtsA and ZipA are recruited to mid-cell where they bind the conserved C-terminal peptide (CTP) domain of FtsZ to anchor it to the inner membrane, allowing the Z-ring to coalesce at mid-cell (Pichoff and Lutkenhaus 2005; Haney et al. 2001). FtsA is also required for the recruitment and regulation of other downstream divisome proteins such as FtsN (Pichoff et al. 2015; Liu et al. 2015). FtsEX are the final core divisome proteins recruited during the first

stage. They regulate key cell wall events such as PG septal synthesis and hydrolysis (Yang et al. 2011) and stimulate the FtsA-mediated recruitment of downstream divisome proteins to mid-cell (Du et al. 2016).

The predicted divisome of *A. tumefaciens* consists of the putative early proteins FtsZ, FtsA and FtsEX, but lacks the FtsZ membrane anchor ZipA (Figure 1-4A-B) (Cameron et al. 2014). Remarkably, the genome of *A. tumefaciens* contains three homologs of *ftsZ* (Atu2086, Atu4673 and Atu4215). Atu2086 (hereafter termed FtsZ) is syntenic with conserved genes that encode essential members of the cell division machinery, including FtsA (Atu2087) and FtsQ (Atu2088) (Cameron et al. 2014), suggesting that FtsZ encodes the major cell division scaffolding protein of *A. tumefaciens*. FtsZ is a tubulin homolog composed of a short N-terminal domain of unknown function, a GTPase domain necessary for the hydrolysis of GTP, a C-terminal linker (CTL) which influences the membrane tethering of FtsZ (Zupan et al. 2013). The role of the two polymer structure and dynamics, and a conserved C-terminal peptide (CTP) required for additional homologs of FtsZ in *A. tumefaciens* has not been determined; however, one of these copies lacks both the CTL and CTP domains and the other is truncated in the GTPase domain (Zupan et al. 2013), suggesting that they may have distinct functions from FtsZ. Indeed, a saturating transposon mutagenesis screen suggests that only FtsZ is essential for viability (Curtis and Brun 2014). FtsZ localizes at the new pole during elongation and at mid-cell during cell

Figure 1-4. Predicted divisome components of *A. tumefaciens*.

(A) Predicted temporal order of assembly of candidate proteins involved in cell division and cell separation in *A. tumefaciens*. (1) FtsZ monomers migrate to mid-cell to form a Z-ring at the future site of cell division. (2) Recruitment of early divisome proteins such as FtsA and FtsEX to mid-cell. At this time, the Z-ring is tethered to the inner membrane by membrane-associated proteins such as FtsA, FzIA, or FzIC. (3) The late divisome proteins (FtsQB, FtsI, and FtsN) are recruited to mid-cell leading to the maturation of the divisome and activation

septal PG biosynthesis. (4) PG hydrolases and their regulators are recruited to mid-cell to hydrolyze septal PG. (5) Finally, cell separation is completed upon the action of PG hydrolases. The presence of a question mark (?) indicates uncertainty in the localization or function of the protein during cell division. (B) Predicted spatial organization of the mature core *A. tumefaciens* divisome. Essentiality of the core divisome proteins is shown: ^Eindicates essential for survival, ^Uindicates unresolved essentiality, and the absence of superscript indicates that the protein is not essential (Curtis and Brun 2014).

division (Brown et al. 2012; Zupan et al. 2013; Grangeon et al. 2015; Cameron et al. 2015; Howell et al. 2017a). Moreover, recruitment of FtsZ to mid-cell is coordinated with the initiation of constriction at the future site of cell division (Brown et al. 2012). Time-lapse microscopy of FtsZ-depleted cells exhibit gross morphological defects such as branching, multipolar elongation, and tip splitting, suggesting that FtsZ is essential for cell division, viability, and establishment of cell shape and length (Howell and Brown 2016). Overall, these results suggest that FtsZ is the major cell division scaffolding protein necessary for the recruitment of the divisome to mid-cell in *A. tumefaciens* (Figure 1-4A).

In *A. tumefaciens*, localization studies place FtsZ at the site for cell division before the arrival of FtsA to mid-cell (Grangeon et al. 2015; Cameron et al. 2014) (Figure 1-4A), indicating that FtsA may not be the only anchor of FtsZ. One candidate FtsZ anchor is FzlC. In *C. crescentus*, FzlC_{CC} localizes to the incipient cell division site with the early wave of cell division proteins (Goley et al. 2010) and interacts with the CTP domain of FtsZ to anchor it to the inner membrane (Meier et al. 2016). In *A. tumefaciens*, FzlC_{AT} is present (Cameron et al. 2014) and shares 56% similarity with FzlC^{CC} suggesting that FzlC_{AT} may anchor FtsZ at mid-cell.

Another candidate for tethering FtsZ is FzlA, a putative glutathione S-transferase (GST) protein. In *C. crescentus*, FzlA_{CC} is an essential protein that localizes to mid-cell in an FtsZ-dependent manner, directly interacts with FtsZ *in vitro*, and regulates FtsZ protofilament curvature (Lariviere et al. 2018; Goley et

al. 2011). In *A. tumefaciens*, FzIA_{AT} is predicted to be an essential protein (Curtis and Brun 2014), and shares 67% similarity to FzIA_{CC}, suggesting that FzIA_{AT} could interact with FtsZ. The final candidate for the tethering of FtsZ to the membrane is FtsE. FtsE is part of the early wave of divisome proteins and interacts with FtsZ (Huang et al. 2013; Goley et al. 2011). In addition to possible roles in stabilization of the FtsZ rings, FtsA and FtsEX likely contribute to the proper regulation of PG biosynthesis at mid-cell; however, the contributions of these proteins to cell division have not yet been explored in *A. tumefaciens*.

Divisome Maturation and Initiation of Septal PG Biosynthesis

The second stage of divisome assembly involves the recruitment of late cell division proteins to regulate PG biosynthesis at mid-cell. In *E. coli*, the proteins sequentially recruited to mid-cell are: FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, and FtsN (Du and Lutkenhaus 2017). FtsK has multiple functions, including the transport and decatenation of DNA, and the localization of FtsQLB to mid-cell (Massey et al. 2006). FtsQ, FtsL and FtsB are membrane proteins that serve as scaffolds for other divisome proteins (Gonzalez et al. 2010). The complex formed by FtsQLB is activated by divisome maturation and regulates PG biosynthesis by modulating the activities of FtsI and FtsW (Tsang and Bernhardt 2015). The precise mechanism by which FtsQLB is activated is unknown, but it is hypothesized that FtsN may trigger the activation of the complex. Next, FtsW, a polytopic membrane protein with glycosyltransferase activity, is recruited to mid-cell and subsequently recruits of FtsI (PBP3) to mid-cell (Cho et al. 2016).

Together, FtsW and FtsI are major contributors to PG biosynthesis at mid-cell. Lastly, FtsN is the last essential divisome protein recruited to mid-cell (Weiss 2015). The arrival of FtsN to mid-cell signals the completion of divisome maturation and septal PG biosynthesis is initiated. FtsN also recruits enzymes involved in the hydrolysis of septal PG to mid-cell enabling pole remodeling and cell separation (Weiss 2015).

The late cell division proteins predicted to be encoded in the *A. tumefaciens* genome include two copies of FtsK, FtsQ, FtsB, FtsW, two copies of FtsI (PBP3a and PBP3b) and FtsN (Figure 1-4A-B) (Cameron et al. 2014). Neither FtsK homolog is predicted to be essential (Curtis and Brun 2014) although the potential for redundancy means that one or both copies of FtsK could play important role in chromosome segregation, cell division, or both processes. The functions of the FtsK proteins have not yet been explored in *A. tumefaciens*. The FtsQLB complex plays an important role in cell division of *E. coli* (Gonzalez et al. 2010; Tsang and Bernhardt 2015); however, in *A. tumefaciens* only FtsQ is predicted to be essential and a homolog of FtsL cannot be readily identified in the genome (Cameron et al. 2014; Curtis and Brun 2014). Thus, it will be of interest to determine if FtsQ and FtsB form a complex important for recruitment of other divisome proteins and contribute to regulation of septal PG biosynthesis. PBP3a (ATU2100) and PBP3b (ATU1067) localize to mid-cell, consistent with a role in cell division (Cameron et al. 2014) and PBP3a is essential for cell survival (Curtis and Brun 2014). A deletion strain for PBP3b was

constructed, but no phenotypic changes were observed (Cameron et al. 2014). Together, these results suggest that in *A. tumefaciens*, PBP3a is the major septal biosynthesis PBP. The predicted FtsN in *A. tumefaciens* has an extended N-terminal region giving rise to an atypical protein topology when compared to *E. coli* FtsN. The N-terminus is predicted to be periplasmic, followed by a cytoplasmic loop, and a periplasmic C-terminus (Figure 1-4B). Only the C-terminal region (amino acids 675-1008) of the *A. tumefaciens* FtsN, which contains a 3-helix region, a glutamine-rich linker, and a SPOR domain, shares similarity with the *E. coli* FtsN. In *E. coli*, the cytoplasmic tail of FtsN interacts with FtsA, the 3-helix region may interact with the FtsQLB complex, and the SPOR domain binds PG and is important for recruiting FtsN to mid-cell (Weiss 2015). Although the function of FtsN is unknown in *A. tumefaciens*, it is exciting to consider how the altered protein structure may alter the functions and regulatory abilities of FtsN during cell division and cell separation.

Pole Remodeling and Cell Separation

In *E. coli*, the cell separation process is mediated by PG hydrolases including lytic transglycosylases (LTGs) and amidases. LTGs are enzymes that cleave β -1,4 glycosidic linkages in the glycan strands, leading to the formation of terminating sugars with 1,6-anhydromuramic acid rings. The degradation of the PG by LTGs initiates the early steps in cell-wall recycling as the product of LTG activity is internalized and converted to lipid II in the cytoplasm (Johnson et al. 2013; Park and Uehara 2008; Vollmer et al. 2008). Most bacteria possess

multiple LTGs and inactivation of all seven in *E. coli* is not tolerated, suggesting that at least some of the enzymes are redundant and have critical functions (Heidrich et al. 2002). *A. tumefaciens* has at least five LTGs (Cameron et al. 2014), and individual LTGs are not required for cell viability (Curtis and Brun 2014); however the function of LTGs in cell division and peptidoglycan recycling remains to be explored.

Amidases are hydrolytic enzymes that specifically cleave the amide bond that links the NAM sugar with the peptide stem (van Heijenoort 2011). LytM-containing proteins typically function as endopeptidases; however, in *E. coli* catalytically-inactive LytM proteins (dLytM) function as regulators of amidase activity. dLytMs are recruited to mid-cell by FtsN and FtsEX and activate amidases (Peters et al. 2011; Yang et al. 2011; Uehara et al. 2009; Uehara et al. 2010). In *E. coli*, a block of septal PG synthesis using cephalixin affects the localization of amidases but not dLytM factors, suggesting that ongoing PG synthesis may be required to modulate amidase activity (Peters et al. 2011). *A. tumefaciens* contains a single LytC-type amidase (AmiC) and two dLytM proteins (EnvC and DipM) (Cameron et al. 2014) (Figure 1-4). The prediction that AmiC will function during septum cleavage and its activity is regulated by DipM remains to be tested experimentally. In addition, genes encoding the targets of EnvC regulation, AmiA and AmiB, are not present in the *A. tumefaciens* genome suggesting that EnvC may regulate other enzymes involved in cell separation. During the final step of cell separation, the Tol-Pal complex constricts the outer

membrane as the septum is cleaved (van Heijenoort 2011; Egan 2018). The Tol-Pal system connects the bacterial outer membrane, periplasm, and cytoplasm through a series of protein-protein interactions. During cell division, this system is thought to provide the energy required for constricting the outer membrane, as septum synthesis and hydrolysis shape the new daughter cell poles (Egan 2018). In *A. tumefaciens*, the Tol-Pal complex is present (Cameron et al. 2014)(Figure 1-4B) and likely essential for cell viability (Curtis and Brun 2014) suggesting that these proteins may have an important function in outer membrane constriction and cell separation. Whereas the presence of genes encoding PG biosynthetic enzymes, PG hydrolases, and putative regulators allow a preliminary model of the *A. tumefaciens* divisome to emerge (Figure 1-4B), the functions of these proteins need to be resolved to paint a clear picture of the processes of mid-cell PG biosynthesis, septum cleavage, pole remodeling, and cell separation in *A. tumefaciens*.

CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

Although the mechanisms by which polar growth and cell division take place in *A. tumefaciens* are largely unknown, significant advances in the field have recently been accomplished. *A. tumefaciens* has become a model organism for the study of polar growth and the development of new genetic tools to deplete essential proteins in *A. tumefaciens* (Figuerola-Cuilan et al. 2016; Grangeon et al. 2017) should enable mechanistic studies in the future. These genetic tools coupled with advances in PG compositional analysis (Cava and de Pedro 2014) and the ability

to visualize sites of PG synthesis (Kuru et al. 2012; Kuru et al. 2015), provide the resources needed to tackle outstanding questions about the spatial and temporal regulation of PG biosynthesis in *A. tumefaciens*. Just a few of the many remaining questions are summarized below in hopes of encouraging future investigations to improve our understanding of mechanisms underlying the coordination of polar growth and cell division in *A. tumefaciens*.

First, is the PG biosynthesis machinery conserved among polar growing bacteria? Characteristic features have been identified among polar growing bacteria, including an increased abundance of overall mucopeptide cross-linking and an enrichment in of LD-crosslinks (Brown et al. 2012). These observations suggest that the PG biosynthesis machinery of polar growing bacteria may require a conserved subset of enzymes to direct polar elongation. Second, what proteins are required for polar elongation? In *A. tumefaciens*, the predicted elongasome complex is only composed of PBP1a, a bifunctional PBP which is predicted to be essential (Curtis and Brun 2014) and has a polar localization pattern (Cameron et al. 2014). Whereas PBPs are expected to be important contributors to polar PG biosynthesis, other enzymes are also likely contributors. Consistent with the increased abundance of LD-cross-links, the genomes of polar growing bacteria are enriched in genes which are predicted to encode LDTs. Interestingly, a subfamily of LDTs, YbiS, is Rhizobiales-specific and two members of this subfamily of LDTs exhibit polar localization (Cameron et al. 2014); however, the contribution of LDTs to polar elongation is still unknown.

Furthermore, additional proteins are likely necessary either to target the enzymes to the growth pole or restrict their activity to the growth pole. Third, how does FtsZ find the middle in *A. tumefaciens*? In *E. coli*, FtsZ negative regulators prevent the polymerization of FtsZ at the cell poles and over the nucleoid, allowing the Z-ring to form only at mid-cell. In bacteria, two well-known systems prevent FtsZ polymerization: the Min system and Nucleoid Occlusion (NO) (Rowlett and Margolin 2013; Wu and Errington 2011). In *A. tumefaciens*, only the Min system is readily identifiable in the genome; however, the Min system is predicted to be dispensable for viability (Curtis and Brun 2014), and absence of the Min system does not cause significant cell division defects (Flores et al. 2018), suggesting that another mechanism must contribute to proper FtsZ positioning. In addition to understanding how FtsZ is properly positioned, it is necessary to understand how the placement of FtsZ at mid-cell is coordinated with the termination of polar growth. Fourth, how is the divisome assembled in *A. tumefaciens*? To begin to address this question, it will be necessary to determine how FtsZ is tethered to the membrane at mid-cell. FtsA arrives at mid-cell after FtsZ (Cameron et al. 2014; Grangeon et al. 2015), indicating that FtsA may not be the only anchor of FtsZ. Other candidates for tethering FtsZ to the membrane include FzIA and FzIC. Next, it will be necessary to confirm and identify the components of the divisome and characterize the contribution of each protein component to septum formation at mid-cell. Finally, how is the activity of autolysins, including amidases and LTGs, coordinated to enable pole remodeling and cell separation? The use of new and existing genetic and biochemical tools

for *A. tumefaciens* should help answer these questions and will reveal how essential processes including DNA replication, PG biosynthesis, chromosome segregation, cell division, and cell separation are coordinated during the cell cycle.

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CHAPTER 2

Mini-Tn7 Insertion in an Artificial *att*Tn7 Site Enables Depletion of the Essential Master Regulator CtrA in the Phytopathogen *Agrobacterium tumefaciens*

Author Contributions

WFC, JD, AS and MH conducted the experiments. WFC, JD, MH and PB designed the experiments. WFC, JD and PB analyzed data and WFC and PB wrote and edited the paper.

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ABSTRACT

Mechanistic studies of many processes in *Agrobacterium tumefaciens* have been hampered by a lack of genetic tools for characterization of essential genes. In this study, we use a *tn7*-based method for inducible control of transcription from an engineered site on the chromosome. We demonstrate that this method enables tighter control of inducible promoters than plasmid-based systems and can be used for depletion studies. This method enables construction of depletion strains to characterize the role of essential genes in *A. tumefaciens*. Here, we use this strategy to deplete the alphaproteobacterial master regulator, CtrA, and find that depletion of this essential gene results in a dramatic rounding of cells that become nonviable.

IMPORTANCE

Agrobacterium tumefaciens is a bacterial plant pathogen and natural genetic engineer. Thus, studies of essential processes including cell cycle progression, DNA replication and segregation, cell growth and division may provide insights for limiting disease or improving biotechnology applications.

INTRODUCTION

Members of the genus *Agrobacterium* are common soil-dwelling bacteria. Most are saprophytic and survive primarily by consuming decaying organic material; but some cause phytopathic diseases, including *Agrobacterium rhizogenes* (hairy root disease), *Agrobacterium rubi* (cane gall disease), *Agrobacterium vitis* (crown

gall of grape) and *Agrobacterium tumefaciens* (crown gall disease). During infection, *A. tumefaciens* transfers a portion of its DNA (T-DNA) to plants resulting in the formation of crown galls in susceptible flowering plants. Infected plants form tumors that limit transport of water and nutrients, leading to decreased vigor and crop yield (1, 2). Because of the ubiquity of *A. tumefaciens* and its ability to evade plant defenses, crown gall disease continues to be a problem, with documented economic impact on fruit and nut trees, particularly in nurseries (2). Thus *A. tumefaciens* has become a model for the study of host-pathogen interactions (3-5) and processes related to pathogenicity including type IV secretion (6), quorum sensing (7), and biofilm formation (8). Finally, mechanistic studies of *A. tumefaciens*-mediated DNA transfer into host cells have been harnessed for the genetic engineering of plants, fungi, and mammals (5, 9-11).

While the processes related to phytopathogenicity have been subject to intense scrutiny, processes essential for *A. tumefaciens* survival in the environment, including both the rhizosphere and in association with hosts are poorly understood. Recent studies have shown that *A. tumefaciens* cells exhibit a striking pattern of polar cell growth (12-14) leading to a vast array of experimental questions (15). Proper spatial and temporal regulation of the cell cycle must be required in order to coordinate key processes such as chromosome replication and segregation, cell wall biogenesis, and cell division. Understanding the mechanisms underlying these essential processes will provide key insights into

the basic biology of *A. tumefaciens* that can be exploited to limit pathogenesis or promote genetic engineering.

A. tumefaciens is a genetically tractable bacterium and current methods exist for the construction of transposon mutants and deletion of non-essential genes (16). A plasmid-based complementation system using a reengineered *lac* promoter is available and has been shown to provide tight regulation of target genes (17). Despite rigorous efforts, depletion of predicted essential genes of *A. tumefaciens* using these vectors have failed suggesting that some proteins are expressed at biologically relevant levels even when repressed. Thus, a limitation of the molecular approaches currently available is the inability to construct depletion mutants for the characterization of essential genes. Here, we report the construction of vectors that integrate into a single engineered site on the *A. tumefaciens* chromosome, enabling single copy gene complementation and depletion studies. Advantages of the chromosomal insertion system include bypassing the need for continued antibiotic selection, tighter regulation, and single-copy complementation.

Transposon Tn7-based plasmid systems have been described and used for single copy integration in many bacterial species (18-23). The Tn7 transposon integrates at high frequency and specificity at the *attTn7* site downstream of the *glmS* gene, which encodes an essential glucosamine-fructose-6-phosphate aminotransferase (18, 24). Many bacteria contain a single *glmS* gene and therefore a single *attTn7* site for insertion of the Tn7 element, which is presumed to be a neutral site for insertion. We find that *A. tumefaciens* has a native *attTn7*

site; however, this site is not neutral for insertion. Thus, we have engineered a strain with a second artificial *attTn7* site (*a-attTn7*) and show that Tn7 insertion is highly biased toward *a-attTn7*. In this work we illustrate that mini-Tn7 vectors can serve as a valuable molecular tool for *A. tumefaciens*, enabling single copy gene complementation of complex phenotypes such as biofilm formation and motility. The degree of complementation and tightness of regulation can be tuned using different promoters on the mini-Tn7 vectors. In this work, we utilize two isopropyl β -D-1-thio-galactopyranoside (IPTG) inducible promoters, *lac* and *tac*. The *lac* promoter from *Escherichia coli* provides tight regulation and a relatively low level of expression (17, 25), whereas the hybrid *tac* promoter (derived from the *lacUV5* and *trp* promoters) results in approximately 5-10x stronger expression (18, 26, 27). We find that the single-copy expression of GFP driven from the *lac* or *tac* promoter is dependent on the presence of inducer. Furthermore, we can rapidly deplete GFP from *A. tumefaciens* cells following the removal of inducer.

Using the mini-Tn7 system in our engineered *A. tumefaciens* strain, we have depleted an essential gene in *A. tumefaciens*. Based on evidence from a saturating transposon mutagenesis screen (28) and an inability to construct a deletion strain (29), we hypothesized that the master regulator protein CtrA is essential for *A. tumefaciens* cell survival. We introduced *ctrA* under the control of the *tac* promoter into the *a-attTn7* and subsequently deleted the native *ctrA* locus. In the presence of inducer, the cells produce CtrA, are viable and exhibit normal rod-shaped morphology. In the absence of inducer, the cells are depleted of CtrA, non-viable, and have a swollen round shape. Our work has validated the

utility of the mini-Tn7 system for the construction of *A. tumefaciens* depletion mutants.

RESULTS AND DISCUSSION

Construction of *A. tumefaciens* with an *a-attTn7* enables neutral insertion of miniTn7 vectors. We have engineered a Tn7-based method for inducible control of transcription from an engineered site on the chromosome of *A. tumefaciens* (Figure 2-1). Transposon Tn7-based plasmid systems have been described and used for single copy gene integration in many bacterial species (18-23, 32). In most bacterial species, a single neutral site of Tn7 insertion (*attTn7*) is present in an intergenic region downstream of the *glmS* gene (18). We attempted to use the Tn7-based system in *A. tumefaciens*; however, the native *attTn7* is not neutral (Figure 2-1A). In *A. tumefaciens*, the *glmS* gene overlaps with the adjacent gene (Figure 2-1A) and insertion of a mini Tn7 vector into this operon causes reduced transformation efficiency, small colonies, and slow growth. Thus, we engineered an artificial *attTn7* site (*a-attTn7*) at a cryptic tetracycline locus in the *A. tumefaciens* genome (Figure 2-1B) using a standard allelic exchange protocol (16). Replacement of the *tetRA* locus with *a-attTn7* and subsequent integration of a Tn7 cassette do not impact growth, motility, or biofilm formation of *A. tumefaciens* (Figure 2-1C; Supplemental Figure 2-1), suggesting that our engineered site is neutral. Indeed, the engineered strain contains two *attTn7* sites, but PCR-based assays for transposon insertion at both sites reveals that insertion is strongly biased toward the engineered site with over 97% (72/74)

Figure 2-1. Construction of an *a-attTn7* site in *A. tumefaciens*.

A. The native *att* site (underlined in red) is located at the 3' end of the *glmS* gene (Atu1786) in *A. tumefaciens*. Insertion of Tn7 constructs into the native *att* site is not neutral, presumably due to the overlap of the *glmS* stop codon (red bar) with the start codon (blue bar) of the downstream gene encoding an acetyltransferase (AT; Atu1785). B. The entire boxed sequence containing the *att* site in panel A was introduced into a cryptic tetracycline locus on the *A. tumefaciens*

chromosome. Allelic exchange was used to replace the cryptic tetracycline locus (*tetRA*; Atu4205-4206) with the artificial attachment site (*a-attTn7*). The mini *Tn7* cassette contains an antibiotic resistance gene, the *lacI* repressor, and either a strong (P_{tac}) or weak (P_{lac}) promoter to drive a gene of interest (GOI). C. Replacement of the *tetRA* locus with *a-attTn7* (black dashed line) and subsequent insertion of a mini *Tn7* cassette (grayline) do not impact cell growth in ATGN compared to the wildtype parental strain (C58; solid black line).

integration events detected only at the *a-attTn7* site.

Single copy chromosomal complementation of complex phenotypes is tighter than plasmid-based complementation. Single copy chromosomal complementation provides several advantages in the complementation of complex phenotypes. First, genes inserted in the chromosome are stably maintained in the absence of selection, enabling complementation studies in the absence of antibiotics which may slow cell growth. Second, the genes are present in single copy, reducing the possible gene dosage effect that can occur when multicopy plasmids are used. Third, the degree of leakiness (expression in the absence of inducer) should be reduced in single copy. Finally, the level of expression from multicopy plasmids using *lac* promoter derivatives under inducing conditions varies in individual cells (33), and chromosomal insertion reduces the genetic noise. Here, we compare single-copy chromosomal complementation and multi-copy plasmid-based complementation with genes under control of the same promoter in the restoration of two complex phenotypes, motility and biofilm formation.

In the Rhizobiales, a regulatory hierarchy controls the ordered assembly of the basal body, the hook, and the filament to produce functional flagella and drive motility (34-37). Rem (regulator of exponential growth motility) activates the flagellar genes and is required for assembly of the flagella and swimming. In order to assay for motility, swim ring diameters of the wild-type, engineered strain, Δrem , and complementation strains were measured after 5 days on ATGN

soft agar (0.3%) (Figure 2-2). In *A. tumefaciens*, the Δrem strain retains only ~10% of the motility when compared to the wildtype or *a-attTn7* strains (Figure 2-2B). When a single copy of *rem*-HA is introduced at the *a-attTn7* site under the control of the *lac* promoter, partial complementation is observed; motility is restored to ~30% in the presence of IPTG (Figure 2-2B). Notably, in the absence of inducer, motility is not restored indicating that the partial complementation requires the presence of the inducer (Figure 2-2B). Plasmid-based complementation of Δrem in the presence of IPTG restores motility to about ~75% of the wildtype strain; however, partial complementation to ~40% occurs in the absence of IPTG (Figure 2-2C). Taken together, these results indicate that single-copy complementation of motility on the chromosome is partial, but limits leaky expression. A reduction in protein expression from the single copy complementation likely accounts for the relatively low level of complementation when compared to the plasmid-based complementation. In addition, since motility is subject to cell-cycle regulation in *A. tumefaciens* (38), it is possible that the constitutive expression of Rem from the inducible promoter (in either the chromosomal or plasmid-based complementation) results in partial complementation.

Figure 2-2. Chromosomal and plasmid-based complementation of motility in Δrem .

A. Schematics of strains used comparing single-copy chromosomal complementation (strains 1 – 4) and multiple copy plasmid complementation (strains 5 -8). B. Strains 1 – 4 were assayed for swimming motility on ATGN soft agar plates in the presence (black bars) or absence (white bars) of the inducer, IPTG. Data are normalized to results obtained in the wild-type strain (strain 1) and are shown as the %WT $\pm$ standard error of the mean (S.E). C. Strains 5 – 8 were assayed for swimming motility on ATGN soft agar plates containing kanamycin to maintain the plasmid in the presence (black bars) or absence

(white bars) of the inducer, IPTG. In the absence of inducer, we repeatedly observe an increase in the mucoidy at the center of the swim rings. In the Δrem strains, which are non-motile, this increase in mucoidy leads to a slightly larger measurement of the swim ring diameter. Data are normalized to results obtained in the wild-type strain containing an empty vector (strain 5) and are shown as the %WT $\pm$ S.E. Swim ring diameters were measured after 5 days of incubation at room temperature. Data shown is the mean of three independent experiments completed in triplicate.

In order to determine if the single-copy chromosomal complementation generally provides tighter regulation, we have assayed the complementation of a mutant impaired in biofilm formation. In *A. tumefaciens*, biofilm formation is dependent at least in part on the ability to extrude a unipolar polysaccharide (UPP) (38, 39). The UPP plays an essential role in attachment to surfaces and subsequent biofilm formation (37, 40). Here, we compare the abilities of chromosome-based and plasmid-based strategies to complement the biofilm formation of a $\Delta uppX$ mutant, which fails to produce a UPP. Adherent biofilm formation on plastic coverslips was measured after 48 hours of incubation for wildtype, *a-attTn7*, $\Delta uppX$, and complementation strains (Figure 2-3). We find that the $\Delta uppX$ mutant retains only ~25% of the biofilm formation when compared to the wildtype or *a-attTn7* strains. When *uppX*-HA was introduced in a single copy at the *a-attTn7* site under the control of the *lac* promoter, IPTG-dependent partial complementation (~50%) was observed (Figure 2-3B). Introduction of *uppX*-HA under the control of the *lac* promoter on a plasmid resulted in a similar degree of complementation in the presence of inducer (~50%); however, partial complementation was also observed in the absence of inducer (~40%) (Figure 2-3C). These observations suggest that single-copy chromosomal insertion enables tight regulation of target genes under the control of the *lac* promoter, potentially enabling the depletion of target genes.

Figure 2-3. Chromosomal and plasmid-based complementation of biofilm formation in $\Delta uppX$.

A. Schematics of strains used comparing single-copy chromosomal complementation (strains 1 – 4) and multiple copy plasmid complementation (strains 5 -8). B. Strains 1 – 4 were assayed for biofilm formation on vertical plastic coverslips immersed in ATGN media in the presence (black bars) or absence (white bars) of the inducer, IPTG. Data are normalized to results obtained in the wild-type strain (strain 1) and are shown as the %WT $\pm$ S.E. C.

Strains 5 – 8 were assayed for biofilm formation in ATGN media containing kanamycin to maintain the plasmid in the presence (black bars) or absence (white bars) of IPTG. Data are normalized to results obtained in the wild-type strain containing an empty vector (strain 5) and are shown as the %WT $\pm$ S.E. For all strains, coverslips were removed after 48 hours of incubation at room temperature and rinsed to remove any loosely associated cells. Adherent biomass was determined as the absorbance of solubilized crystal violet (A_{600}) and the optical density of the planktonic culture (OD_{600}) was measured. Biofilm scores were calculated as the ratio of A_{600}/OD_{600} and data was normalized. Data shown is the mean of three independent experiments completed in triplicate. Representative coverslips prior to crystal violet solubilization are shown for each strain.

MiniTn7 vectors expressing sfGFP in *a-attTn7* are tightly regulated. To assess the ability of the mini-Tn7 system to enable depletion studies, we introduced the gene encoding superfolder GFP (41-43) into the *a-attTn7* site under the control of the *lac* and *tac* promoters (Figure 2-4A). As expected, we observed that the *tac* promoter drives a much higher level of expression compared to the *lac* promoter as assessed by detection of GFP levels in Western blots and the relative amount of fluorescence emitted by GFP in live cells when IPTG is present (Figures 2-4B-C). Similarly, we find that expression of *sfGFP* under the control of *lac* or *tac* promoter from the chromosomal integration is much lower than from the same promoter introduced on a plasmid (Supplemental Figure 2-2). These observations suggest that the *tac* and *lac* promoters can drive relatively high or low levels of expression as needed for specific target genes.

Next, we wanted to determine if the sfGFP could be depleted from cells when the inducer is removed. Cells grown in the presence of IPTG were washed to remove inducer and the depletion of GFP from cell populations was monitored by Western blot (Figures 2-4B and Supplemental Figure 2-2) and fluorescence microscopy (Figure 2-4C). Chromosomal insertion of *sfGFP* under control of the *tac* and *lac* promoters enables rapid depletion of sfGFP in the absence of inducer (Figure 2-4B, Supplemental Figure 2-2). Under control of the *tac* promoter, the levels of GFP begin to deplete within 2-3 hours and are barely detectable in Western blots or live cell imaging after 5 hours. Depletion occurs more rapidly (within 2 hours) under the *lac* promoter, presumably due to the reduced level of initial expression. Together, these data suggest expression of genes of interest

Figure 2-4. GFP driven from P_{tac} or P_{lac} in the a -att site rapidly depletes.

A. Schematics of strains used to compare expression of *sfgfp* from P_{tac} (top) and P_{lac} (bottom). B. Western blots illustrate the depletion of GFP following the removal of the inducer (IPTG) as described in the materials and methods. Expression of GFP driven from P_{tac} (top) is higher than expression of GFP driven from P_{lac} (bottom). C. Depletion of GFP signal driven from P_{tac} (top) and P_{lac} (bottom) following the removal of the inducer (IPTG) is monitored in live cells using fluorescence microscopy.

from the *lac* or *tac* promoters should enable depletion of target proteins following removal of the gene at the native locus. Depletion of CtrA decreases viability and induces rounding of *A. tumefaciens* cells. Coordination of DNA replication and segregation, cell elongation, and cell division must be properly regulated during the cell cycle of *A. tumefaciens*. Yet, little is known about the mechanisms of essential processes driving cell cycle progression in *A. tumefaciens* due to the limited ability to characterize essential genes. In many Alphaproteobacteria, the response regulator protein, CtrA, plays an important role as a transcription factor to mediate proper cell cycle progression (44-49). In *A. tumefaciens*, CtrA is essential for cell survival (28) and remains uncharacterized. Here, we deplete *ctrA* as a validation of the miniTn7 system for depletion of essential genes.

In order to deplete CtrA from *A. tumefaciens* cells, *ctrA* was introduced under the control of the *tac* promoter at the *a-attTn7* site. In the presence of IPTG, *ctrA* was deleted from the native location by allelic exchange using a standard protocol (16) (Figure 2-5A). Growth curve analysis of the *ctrA* depletion strain in the presence of IPTG indicates that growth is comparable to wildtype. In contrast, in the absence of IPTG indicate the optical density of cells depleted of CtrA increases for several hours before reaching a plateau (Figure 2-5B); however, the observed increase in cell density is not a result of net population growth, but rather due to the elongation and swelling of individual non-viable cells (Figures 2-5C-D). In order to assess the ability of cells depleted of CtrA to recover, we conducted colony forming assays after 2, 4, 8, and 24 hours of incubation in the presence or absence of IPTG. We find the *ctrA* depletion rapidly

results in a loss of cell viability with no increase in the number of viable cells (Figure 2-5C). In the presence of the IPTG, the *ctrA* depletion strain is capable of cell elongation and division resulting in the formation of a microcolony within 24 hours (Figure 2-5D, top panel). In contrast, in the absence of IPTG, the depletion of CtrA causes the cells to elongate without dividing (Figure 2-5D, bottom two panels). After 10 hours of *ctrA* depletion, most cells begin to swell, resulting in a terminal block in cell division (Figure 2-5D, bottom two panels). Between 10 and 24 hours of *ctrA* depletion, although some cells generate constrictions (Figure 2-5D, bottom panel) only 11% (17/149) of individual cells complete cell division. Notably, the rare cell division events that occur are atypical and often occur at asymmetric sites resulting in production of mini cells. The cells continue to swell from the mid-cell and ultimately lyse. These observations are consistent with an expected role of CtrA in the regulation of cell division as described in the closely related bacterium *Sinorhizobium meliloti* (48).

CONCLUSIONS

We have successfully engineered *A. tumefaciens* with an artificial *attTn7* site at a neutral locus in the chromosome and constructed a set of mini-Tn7 vectors that allow integration and inducible expression of target genes from the *a-attTn7* site. This approach allows for more precise regulation of target genes than multi-copy plasmids using the same promoters, enabling complementation studies that require tight regulation of gene expression. Furthermore, this method enables rapid depletion of target proteins. As proof-of-concept, we have depleted CtrA

A C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-ctrA$ $\Delta ctrA$

B

C

D

Figure 2-5. Depletion of the essential cell cycle regulator CtrA results in the production of non-viable, rounded cells.

A. Schematic of the *ctrA* depletion strain. B. Growth curve of the wildtype parent strain (dashed black line) and the *ctrA* depletion strain in the presence (solid black line) and absence of IPTG (gray line). C. Cell viability was assessed as the ability to produce colony forming units on ATGN plates containing IPTG after the *ctrA* depletion strain was cultured in the presence (black bars) or absence (white bars) of IPTG for each indicated timepoint. The cell viability score was calculated as the number of colony forming units at the indicated timepoint/number of colony forming units at the onset of the experiment. D. Select images from 24 hours of timelapse microscopy of individual *ctrA* depletion cells grown on ATGN agarose pads in the presence (top panel) or absence (bottom two panels) of IPTG.

and find that cells fail to divide, undergo dramatic changes in morphology and lose viability. To the best of our knowledge, this is the first depletion of an essential gene in *A. tumefaciens*. This method provides a necessary advance in our ability to study essential processes such as cell cycle progression, cell growth, and cell division in *A. tumefaciens*.

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 2-1. A list of DNA primers and DNA fragments is provided in Table 2-2. *Agrobacterium tumefaciens* C58 and derived strains were grown in ATGN media (30) without exogenous iron at 28°C with shaking. When appropriate, antibiotics were used at the following working concentrations: kanamycin 300 µg/ml and gentamycin 200 µg/ml. When indicated, IPTG was used as an inducer at a concentration of 1 mM.

E. coli DH5α and S17-1 λ *pir* were routinely cultivated in Luria-Bertani (LB) medium at 37°C with shaking. When necessary antibiotics were used at the following concentrations: kanamycin 50 µg/ml and gentamycin 20 µg/ml.

Construction of an artificial *attTn7* (a-*attTn7*) site in *A. tumefaciens*.

A list of all synthesized DNA primers and gene fragments used in this study is provided in Table 2-2. All custom made DNA was made by Integrated DNA Technologies, Inc. The artificial attachment site was introduced into the *tetRA* locus using allelic replacement as described (16). A synthetic double stranded

373 base pair gBlock® gene fragment, att miniTn7 tet locus, was ligated into pMEH0125 with the restriction endonuclease sites NdeI and NcoI. The resulting construct, pNPTS139 $\Delta tetRA::a-attTn7$, was used to replace the *tetRA* locus with the *a-attTn7* site. PCR amplification was used to verify the insertion by using primers Tet locus P5 and P6.

Insertion of mini-Tn7 vectors in *A. tumefaciens*.

Standard electroporation protocols (16) were used to introduce mini-Tn7 vectors along with the helper plasmid, pTNS3, into *A. tumefaciens* cells. Briefly, 40 μ l of electrocompetent cells were mixed with 800 ng mini-Tn7 plasmid and 50 ng pTNS3, electroporated at 25 volts/cm, after addition of fresh media, transformed cells were incubated with shaking at 28°C for 4 hours, and plated on selective media. Insertion was verified by colony PCR using the upstream *tetRA* locus primer, tet forward, and the right end Tn7 specific primer, Tn7R109 (Table 2-2).

Deletion of target genes in *A. tumefaciens*.

Nonpolar, markerless deletions of the *A. tumefaciens rem* (Atu0573), *uppX* (Atu0480), and *ctrA* (Atu2434) genes were generated using the plasmids pNPTS138 Δrem , pNPTS138 $\Delta uppX$, and pNPTS138 $\Delta ctrA$ and following an established allelic replacement protocol (16). Primary integrants were made by conjugation with *E. coli* S17-1 cells as the donor strain for pNPTS138/139. Primers used for PCR verifying deletions were; Atu0573 P5/Atu0573 P6, Atu0480 P5/Atu0480 P6, and CtrA P5/CtrA P6 (Table 2-2). For the *ctrA*

depletion, two strains containing a chromosome-based complementation copy of CtrA with either an inducible *lac* or *tac* promoter were used with a modified protocol adding 1 mM IPTG to the media at all steps to maintain expression. Only the strain containing the complementing *tac* promoter driven copy of *ctrA* was successful for deleting the chromosomal copy of *ctrA*.

Construction of complementation plasmids.

Complementation plasmids of the *A. tumefaciens rem* (Atu0573), *uppX* (Atu0480), and *ctrA* (Atu2434) genes were made by PCR amplification of genomic DNA using the primers; Atu0573 NdeI F/Atu0573 BamHI R, Atu0480 NdeI F/Atu0480 BamHI R, and CtrA NdeI F/ CtrA BamHI R (Table 2-2). Amplified gene fragments were digested and ligated into plasmids digested with NdeI / BamHI to make the corresponding plasmids listed for each gene in Table 2-1. Additional information on plasmid modification can be found in supplementary methods.

Western blot analysis. For monitoring of cytoplasmic GFP expression, 40 mL of cells were grown to an $OD_{600}=0.6$ in the presence of IPTG. A 2mL aliquot of culture was removed and the pellet was stored to serve as the time 0 (+IPTG) point. The remaining 38mL culture washed with ATGN via centrifugation at 4,000RPMs for 10min three times to remove the IPTG. The washed pellet was resuspended in 20mL of fresh ATGN, normalized to an $OD_{600}= 0.4$. Aliquots were collected hourly to monitor the levels of GFP expression during the depletion.

Cell pellets were stored at -80°C until further analysis. Cell pellets were incubated with 100 µL of a Master Mix containing 1 mL of BugBuster[®] Protein Extraction Reagent (Novagen), supplemented with 1X protease inhibitors cocktail EDTA-free (Sigma), 10 µL of lysonase (Novagen), 2,500 U/ml of DNase I (Thermo Scientific) and 1 mM DTT (Thermo Scientific) for 25 min with shaking at room temperature to lyse the cell pellets. Whole cell lysates were clarified by centrifugation at 10,000 RPMs for 15 min. A final concentration of 1X Laemmli buffer was added to cleared cell lysates. Samples were boiled at 100°C for 5 min prior loading on a 12% SDS-PAGE gel. Separated proteins were electroblotted onto PVDF membranes (BioRad) and blocked overnight in 5% non-fat dried milk powder, solubilized in 1% TBST (1X TBS, 1% Tween-20). Blocked PVDF membranes were probed with anti-GFP (1:3,000) monoclonal antibody for 1 hr in 2.5% milk/TBST, followed by incubation with a donkey anti-mouse horseradish peroxidase-conjugated secondary antibody for 1 hr in 2.5% milk/TBST. Secondary antibody was detected using the ECL Plus HRP Substrate (Thermo Scientific Pierce).

Swim plate assays. Swim plate assays were conducted as described (31). Briefly, 5 µl of exponential phase cells normalized to an OD₆₀₀ = 0.6 were spotted on 0.3% AT agar plates and incubated in a sealed humid chamber for 5 days. The diameters of the swim rings were measured on days 3 and 5. Each strain was tested in triplicate during three independent experiments.

Biofilm assays. Biofilm assays were conducted as described (31). Bacteria were grown to mid-log phase in ATGN media and diluted to $OD_{600} = 0.05$. 3 ml of diluted bacteria was placed in the wells of 12-well polystyrene plates containing polyvinyl chloride coverslips placed vertically each well. Plates were incubated at room temperature for 48 hours. After incubation, coverslips were removed and rinsed with dH_2O to remove planktonic cells. Coverslips were stained with 0.1% crystal violet for 5-10 minutes and destained with 33% acetic acid. A_{600} of the solubilized crystal violet and OD_{600} of cultures from each well were measured. The biofilm score was calculated as the A_{600}/OD_{600} to allow for normalization of strains. Three biological replicates were completed in triplicate for each experiment.

Growth curve analysis. For the CtrA depletion strain, cells were grown in ATGN media in the presence of 1 mM IPTG to an $OD_{600} = 0.4$. The cells were washed 3 times by centrifugation in ATGN media to remove the inducer and resuspended to an $OD_{600} = 0.05$ in ATGN media with or without IPTG. For each condition, 200 μ l of sample was added in triplicate into a 96 well plate. The OD_{600} was measured in a BioTek Synergy H1 Hybrid Reader every 15 minutes for 48 hours at 28°C with 1 minute shaking before reads.

Cell viability assays. For the CtrA depletion strain, cells were grown in ATGN media in the presence of 1 mM IPTG to an $OD_{600} = 0.4$. The cells were washed 3 times by centrifugation in ATGN media to remove the inducer and resuspended

to an $OD_{600} = 0.05$ in ATGN media with or without IPTG. Serial dilutions of the cell culture were plated on ATGN media containing 1 mM IPTG immediately after washing to determine the number of colony forming units at the onset of the experiment. Serial dilutions from cultures with and without IPTG were plated after 2, 4, 8, and 24 hours of induction or depletion to obtain counts of colony forming units. Relative cell viability scores were calculated as the number of colony forming units at the indicated timepoint (2 hr, 4 hr, 8hr, or 24 hr)/number of colony-forming units at the onset of the experiment (0 hr). Each condition was tested in duplicate during three independent experiments.

Fluorescence and timelapse microscopy. A small volume ($\sim 0.8 \mu\text{l}$) of cells in exponential phase was applied to a 1% agarose pad as described previously (12). For timelapse imaging, cells were imaged every 10 minutes for the duration of the experiment. Differential interference microscopy and epifluorescence microscopy was performed with an inverted Nikon Eclipse TiE with a QImaging Rolera em-c² 1K EMCCD camera and Nikon Elements Imaging Software.

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TABLES

Table 2-1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference /Source
<i>Plasmids</i>		
pTNS3	Ap ^r ; helper plasmid encoding the site-specific TnsABCD Tn7 transposition pathway	(32)
pUC18-mini-Tn7T-GM-LAC	Ap ^r Gm ^r ; mini-Tn7 vector containing lacI ^q and <i>tac</i> promoter	(18)
pUC18-mini-Tn7T-GM-Plac-HA	Ap ^r Gm ^r ; mini-Tn7 vector containing lacI ^q and <i>lac</i> promoter	This study
pUC18-mini-Tn7T-GM-Ptac-HA	Ap ^r Gm ^r ; mini-Tn7 vector containing lacI ^q and <i>tac</i> promoter	This study
pUC18-mini-Tn7T-GM-Ptac-sfgfp	Ap ^r Gm ^r ; mini-Tn7 vector containing sfGFP under control of the <i>tac</i> promoter	This study
pUC18-mini-Tn7T-GM-Plac-sfgfp	Ap ^r Gm ^r ; mini-Tn7 vector containing sfGFP under control of the <i>lac</i> promoter	This study
pNTPS138/139	Km ^r ; Suicide vector containing <i>oriT</i> and <i>sacB</i>	D. Alley
pMEH0125	Suicide plasmid for allelic exchange of <i>tetRA</i> locus	Fuqua Lab
pNTPS139 $\Delta tetRA::a-attTn7$	Suicide plasmid for allelic exchange of <i>tetRA</i> locus with artificial <i>attTn7</i> site	This study
pSRKKm	Km ^r ; broad host range vector containing lacI ^q and <i>lac</i> promoter	(17)
pSRKKm-Plac-HA	pSRKKm vector containing lacI ^q and <i>lac</i> promoter with HA tag	This study
pSRKKm-Plac-sfgfp	pSRKKm vector containing lacI ^q and <i>lac</i> promoter with sfGFP	This study
pSRKKm-Ptac-sfgfp	pSRKKm vector containing lacI ^q and <i>tac</i> promoter with sfGFP	This study
pNTPS138 Δrem	Km ^r Suc ^s ; deletion plasmid for <i>rem</i>	Fuqua Lab
pSRKKm-Plac- <i>rem</i> -HA	Km ^r ; complementation vector containing <i>rem</i> -HA under control of <i>lac</i> promoter	This study
pUC18-mini-Tn7T-GM-Plac- <i>rem</i> -HA	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>rem</i> -HA under control of the <i>lac</i> promoter	This study
pNTPS138 $\Delta uppX$	Km ^r Suc ^s ; deletion plasmid for <i>uppX</i>	Fuqua Lab
pSRKKm-Plac-	Km ^r ; complementation vector containing	This study

<i>uppX</i> -HA	<i>uppX</i> -HA under control of <i>lac</i> promoter	
pUC18-mini-Tn7T-GM-Plac- <i>uppX</i> -HA	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>uppX</i> -HA under control of the <i>lac</i> promoter	This study
pJEH005 (pNTPS138Δ <i>ctrA</i>)	Km ^r Suc ^s ; deletion plasmid for <i>ctrA</i>	Fuqua Lab
pUC18-mini-Tn7T-GM-Plac- <i>ctrA</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>ctrA</i> under control of the <i>lac</i> promoter	This study
pUC18-mini-Tn7T-GM-Ptac- <i>ctrA</i>	Ap ^r Gm ^r ; mini-Tn7 vector containing <i>ctrA</i> under control of the <i>tac</i> promoter	This study
pKC129	Source of sfGFP	Huang Lab
pRVGFPC-2	Source of multiple cloning site fragment.	(50)
<i>E. coli</i> strains		
DH5α	Cloning strain	Life Technologies
S17-1	Sm ^r ; RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	(51)
<i>A. tumefaciens</i> strains		
C58	Nopaline type strain; pTiC58; pAtC58	(52)
C58 pSRKKm-Plac-HA	C58 with empty pSRKKm plasmid	This study
C58Δ <i>tetRA</i> ::a- <i>attTn7</i>	Replacement of the <i>tetRA</i> locus with an artificial <i>attTn7</i> site	This study
C58Δ <i>tetRA</i> ::a- <i>attTn7</i> pSRKKm-Plac-HA	C58Δ <i>tetRA</i> ::a- <i>attTn7</i> with empty pSRKKm-Plac-HA plasmid	This study
C58Δ <i>tetRA</i> ::a- <i>attTn7</i> pSRKKm-Plac-sf <i>gfp</i>	C58Δ <i>tetRA</i> ::a- <i>attTn7</i> with pSRKKm expressing sfGFP under the control of the <i>lac</i> promoter	This study
C58Δ <i>tetRA</i> ::a- <i>attTn7</i> pSRKKm-Ptac-sf <i>gfp</i>	C58Δ <i>tetRA</i> ::a- <i>attTn7</i> with pSRKKm expressing sfGFP under the control of the <i>tac</i> promoter	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-LAC	Mini-Tn7T-GM-LAC inserted into a- <i>attTn7</i> site	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Plac-sf <i>gfp</i>	Mini-Tn7T-GM-Plac-sf <i>gfp</i> inserted into a- <i>attTn7</i> site to express sfGFP under the <i>lac</i> promoter	This study
C58Δ <i>tetRA</i> ::mini-	Mini-Tn7T-GM-Ptac-sf <i>gfp</i> inserted into a-	This study

Tn7T-GM-Ptac- <i>sfgfp</i>	<i>attTn7</i> site to express sfGFP under the <i>tac</i> promoter	
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>rem</i>	Δ <i>rem</i>	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Plac- <i>rem</i> -HA Δ <i>rem</i>	Chromosome-based complementation of Δ <i>rem</i> with Rem-HA	This study
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>rem</i> pSRKKm-Plac-HA	Empty pSRKKm-Plac-HA plasmid in Δ <i>rem</i>	This study
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>rem</i> pSRKKm-Plac- <i>rem</i> -HA	Plasmid based complementation of Δ <i>rem</i> with Rem-HA	This study
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>uppX</i>	Δ <i>uppX</i>	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Plac- <i>uppX</i> -HA Δ <i>uppX</i>	Chromosome-based complementation of Δ <i>uppX</i> with UppX-HA	This study
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>uppX</i> pSRKKm-Plac-HA	Empty pSRKKm-Plac-HA plasmid in Δ <i>uppX</i>	This study
C58Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>uppX</i> pSRKKm-Plac- <i>uppX</i> -HA	Plasmid based complementation of Δ <i>uppX</i> with UppX-HA	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Plac- <i>ctrA</i>	Mini-Tn7T-GM-Ptac- <i>ctrA</i> inserted into a- <i>attTn7</i> site to express CtrA under the <i>lac</i> promoter	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Ptac- <i>ctrA</i>	Mini-Tn7T-GM-Ptac- <i>ctrA</i> inserted into a- <i>attTn7</i> site to express CtrA under the <i>tac</i> promoter	This study
C58Δ <i>tetRA</i> ::mini-Tn7T-GM-Ptac- <i>ctrA</i> Δ <i>ctrA</i>	Chromosome-based complementation of Δ <i>ctrA</i> with CtrA	This study

Table 2-2. Synthesized DNA primers and gene fragments used in this study.

Synthesized DNA	Sequence
Primers	
Tet forward	5'- ACA TGT TGT ATA CCG GAA ACT GAT TGC AC -3'
Tn7R109	5'- CAG CAT AAC TGG ACT GAT TTC AG -3'
sfGFP HindIII F	5'- GCA GAG AAG CTT ATG GTG AGT AAA GGT GAA-3'
sfGFP NheI R	5'- GCT AGC TAG CCT ATT TGT AGA GCT CAT CCA TGC C-3'
Linker SacIBamHI F	5'- CCG GGA TCC GCT GGC TCC GCT GCT GGT TCT GGC A-3'
Linker Hind R	5'- AGC TTG CCA GAA CCA GCA GCG GAG CCA GCG GAT CCC GGA GCT-3'
HA BamHI	5'- GA TCC TAC CCA TAC GAT GTT CCA GAT TAC GCT TAA TGA G-3'
HA NheI	5'- CTA GCT CAT TAA GCG TAA TCT GGA ACA TCG TAT GGG TAG-3'
pTAC BstBI F	5'- GCG ATG TTC GAA TCA CTG CCC GCT TTC CAG TC-3'
pTAC NdeI R	5'- GCG TGC CAT ATG TTT CCT GTG TGA AAT TG-3'
BBMiniTn7 NheI F	5'- GCG TTA GCT AGC TAA TGA AGG CCA ACC AGA TAA GTG-3'
BBMiniTn7 NdeI R	5'- GCG TTA CAT ATG GTG CAC TCT CAG TAC-3'
Gm SpeI F	5'- GGC AAT ACT AGT GCG GCG TTG TGA CAA TTT AC-3'
Gm SalI R	5'- GGC ACT GTC GAC TTG ACA TAA GCC TGT TCG GTT-3'
LacR SalI F	5'- GCT AGT CGA CGA ATC AGG GGA TCT TGA AGT TC-3'
LacR AgeI R	5'- GCT AAC CGG TCG TTG ACA CCA TCG AAT GG-3'
Atu0573 NdeI F	5'- CGC ACG CAT ATG ATC GTA GTG GTT GAT GAG-3'
Atu0573 BamHI R	5'- CGC TGG ATC CTT GCC AAT CAA TGC TGT AGC CG-3'
Atu0573 P5	5'- TAC CCA CTA TAT ATA GAT CAA ATC GGC A-3'
Atu0573 P6	5'- ACC CAT CCT GAA CCA CGA AGA AAC CGT TG-3'
Atu0480 NdeI F	5'- CGC ACG CAT ATG TCC AGC GCT GCC CAG CTC-3'

Atu0480 BamHI R	5'- CGC TGG ATC CTT TGG CTG GCG CGA AAA CC-3'
Atu0480 P5	5'-GAA GCG CAG TCG TTG TTG GAT TTG TC-3'
Atu0480 P6	5'- TAT GAG CCG ATG TAT GAG GCG TTG AAG-3'
CtrA NdeI F	5'- GTC GCT CAT ATG CGG GTT CTA CTG ATT-3'
CtrA BamHI R	5'- GTC GCT GGA TCC TCA GGC GGT TTC GAG-3'
CtrA P5	5'- GCT GCA ACT GGT TAA GCT GCC-3'
CtrA P6	5'- CGA GAC GTA GCT GAC CAG TGT C-3'
Tet locus P5	5'- CTA CGT CGA AAT CAG GTG GT-3'
Tet locus P6	5'- CTT CGT TTA CCT GAT TCT GTC CG-3'
Gene Fragments	
miniTn7 MCS	5'- cggattcattcggatattaatcgggtgtgaaataccgcacagatgcgt aaggagaaaataccgcatcaggcgcca ttcgccattcaggctgcgcaactgttggaagggcgatcgggtcg ggcctcttcgctattacgccagctagaggaccagccgcgtaacct ggcaaaatcggttacgggtgagtaataaatggatgccctgcgtaa gcgggtgtggcggaataaagtcttaactgaacaaaataga tctaaactatgacaataaagtcttaactagacagaatagttgtaa actgaaatcagtcagttatgctgtgaaaaagcatactggactttgt tatggctaaagcaaactctcattttctgaagtgcaaattgccgctg tattaaagaggggctggtgggttcgaggacgacggtatggataag gtagcttaattagctgagcttgactcctgttgatagatccagtaatg acctcagaactccatctggattgttcagaacgctcgggtgccgccg ggcgtttttattggtgagaatccaagctagactgcgatgagtgga ggcgggggcgtaatttttaaggcagttattggtgccctaaacgcc tggggtaatgactctctagcttgaggcatcaataaaacgaaagg ctcagtcgaaagactgggccttctgtttatctgttgttcgggtgaa gctctcctgagtaggacaaatccgccgctaggagctgcggcccg gacgatgcgaattgggactagtttaggtatcccgggaatcctaggt gtcgactgatcgatggcaccggccgcaaagtattgccgctgcctt actggagctatagctggtcgggtactcgaaggtacagggaccgg tcctaagcgggccgccaacttggttagctatctgatcacgggtgaacg gc -3'
Lac promoter	5'- gctcagccatcaagggtgatgcaacatggatgcatgggaccggtc actagctgagcttgactcctgttgatag atccagtaatgacctcagaactccatctggattgttcagaacgctc ggtgccgcccggcggtttttattggtgagaatccaagctagactgc gatgagtggcagggcgggcgtaatttttaaggcagttattggtg ccctaaacgcctggggtaatgactctctagcttgaggcatcaaat

	aaaacgaaaggctcagtcgaaagactgggcctttcgtttatctgtt gtttgtcgggtgaacgctctcctgagtaggacaaatccgccgctagg agcttgcggtacaggccgattcattaatgcagctggcacgacag gtttcccgaactggaaagcgggcagtgagcgcaacgcaattaatgt gagttagctcactcattaggcaccccaggctttacactttatgcttcc ggctcgatgttggtggaattgtgagcggataacaatttcacacag gaaacagcatatgcctgcaggcgccttaattaatatgcatggtacc ttaagatctcgagctccgggatccaatccggtatgggctagcgagt tactgagtggttccatgtc-3'
att miniTn7 tet locus	5'- cgtcgatgctatgcggctagccgagccgctgccgatccagctgct cgcctatcacaccgccgtcttcatgggc acggatgtggaccagccgcgcaatctggcgaaatcggtgaccgt ggaatgatcatccggccggagcggcaggggtgatgaagaggcga tcgctcgcgttacggaagatgccttccgtaacgtcgaacaccgtag cgggacagaacaactgatcgtcgcgcggtgcgggacgcgag gcatcaaaataaacgaaaggctcagtcgaaagactgggcctttc gtttatctgttgttcgggtgaacgctctcctgagtaggacaaatcc gccgcccatggatgttccgtagcgtagctgcg-3'

SUPPLEMENTARY MATERIAL

Mini-Tn7 insertion in an artificial *att*Tn7 site enables depletion of the essential master regulator CtrA in the phytopathogen *Agrobacterium tumefaciens*

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Running Title: Depletion of an essential gene in *Agrobacterium tumefaciens*

SUPPLEMENTARY METHODS

Modification of complementation plasmids. The backbones of pSRKKm and pUC18-mini-Tn7T-GM-LAC were modified to share a common multiple cloning site (MCS) and add additional promoters. To add a new MCS, pSRKKm was digested with NdeI/NheI, backbone purified, and ligated with NdeI/NheI digested fragment from pRVGFPC-2 (x). For sfGFP expression, the resulting plasmid was cut with SacI/NheI and double ligated with PCR amplified *sfgfp* from pKC129 with primers sfGFP HinkIII F/ *sfgfp* NheI R and annealed primers Linker SacIBamHI F/ Linker Hind R. The resulting plasmid was named pSRKKm-Plac-*sfgfp*. To add a HA tag, pSRKKm-Plac-*sfgfp* was cut with BamHI/NheI and ligated with the annealed primers HA BamHI /HA NheI to make pSRKKm-Plac-HA. The promoter was changed by PCR amplifying the *tac* promoter and repressor from pUC18-mini-Tn7T-GM-LAC with the primers pTAC BstBI F/ pTAC NdeI R. The fragment was then ligated into BstBI / NdeI digested and purified pSRKKm-Plac-*sfgfp* and pSRKKm-Plac-HA backbones. The resulting *tac* promoter plasmids were named pSRKKm-Ptac-*sfgfp* and pSRKKm-Ptac-HA. Modification of pUC18-mini-Tn7T-GM-LAC was done by PCR amplifying the backbone of the plasmid with primers BBMiniTn7 NheI F / BBMiniTn7 NdeI R. The digested backbone was ligated with the gene fragment, miniTn7 MCS, which was digested with AseI / NheI. The gentamycin cassette and *lacI*Q gene from pUC18-mini-Tn7T-GM-LAC were PCR amplified using the primers Gm SpeI F / Gm SalI R and LacR SalI F / LacR AgeI R. Each was separately ligated into the plasmid with corresponding digestion sites. This plasmid was then digested with AgeI / NheI and ligated with the gene

fragment, lac promoter. A HA tag was added as previously described and the construct was named pUC18-mini-Tn7T-Gm-Plac-HA. To replace the HA tag with *sfgfp*, pSRKKm-Plac-*sfgfp* was digested with BamHI / NheI to release *sfgfp* which was ligated into BamHI / NheI digested pUC18-mini-Tn7T-Gm-Plac-HA, resulting in construct pUC18-mini-Tn7T-Gm-Plac-*sfgfp*. The two plasmids pUC18-mini-Tn7T-Gm-Plac-*sfgfp* and pUC18-mini-Tn7T-Gm-Plac-HA were both digested with Sall / NdeI and ligated with PCR amplified fragment from pSRKKm-Ptac-*sfgfp* containing the *tac* promoter, using primers LacR Sall F / pTAC NdeI R. These *tac* promoter plasmids were named pUC18-mini-Tn7T-Gm-Ptac-*sfgfp* and pUC18-mini-Tn7T-Gm-Ptac-HA.

SUPPLEMENTARY FIGURES

Supplemental Figure 2-1. Replacement of the *tetRA* locus with *a-attTn7* and subsequent integration of a Tn7 cassette do not impact motility or biofilm formation of *A. tumefaciens*.

A. Schematics of strains used to determine impact of *a-attTn7* and Tn7 insertion on motility and biofilm formation. B. Strains 1 – 3 were assayed for swimming motility on ATGN soft agar plates in the presence (black bars) or absence (white bars) of the inducer, IPTG. Data are normalized to results obtained in the wild-type strain (strain 1) and are shown as the %WT $\pm$ standard error of the mean (S.E). Swim ring diameters were measured after 5 days of incubation at room

temperature. Data shown is the mean of three independent experiments completed in triplicate. Data for strains 1 and 2 is also shown in Figure 2-2C. Strains 1 – 3 were assayed for biofilm formation on vertical plastic coverslips immersed in ATGN media in the presence (black bars) or absence (white bars) of the inducer, IPTG. Data are normalized to results obtained in the wild-type strain (strain 1) and are shown as the %WT $\pm$ S.E. Coverslips were removed after 48 hours of incubation at room temperature and rinsed to remove any loosely associated cells. Adherent biomass was determined as the absorbance of solubilized crystal violet (A_{600}) and the optical density of the planktonic culture (OD_{600}) was measured. Biofilm scores were calculated as the ratio of A_{600}/OD_{600} and data was normalized. Data shown is the mean of three independent experiments completed in triplicate. Representative coverslips prior to crystal violet solubilization are shown for each strain. Data for strains 1 and 2 are also shown in Figure 2-3.

Supplemental Figure 2-2. Comparison of plasmid and chromosome-based expression of *sfgfp* from P_{tac} and P_{lac} promoters.

Western blots illustrate the depletion of GFP following the removal of the inducer (IPTG) in each case at 2, 4, and 8 hours post-depletion. Exposure times are shown on the top right corner of each blot. Doubling the exposure time allowed detection of GFP when expressed from the chromosomal P_{lac} promoter.

CHAPTER 3

Peptidoglycan (PG) Hydrolases are Involved in the PG Recycling and Beta-lactam Resistance Pathways of *Agrobacterium tumefaciens*

Author Contributions

WFC and CR conducted the experiments. WFC and PB designed the experiments. WFC, JB, CR and PB analyzed data and WFC and PB wrote and edited the dissertation chapter.

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Peptidoglycan Hydrolases Involved in the beta-lactam Resistance Pathway of

A. tumefaciens

ABSTRACT

In Gram-negative bacteria, a predominant mechanism for the destruction of beta-lactam antibiotics is the inducible production of beta-lactamases. Production of inducible beta-lactamases is indirectly correlated with the activities of peptidoglycan hydrolases. Here, we show that inactivation of the most highly expressed lytic transglycosylase, Atu3779, in the plant pathogen *A. tumefaciens*, makes the cells significantly more susceptible to beta-lactams. In addition, we found that the cytoplasmic amidase, AmiD, leads to a significant increase in the resistance to beta-lactams due to increased production of beta-lactamases. In addition, inactivation of AmiD improves the overall fitness of *A. tumefaciens* as the viability of $\Delta amiD$ is improved under all the conditions tested compared to WT cells. Together, these results begin to elucidate the pathway responsible for the high level of beta lactam resistance in *A. tumefaciens*.

Keywords

Antibiotic resistance, *Agrobacterium tumefaciens*, Amidases, Lytic transglycosylases

IMPORTANCE

Here we report two PG hydrolases, AmiD and Atu3779, are involved in the beta-lactam resistance of *A. tumefaciens*. Knowledge about proteins that promote or inhibit beta-lactam resistance may help in the development more efficient tools to improve the efficiency of *Agrobacterium*-mediated plant genetic transformations

by enabling efficient removal of *Agrobacterium* from transformed plant tissue, potentially maximizing crop yield and food production.

INTRODUCTION

The phytopathogen *Agrobacterium tumefaciens* is one of the top three most important economic and scientific plant pathogens (1). *A. tumefaciens* causes Crown Gall disease to flowering plants, nut trees and woody shrubs. In nature, *A. tumefaciens* infects wounded plants by adhering to the wound site and delivering a section of the bacterial Ti plasmid, or T-DNA, into the plant chromosomes (2). The sugars and phenolic compounds secreted at the plant wound site activate the expression of virulence genes that reside on the Ti plasmid, which lead to constitutive expression of plant hormones (3). The high abundance of phytohormones serve as custom food source for *A. tumefaciens*, but also triggers the local tissue proliferation or gall formation (2). Gall formation on plants and trees leads to crop damage, and significant economic losses have been attributed to this issue every year (4, 5). Remarkably, the *A. tumefaciens*' ability of inter-kingdom DNA transfer has allowed the production of transgenic plants through plant genetic engineering (2). One challenge for *Agrobacterium*-mediated transformation is the clearance of *Agrobacterium* from the potentially transformed plant tissue. It is also important to remove *Agrobacterium* from plants to reduce the risk of releasing engineered *Agrobacterium* into the environment. Beta-lactam antibiotics are frequently applied during plant transformation to kill the bacteria; however, *Agrobacterium* is resistant to many

beta-lactam antibiotics (6). Thus, understanding the precise role of chromosomally-encoded proteins that induce beta-lactam resistance in *A. tumefaciens* will help us develop more efficient tools to improve the efficiency of plant genetic transformations (6) and to maximize crop yields and food production. Here, we elucidated the role of genes involved in the beta-lactam resistance and cell wall recycling pathways of *A. tumefaciens*.

The bacterial cell wall consists of alternating NAG and NAM sugars, which form glycan strands. The glycan strands are linked together through crosslinked peptide stems (7–10). Although the peptidoglycan (PG) cell wall is sufficient for cell shape maintenance (11, 12), its expansion and remodeling requires cell wall synthesis and hydrolysis (13) accomplished by a large set of dedicated enzymes namely, PG synthases and PG hydrolases. As PG synthases build the PG cell wall, the remodeling and recycling of the cell wall depends on the action of hydrolytic enzymes (13–16). Among these enzymes, the lytic transglycolylases (LTs), specifically cleave the NAG and NAM glycan strands with the concomitant formation of anhydro-ring (15, 17) (Figure 3-1A). The action of LTs, thus, leads to the liberation anhydromuropeptide fragments from the bacterial cell wall (Figure 3-1A, Step 1). These fragments are subsequently translocated into the cytoplasm

Figure 3-1. Simplified models of PG recycling and beta-lactamase induction.

(A) During growth, the turnover of peptidoglycan fragments is mediated by lytic transglycosylases (LTs). (1) LTs cleave the cell wall between the NAG and NAM sugar, generating an anhydromuropeptide. (2) Anhydromuropeptides are translocated into the periplasm via inner membrane permeases. (3) Once in the cytoplasm, anhydromuropeptides are digested by carboxypeptidases (CPases). CPases cleave the terminal amino acid. (4) Amidases further digest the anhydromuropeptide by removing the peptide stem. (5) Other PG hydrolases, such as glucosaminidases, cleave the sugar moieties, which are subsequently recycled for use in peptidoglycan biosynthesis. (B) Anhydromuropeptides are produced and transported to the cytoplasm as described in steps 1-3 above. (4-5) In the absence of amidases, anhydromuropeptides accumulate in the cytoplasm leading to activation of beta-lactamase production and increase beta-lactam resistance.

via the activity of inner membrane permeases (16, 18, 19) (Figure 3-1A, Step 2). Once in the cytoplasm, these fragments are digested by amidases and carboxypeptidases (Figure 3-1A, Steps 3 and 4) and subsequently recycled for using in nascent PG biosynthesis (18) (Figure 3-1A, Step 5).

The PG cell wall recycling and beta-lactam resistance pathways are intimately interconnected in most bacteria. Failure in the recycling of LT degradation products in the cytoplasm, activates the beta-lactam resistance mechanism by inducing chromosomally-encoded beta-lactamases (20–22). Currently, two accepted models describe the mechanism by which chromosomally-encoded beta-lactamases are activated in the cell and lead to beta-lactam resistance. The first mechanism involves the inactivation of amidases in the cytoplasm (Figure 3-1B, Step 4), leading to the cytoplasmic build-up of anhydromuropeptides (22–24) (Figure 3-1B, Step 5), this due to a failure in anhydromuropeptide digestion by amidases, and recycling. The build-up of anhydromuropeptides leads to the activation of beta-lactamase, the beta-lactam cleaving enzymes. The second mechanism involves the exposure of cells to beta-lactam antibiotics. Beta-lactam antibiotics target the PG cell wall synthases penicillin-binding proteins (PBPs). More specifically, beta-lactams inhibit the PBP-mediated crosslinking and insertion of new PG monomers by mimicking the natural D-Ala-D-Ala substrate found on PG stems (25). Inhibition of the crosslinking (transpeptidation) activity of PBPs results in a block of cell wall extension with increased remodeling by cell wall lytic transglycosylases (LTs).

The extensive cleavage of glycan strands by LTs, leads to the build-up of anhydromuropeptides in the bacterial cytoplasm and the induction of beta-lactamases (Figure 3-1B). Among all the chromosomally-expressed beta-lactamases, we can find AmpC. The expression of *ampC* is regulated by the transcriptional regulator, AmpR, a bifunctional protein that can serve as a transcriptional repressor or activator (20). Indeed, in the absence of beta-lactam antibiotics, or low anhydromuropeptide concentration in the cytoplasm, AmpR represses the expression of *ampC*. However, exposure to beta-lactam antibiotics and/or the subsequent increase of anhydromuropeptides in the cytoplasm, displaces AmpR from the DNA binding site, leading to AmpC beta-lactamase induction (19) and increased bacterial resistance to beta-lactams antibiotics. Another gene involved in the induction of AmpC are cytoplasmic amidases such as AmpD (19, 21, 22, 24). AmpD has been shown to only process 1,6-anhydromuropeptides in MurNAc-tripeptides and tetrapeptides, and has no hydrolytic effect on PG biosynthesis precursors (15, 26) (Figure 3-1). In contrast to AmpD, the type 2 amidase AmiD has an extended substrate specificity for 1,6-anhydromuropeptides and MurNAc-L-Ala bonds on the cell wall (23, 24). In some bacterial organisms such as *E. coli*, deletion of the type 2 family of amidases activates the beta-lactam resistance pathway leading to resistance to this type of antibiotics upon expression of beta-lactamases (21, 22). Currently, the contributions of cell wall hydrolases to cell wall integrity, remodeling and beta-lactam resistance in *A. tumefaciens* is unknown. Here we show that deletion of a single LT, the MltB Atu3779, is sufficient to cause a significant decrease in beta-

lactam resistance. Moreover, we find that the cytoplasmic amidase AmiD is involved in PG recycling and beta-lactam resistance.

RESULTS AND DISCUSSION

3.1 Wide diversity of lytic transglycosylases (LTs) in the genome of *A. tumefaciens*

In bacteria, lytic transglycosylases (LTs) can be subdivided into three families. Family 1, or soluble lytic transglycosylases (SLTs), have been shown to be involved in bacterial cell separation (27, 28), PG recycling, beta-lactam resistance and outer membrane permeability (29–31). Family 2 corresponds to the membrane-bound lytic transglycosylases A (MltA). In *E. coli*, deletion of MltA leads to cell separation defects, and overexpression causes severe cell lysis and spheroplasting (32, 33). In addition to hydrolyzing crosslinked PG, MltA is the only LT that can accept soluble naked glycan chains as substrates. Deletion of MltA does not affect growth or morphology in *E. coli* (32, 34). Lastly, family 3, or the membrane-bound lytic transglycosylases B, MltB has been shown to be involved in exolytic and endolytic PG cleavage reactions (15, 34, 35). In addition, depending on the bacterial model organism, deletion of the MltB family results in beta-lactam resistance or hypersensitivity and increased outer membrane permeability (30, 34).

We began our study by performing a bioinformatic analysis using the CAZyme (Carbohydrate Active Enzyme) database (CAZy, <http://www.cazy.org>)

and the BLAST server (36). Our analysis revealed the presence of genes encoding 11 lytic transglycosylases [Atu0572, Atu1022, Atu2112, Atu2117, Atu2451, Atu3092, Atu0009, Atu0092, Atu2122, Atu2489 and Atu3779] in the genome of *A. tumefaciens* belonging to all three families of LTs (Figure 3-2). Eight LTs contain the Slt_1 domain characteristic of LT family 1, a single LT belongs to family 2 (MltA) and four LTs are members of family 3 (MltB), which contain Slt_2 domains (Figure 3-4A). Interestingly, most of the LTs found in *A. tumefaciens* genome contain a signal sequence domain for periplasmic transport, which highlights their putative involvement in a wide array of periplasmic activities in *A. tumefaciens*. A saturating transposon mutagenesis screen performed in *A. tumefaciens* revealed the LTs found in the genome are dispensable for viability suggesting a high level of redundancy and/or a high degree of overlapping functions (37).

To better understand the function of LTs, we assessed the spatial and temporal localization of the LTs in WT cells using C-terminal fusions to sfGFP (Figure 3-2). First, we found that MltBs Atu3779 and Atu0092 display an unusual localization pattern with multiple puncta that localize throughout the cell. The MltBs Atu2122 and Atu2489 display a polar to mid-cell localization in WT cells suggesting that these enzymes may function during polar growth or cell division. The protein localization patterns and phylogeny of the 4 MltB family suggest functional diversification of these (Figure 3-2, MltB Family 3). In

the case of the more diverse family of LTs, the SltS, we observed two different localization patterns within the several tree branches. The SltS from *A. tumefaciens* can be found localizing at mid-cell or dispersed in WT cells. Lastly, we found that MltA is dispersed throughout the cytoplasm. Together, this data shows that LTs in *A. tumefaciens* are diverse and have distinct localization patterns, which may suggest different and/or overlapping functions in the *A. tumefaciens* periplasmic space.

3.2 All LTs are expressed in *A. tumefaciens*

Given the wide diversity and expansion of LTs in *A. tumefaciens* we sought to determine if all of the LTs are routinely expressed in *A. tumefaciens*. Thus, the expression profiles of genes encoding LTs was explored through a transcript abundance analysis of C58 WT cells. In order to compare relative expression of the LTs, we constructed a normalized expression profiling map of 4,405 genes, normalized around median Reads Per Kilobase of transcript per Million mapped reads (RPKM) (Figure 3-3). All LTs from *A. tumefaciens* are expressed, however, the read abundance level varies between them. Indeed, we found that the expression levels for the Slt Atu2112 and Atu3092 are below median expression level (Figure 3-3). In contrast, we found that the MltB family (Atu3779, Atu2122, Atu2489 and Atu0092) expression is substantially higher than median expression (Figure 3-3). Lastly, we observed that the most highly expressed transcripts encode the Slt is Atu2117 and the MltB is Atu3779.

Figure 3-3. All lytic transglycosylases are expressed in WT cells.

Normalized expression profiling of lytic transglycosylases. *cysJ* was used to normalized the expression map. The median Reads Per Kilobase of transcript per Million mapped reads (RPKM) of CysJ is 1111.3. Fold deviance was calculated by dividing the gene of interest RPKM by the CysJ RPKM. The X-axis represents the C58 gene locus. Lines represent the lytic transglycosylases of *A. tumefaciens*. Members of the Slf family are indicated in yellow. Blue is the MltA family and green represents the MltB family.

3.3 LTs are involved in cell length and width maintenance

To assess the functions of LTs, we constructed single deletion mutants. First, we assessed cell viability (Figure 3-4B) and measured cell length and width (Figure 3-4C) of each mutant in LB medium. Single deletion of LTs do not affect cell viability, however, we observed significant changes in cell length and width (Figure 3-4C). Compared to WT, deletion of SltS Atu2117 and Atu3093 displayed a significant increase in cell length and width, whereas deletion of Atu0009, Atu1022 and Atu3779 showed only a significant decrease in cell width. The absence of that the SltS Atu2117, Atu3093, Atu1022 and the MltB Atu3779 led to an overall impact on cell shape maintenance suggesting that they play an important role in cell wall growth and remodeling of *A. tumefaciens*.

3.4 Inactivation of Atu3779 decreases resistance to AMP

LTs have been involved cell shape maintenance through their remodeling activities in the periplasm (34). Our last results revealed that at least four LTs (Atu1022, Atu2117, Atu3093 and Atu3779) were involved in the cell length and width maintenance of *A. tumefaciens*, thus they may play an important role in cell wall remodeling. Cell wall remodeling enzymes, such as LTs, have been shown to play crucial roles in PG recycling and beta-lactam resistance, leading to activation or repression of inducible beta-lactamases (29–31). In an effort to understand if *A. tumefaciens* LTs play important roles in PG recycling and beta-lactam resistance, we determined the resistance of LT deletion mutants to

Figure 3-4. LTs are involved in cell length and width maintenance.

(A) Schematic of the LT amino acid sequence according to families. (B) Cell viability of LT deletion mutants. (C) Violin plots of the cell length and width of the LT deletion mutants. The distribution of the data is indicated with the background dots. Top quartile (Q1) indicates 75% of the population, middle line and top number represent the median, and bottom quartile (Q2) represents the 25% of the population. **** = p-value <0.0001. Data was analyzed by a one-way ANOVA followed by a Dunnett's multiple comparison test. Comparisons are to WT.

Figure 3-5. Inactivation of *Atu3779* decreases resistance to AMP.

(A) Antibiotic susceptibility cell viability assay was performed. Viability of LT-deficient cells spotted on LB solid media or LB containing a low (10µg/ml) and a sublethal (80µg/ml) dose of ampicillin (AMP). Exponentially growing cultures were serially diluted, spotted on solid LB agar and incubated at 28°C for 36 h before imaging.

different concentrations of ampicillin (Figure 3-5A). Compared to WT, inactivation of *Atu3779* (Δ *atu3779*) led to a significant decrease in ampicillin (AMP) resistance on solid LB medium. Indeed, deletion of *Atu3779* causes cells to become sensitive to low levels of AMP (Figure 3-5A). The rest of the LT mutants are not impacted by treatment with sublethal concentrations of AMP (Figure 3-5A). Based on this observation, we hypothesize that the absence of *Atu3779* may prevent the induction of beta-lactamase following ampicillin treatment. In future work, we plan to test this hypothesis using a nitrocefin assay (38) to quantitate beta-lactamase activity of WT and Δ *atu3779* cells in the absence and presence of AMP.

3.5 The cytoplasmic amidase AmiD increases the fitness of WT cells and is required for repression of beta-lactamase activity

Deletion or mutation of cytoplasmic amidases has been shown to activate inducible beta-lactamases (21, 24). Cytoplasmic 1,6-anhydro-*MurNac* L-alanine amidases remove the peptide stems from the anhydromuropeptides in the cytoplasm (Figure 3-1A). Inactivation of these amidases has been shown to increase the pool of anhydromuropeptides in the cytoplasm, leading to derepression of beta-lactamases. In *A. tumefaciens*, a single cytoplasmic amidase was found in the genome (*Atu* 2113) and named AmiD (Figure 3-6A). To understand the role of AmiD in WT cells, we constructed a deletion strain. Compared to WT, deletion of *amiD* in *A. tumefaciens* slightly increases cell

Figure 3-6. The cytoplasmic amidase AmiD increases the fitness of WT cells.

(A) Schematic representation of AmiD. (B) Viability of amidase-deficient cells spotted on LB solid media. pAmiD represents the complementing plasmid in $\Delta amiD$, pEmpty indicates control empty plasmid in $\Delta amiD$. Exponentially growing cultures were serially diluted, spotted on solid LB agar and incubated at 28°C for 36 h before imaging. (C) Viability of $\Delta amiD$ cells spotted on LB solid media containing 2% salt. (D) Outer-membrane permeability using an SDS-based

sensitivity assay shows. (E) Phase-contrast microscopy of WT and *ΔamiD* cells.

(F) Violin plots of the cell length and area of WT, *ΔamiD* and *ΔamiD*

complementing mutant. The distribution of the data is indicated with the

background dots. Top quartile (Q1) indicates 75% of the population, middle line

and top number represent the median, and bottom quartile (Q2) represents the

25% of the population. **** = p-value <0.0001. Data was analyzed by a one-way

ANOVA followed by a Dunnett's multiple comparison test. Comparisons are to

WT.

viability (Figure 3-6B), growth in hypertonic growth conditions (Figure 3-6C) and decreases outer membrane permeability (Figure 3-6D) suggesting that $\Delta amiD$ improves the fitness of *A. tumefaciens* by providing the bacteria a selective advantage. No changes in cell length or area were observed upon deletion of $\Delta amiD$ (Figure 3-6E), however, in an attempt to complement $\Delta amiD$ strain using a low-copy plasmid, expression of pAmiD significantly increased cell length and area of the cells (Figure 3-6F). Taking these results together, we conclude that modulation of the levels of AmiD in nature, may help the cells thrive in harsh environmental conditions.

Since we hypothesized that deletion of *amiD* may derepress beta-lactamase activity, we sought to determine if $\Delta amiD$ is AMP resistant. Indeed, deletion of *amiD* leads increased resistance to AMP (Figure 3-7A) consistent with increased beta-lactamases activity as detected by the nitrocefin assay (Figure 3-7B). Together, these results suggest that AmiD is a cytoplasmic amidase involved PG recycling and beta-lactam resistance in *A. tumefaciens*.

CONCLUSION

Peptidoglycan hydrolases are involved in a wide variety of processes in the cell, from cell growth (33, 39), cell separation (15, 40–42), to PG recycling and beta-lactam resistance (16, 18, 19, 43). The abundance of LTs and their different localization patterns may suggest that they contribute to multiple functions within the cell. We find that the MltB Atu 3779 is highly expressed and absence of this

Figure 3-7. Deletion of *amiD* leads to an increase in beta-lactam resistance.

(A) Cell viability and beta-lactam resistance assay of WT and $\Delta amiD$. (B)

Determination of beta-lactamase production was performed by through a nitrocefin assay (38).

enzyme causes beta-lactam antibiotic sensitivity. On the other hand, the type 2 amidase, AmiD, is involved in PG recycling and the induction of beta-lactamase. Absence of AmiD leads to increased growth rates not only under standard laboratory conditions, but also in harsh environments such as hypertonic environments. We hypothesize that deletion of *amiD* increases anhydromuropeptide levels in the cell leading to activation of the AmpC-type beta-lactamase expression. Although AmpC-type beta-lactamases are not present in all bacteria, we found a region in the genome of *A. tumefaciens* that display high homology to the AmpC/AmpR operons from other bacteria such as *E. coli*. Indeed, AmpC (Atu3077) and AmpR (Atu3078) from *A. tumefaciens* are 47.1% and 66.78% similar to the *P. aeruginosa* AmpC and AmpR, respectively. It remains to be determined whether deletion of AmpC in WT and $\Delta amiD$ backgrounds changes the levels of beta-lactam resistance and beta-lactamase production. In the future, characterization of AmpC and its contribution to beta-lactamase activity will allow us to further understand the high level of beta-lactam resistance in *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Agrobacterium tumefaciens* C58 and derived strains were grown in Lysogeny Broth (LB) medium at 28°C with shaking. When appropriate, the antibiotic kanamycin was added at a concentration of 300 µg/ml. When indicated, IPTG was used as an inducer at a concentration of 1 mM.

E. coli DH5 α and S17-1 were routinely cultivated in LB medium at 37°C with shaking. the antibiotic kanamycin was added at a concentration of 50 μ g/ml.

Construction of expression plasmids and strains. All strains and plasmids used are listed in Table 3-1. Synthesized DNA primers are listed in table 3-2. To construct expression vectors containing any of the LT or AmiD, the corresponding coding sequences were amplified from purified *Agrobacterium tumefaciens* C58 genomic DNA. The amplicons and plasmids pSRKKM-Plac-*sfgfp*, pSRKKM-T7-*sfgfp* were digested overnight and ligated overnight at 4°C using NEB T4 DNA ligase.

Construction of expression plasmids and strains. Gene deletions were achieved by allelic exchange and vectors were constructed as previously described (44). Briefly, 500 bp fragments upstream and downstream of the gene of interest were amplified using primer pairs P1/P2 and P3/P4. Overlapping PCR was used to the amplicons generated by P1/P2 and P3/P4, 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 Kan resistant, sucrose sensitive primary exconjugants. Primary exconjugants 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.

Bioinformatic analyses. Proteins of interest were found using BLAST-TP and Cazy (<http://www.cazy.org/b42.html>), and analyzed using TMHMM (45) and SignalP 4.1 (46). Phylogenetic trees were constructed using a ClustalW protein alignment and creating a PhyML tree, version 3.0, as a Geneious plugin using the Geneious Tree Builder with the following settings: Le Gascuel substitution model with 100 bootstrap models.

Fluorescence and time-lapse microscopy. A small volume (~0.8 μ l) of cells in exponential phase was applied to a 1% agarose pad as described previously (47). For time-lapse imaging, cells were imaged every 10 minutes for the duration of the experiment. Differential interference microscopy and epifluorescence microscopy was performed with an inverted Nikon Eclipse TiE with a QImaging Rolera em-c² 1K EMCCD camera and Nikon Elements Imaging Software.

Cell viability assays. The cell viability assay was performed as described (47). For cell viability spot assays, cultures were grown overnight and diluted to an OD₆₀₀ = 0.05 and serially diluted in LB. 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 48 h before imaging. When appropriate LB plates contained KM 300 μ g/ml, IPTG 1mM as indicated in figure legends. For the DipM or DipM-derived depletion strains, cells were grown in LB media in the presence

of 1 mM IPTG to an $OD_{600} = 0.6$. The cells were washed 3 times by centrifugation in LB media to remove the inducer and resuspended to an $OD_{600} = 0.05$ in LB media. Serial dilutions were performed, and 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 36 h before imaging. Each condition was tested in duplicate during three independent experiments.

Ampicillin susceptibility assay. Antibiotic susceptibility of *A. tumefaciens* was determined on solid cultures using the cell viability protocol.

SDS hypersensitivity/outer membrane integrity assay. For the SDS hypersensitivity assay, exponentially growing cells were diluted to an $OD_{600} = 0.5$. 0.25ml of each culture was aliquoted into 1.5 ml tubes and centrifuged at 7000 x rpms for 5 minutes. Cells were resuspended in 0.25 ml of various concentrations of SDS solubilized in HEPES buffer at a pH 7.4, and incubated for 5 min. Untreated controls were resuspended in 0.25 ml of HEPES buffer only. 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 24 h before imaging. When treating depletion strains with SDS, cells were depleted for a specified amount time, treated with SDS for 5 min and plated in medium containing IPTG and no SDS.

Nitrocefin Assay. Nitrocefin is a chromogenic substrate for measuring beta-lactamase activity. Nitrocefin has an absorbance maxima of 390 nm. Upon hydrolysis of the β -lactam ring by a β -lactamase, the absorbance shifts from 390

nm to 486 nm. By monitoring absorbance at 486 nm over time and using Beer's law ($A=\epsilon lc$) we can directly measure the rate of β -lactamase hydrolytic activity. In two separate experiments wildtype *Agrobacterium tumefaciens* and the $\Delta amiD$ mutant were grown in LB until desired optical densities of 0.5 (OD_{600}) were reached. The cells were then pelleted by centrifugation at 13500 rpm for 5 minutes and the supernatant collected. 25.8 μ l of a nitrocefin stock (1 mg/ml in DMSO) was added to 474.2 μ l of assay buffer (20 mM HEPES, 300 mM NaCl, pH 7.5). This gives 100 μ M nitrocefin solution. 200 μ l of the 100 μ M nitrocefin solution was then added to a cuvette with 200 μ l of supernatant (or LB for blank) and mixed briefly by tapping the cuvette. The cuvette is then placed in the spectrophotometer (Nanodrop 2000c) and the absorbance immediately measured at 486 nm in 5 second intervals for 3 minutes. The change in absorbance was converted to change in concentration of hydrolyzed product by using Beer's law ($A=\epsilon lc$) using the extinction coefficient of 20500 $M^{-1} cm^{-1}$ and path length 1.

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TABLES

Table 3-1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference/ Source
<i>Source Plasmids</i>		
pSRKKm-Plac-sfgfp	pSRKKm vector containing lacI ^q and lac promoter with sfGFP	(48)
pSRKKm-Pcym-sfGfp	pSRKKm vector containing lac promoter flanked by <i>cuO</i> operator site along with the <i>cymR</i> repressor gene	This study
pSRKKm-T7-sfgfp	pSRKKm vector containing T7 and lac promoters with sfGFP	(49)
pNTPS139	Km ^r ; Suicide vector containing <i>oriT</i> and <i>sacB</i>	D. Alley
<i>Replicating Plasmids</i>		
pSRKKm-Pcym- <i>Atu0009-sfgfp</i>	pSRKKm vector containing <i>Atu0009-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu0092-sfgfp</i>	pSRKKm vector containing <i>Atu0092-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu0572-sfgfp</i>	pSRKKm vector containing <i>Atu0572-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu1022-sfgfp</i>	pSRKKm vector containing <i>Atu1022-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu2112-sfgfp</i>	pSRKKm vector containing <i>Atu2112-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu2117-sfgfp</i>	pSRKKm vector containing <i>Atu2117-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu2122-sfGfp</i>	pSRKKm vector containing <i>Atu2122-sfGfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu2451-sfgfp</i>	pSRKKm vector containing <i>Atu2451-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu2489-sfgfp</i>	pSRKKm vector containing <i>Atu2489-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu3093-sfgfp</i>	pSRKKm vector containing <i>Atu3093-sfgfp</i> under control of the cym promoter	This study
pSRKKm-Pcym- <i>Atu3779-sfgfp</i>	pSRKKm vector containing <i>Atu3779-sfgfp</i> under control of the cym promoter	This study
pSRKKm-T7- <i>amiD-sfgfp</i>	pSRKKm vector containing <i>Atu3779-sfgfp</i> under control of the T7 promoter	This study
<i>Deletion plasmids</i>		

pNTPS139Δ <i>Atu0009</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu0009</i>	Cava Lab
pNTPS139Δ <i>Atu0092</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu0092</i>	Cava Lab
pNTPS139Δ <i>Atu1022</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu1022</i>	Cava Lab
pNTPS139Δ <i>Atu2112</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu2112</i>	Cava Lab
pNTPS139Δ <i>Atu2117</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu2117</i>	Cava Lab
pNTPS139Δ <i>Atu2122</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu2122</i>	Cava Lab
pNTPS139Δ <i>Atu3093</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu3093</i>	This study
pNTPS139Δ <i>Atu3779</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu3779</i>	Cava Lab
pNTPS139 Δ <i>amiD</i>	Km ^r Suc ^s ; deletion plasmid for <i>amiD</i>	Cava Lab
<i>E. coli</i> strains		
DH5α	Cloning strain	Life Technologies
S17-1	Sm ^r ; RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	(50)
<i>A. tumefaciens</i> strains		
C58	Nopaline type strain; pTiC58; pAtC58	
C58 Δ <i>0009</i>	Deletion strain for Δ <i>mltA</i>	This study
C58 Δ <i>0092</i>	Deletion strain for Δ <i>mltB1</i> (<i>Atu0092</i>)	This study
C58 Δ <i>1022</i>	Deletion strain for Δ <i>slt1</i> (<i>Atu1022</i>)	This study
C58 Δ <i>2112</i>	Deletion strain for Δ <i>slt2</i> (<i>Atu2112</i>)	This study
C58 Δ <i>2117</i>	Deletion strain for Δ <i>slt3</i> (<i>Atu2117</i>)	This study
C58 Δ <i>2122</i>	Deletion strain for Δ <i>mltB2</i> (<i>Atu2122</i>)	This study
C58 Δ <i>3779</i>	Deletion strain for Δ <i>mltB3</i> (<i>Atu3779</i>)	This study
C58 Δ <i>tetRA</i> ::a- <i>attTn7</i>	Replacement of the Δ <i>tetRA</i> locus with an artificial <i>attTn7</i> site	(48)
C58 Δ <i>tetRA</i> :: a- <i>attTn7</i> Δ <i>3093</i>	Deletion strain for Δ <i>slt4</i> (<i>Atu3093</i>)	This study
C58 Δ <i>tetRA</i> ::a-	C58 with empty pSRKKm-T7-plasmid	This

<i>attTn7</i> pSRKKm-T7- <i>sfgfp</i>		study
C58 $\Delta tetRA::a-attTn7$ $\Delta amiD$	Deletion strain for $\Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7$ $\Delta amiD$ pSRKKm-T7- <i>amiD-sfgfp</i>	$\Delta amiD$ deletion strain with constitutive expression of <i>amiD</i> . Complementation strain for $\Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu0009-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu0009-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu0092-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu0092-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu0572-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu0572-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu1022-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu1022-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu2112-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu2112-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu2117-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu2117-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu2122-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu2122-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu2451-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu2451-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu2489-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu2489-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7$ pSRKKm-	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu3093-sfGFP</i>	This study

Pcym- <i>Atu3093-sfgfp</i>		
C58 $\Delta tetRA::a-attTn7$ pSRKKm-Pcym- <i>Atu3779-sfgfp</i>	C58 $\Delta tetRA::a-attTn7$ strain with cumate-inducible expression of <i>Atu3779-sfGFP</i>	This study

Table 3-2. Synthesized DNA primers used in this study.

Synthesized DNA	Sequence
Primers	
Atu2113 NdeI F	5'- GTCGTCCATATGAAAGAATGTCTGCCGGA T-3'
Atu2113 BamHI R (-STOP)	5'- GTCGTCCGATCCGGCGGCCGAAAATCT-3'
Atu2113 P1	5'- GTCGTCACTAGTCAGATTTTCGATTTC GGACGAA-3'
Atu2113 P2	5'- AAGCTTGGTACCGAATTCCGAACATTCTT CATGCGACG-3'
Atu2113 P3	5'- GAATTCGGTACCAAGCTTCTGCCGAGATT TTCGGCCGCCTGA-3'
Atu2113 P4	5'- GTCGTCCGTACCCACCGAAACCACGGC ATGCGCCA-3'
Atu2113 P5	5'-GCGCAATCGGCGACGCGG-3'
Atu2113 P6	5'-GTCGTCTGCTGCACACTTCGCCGC-3'
Atu3779 P4	5'-CAGGAAATCACCGATGACCAGAAC-3'
Atu3779 P5	5'-AGGCTCATCGCCGACGATATCAC-3'
Atu3779 P6	5'-TATTCCTTGAACCAGACTTCCGTGC-3'
Atu0009 NdeI F	5'- GTCGTCCATATGAATACGCCCTTTTCGAT CGAC-3'
Atu0009 NdeI R (-STOP)	5'- GTCGTCCGATCCTCGCCGATACCTTTCCG CAGC -3'
Atu0572 NdeI F	5'- GTCGTCCATATGCCGGAAGGAATTCTCTA T-3'

Atu0572 BamHI R (-STOP)	5'- GTCGTCGGATCCTCCGTCGCAAAAGTTTT TCGC-3'
Atu0092 NdeI F	5'- GTCGTCCATATGCCACACCGATTTGCTAC C -3'
Atu0092 BamHI R (-STOP)	5'- GTCGTCGGATCCTTTCAGCTTCGCCTTCA GTGT -3'
Atu1022 NdeI F	5'- GTCGTCCATATGGCGGCGGGGCTTGCCG CA -3'
Atu1022 BamHI R (-STOP)	5'- GTCGTCGGATCCCCCGCACGTCCATGG CGCAG -3'
Atu2112 NdeI F	5'- GTCGTCCATATGAACAGGTTTGTGTTGC TGTC -3'
Atu2112 BamHI R (-STOP)	5'- GTCGTCGGATCCGCTCGCCAGCAATGCC TG-3'
Atu2117 NdeI F	5'- GTCGTCCATATGCGAAATTCGGGCAAATT CCGC -3'
Atu2117 BamHI R (-STOP)	5'- GTCGTCGGATCCGTTCCGCAGAACGTGC AGCAT -3'
Atu2122 NdeI F	5'- GTCGTCCATATGCCCACCTTCCGGTCGCG C -3'
Atu2122 BamHI R (-STOP)	5'- GTCGTCGGATCCAAGCGCCTGGAGAAGT GCCTG -3'
Atu2451 NdeI F	5'- GTCGTCCATATGCGTGGCGTATTTGCCGT T-3'
Atu2151 BamHI R (-STOP)	5'- GTCGTCGGATCCGCGATATCCGCACTGCT

	GTAG -3'
Atu2489 NdeI F	5'- GTCGTCCATATGCGGGCAATGAAGGCAAT TCTG -3'
Atu2489 BamHI R (-STOP)	5'- GTCGTCTGGATCCTTGGCCGTCGATCGCCT TGCC -3'
Atu3093 NdeI F	5'- GTCGTCCATATGGCTCTTGCCTTCTATTTG CTT-3'
Atu3093 BamHI R (-STOP)	5'- GTCGTCTGGATCCACGGTTGCGGAACACG GT-3'
Atu3779 NdeI F	5'- GTCGTCCATATGACAAAGACCCTTTCAAAT GTC-3'
Atu3779 KpnI R (-STOP)	5'- GTCGTCTGGTACCATTGCGCAGGGCTCTCA GCAA-3'

CHAPTER 4

Regulation of Peptidoglycan (PG) Hydrolases Involved in the Septal PG Hydrolysis of *Agrobacterium tumefaciens*

Author Contributions

WFC and CD conducted the experiments. WFC, CD and PB designed experiments. CD, WFC and PB analyzed data. WFC and PB wrote and edited the dissertation chapter.

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A. tumefaciens

ABSTRACT

The Alphaproteobacteria are known for their diversity of cell shapes and growth patterns. The plant pathogen *Agrobacterium tumefaciens* is the emerging model for the study of polar growth within the order Rhizobiales. Currently, the mechanisms by which polar growing bacteria regulate peptidoglycan (PG) hydrolysis during cell separation and new growth pole formation remain mostly unknown. In an effort to understand the protein networks that regulate the cell separation process in *A. tumefaciens*, we characterized the function of putative PG hydrolase regulators in *Agrobacterium*. Our results show that DipM is essential for cell survival. Time-lapse DIC microscopy reveals that the absence of DipM causes severe cell division defects, including significant changes in cell length and max mid-cell width. Cell lysis induced by overexpression of DipM requires the amidase, AmiC, suggesting that DipM activates AmiC activity. Changes in PG muropeptide composition in the absence of DipM suggest that this protein regulates lytic transglycosylases. Remarkably, the cell division phenotypes associated with the absence of DipM can be delayed upon deletion of lytic transglycosylases. Together these results suggest that DipM is directly or indirectly regulating a complex network of cell wall hydrolases in *A. tumefaciens*, allowing cell separation to proceed.

Keywords: *Agrobacterium*, peptidoglycan hydrolases, amidases, lytic transglycosylases, LytM, cell separation

IMPORTANCE

Current public health issues such as the emergence of antibiotic drug resistance in bacterial pathogens, call for improved understanding fundamental of processes in the cell biology of bacteria, including the discovery of novel protein targets that participate in essential processes for bacterial survival. PG hydrolases, which are known to mediate the bacterial cell separation process, cause bacterial cell lysis if the precise regulatory mechanisms are not in place. Thus, elucidating the mechanisms that control cell separation process in bacteria may provide additional candidate targets for antimicrobial therapies. Characterization of these essential processes in non-canonical bacterial models will provide a more a comprehensive understanding of the diverse mechanisms that drive essential processes in bacteria.

INTRODUCTION

In bacteria, the peptidoglycan (PG) cell wall plays an essential role in maintaining cell shape, protecting bacteria from environmental stressors, and preventing cell lysis (1–5). The PG cell wall is a net-like structure that consists of glycan strands containing alternating β -1,4-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) sugars crosslinked together through peptide stems (6). While the bacterial PG cell wall is necessary and sufficient to determine bacterial cell shape, expansion and separation of the cell wall requires enzymatic action (7). In order to expand and separate, coordination between PG cell wall

synthesis and hydrolysis is necessary to allow insertion and remodeling of new cell wall material at very specific locations within the cell (8).

Bacterial PG hydrolases are a highly diverse group of enzymes that contribute to many important processes in the cell, including PG biosynthesis (9), separation of bacterial cells (10–15), peptidoglycan recycling, (16–20) and insertion of structures that span across the cell envelope, including flagella and secretion systems (21, 22). In Gram-negative organisms, the cell separation process is specifically mediated by cell wall amidases, lytic transglycosylases (LTs) and regulators of these hydrolyses called LytM factors (14) (Figure 4-1).

In members of the Gammaproteobacteria, including *E. coli*, *P. aeruginosa* and *V. cholera*, inactivation of amidases results in a block in the cleavage of septal PG cell wall, which leads to cells connected through the outer membrane, or cell chaining (15, 20, 23). Interestingly, the block in septal cleavage can be exacerbated by deleting LTs in the same amidase-null deletion background suggesting that LTs contribute to cell separation (14). Finally, overexpression of amidases or LTs is lytic in several Gammaproteobacteria suggesting that a tight regulation of PG hydrolases is required to avoid cell lysis. Indeed in *E. coli* and *Vibrio cholera* the catalytically-inactive endopeptidases, LytM-containing proteins, function as regulators of amidase activity. The catalytically-inactive LytM-factors are recruited to mid-cell by cell division proteins FtsN and FtsEX (24, 25). Once at mid-cell, LytM-factors activate amidases by causing a conformational change

PG hydrolases: Endopeptidases Carboxypeptidases Amidases
Lytic transglycosylases

PG hydrolase regulators: Inactive Endopeptidases

Figure 4-1. Peptidoglycan hydrolases of *A. tumefaciens*.

A. tumefaciens peptidoglycan hydrolases include endopeptidases (blue) and carboxypeptidases (yellow), which cleave LD- and DD-bonds in the peptide stems. *N*-Acetylmuramoyl-L-alanine amidases (pink) cleave the peptide bond that links the NAM sugar with the peptide stem, leading to naked glycan strands or naked NAM sugars. Lytic transglycosylases (LTs, green) cleave β -1,4 glycosidic linkages in the glycan strands. This cleavage event leads to the formation of terminating sugars containing a 1,6-anhydromuramic acid rings. Lastly, two endopeptidases predicted to be catalytically inactive likely function

regulators of PG hydrolases (dark blue, top right).

that displaces an amidase autoinhibitory loop away from the catalytic domain causing hydrolysis of peptide stems (26).

Remarkably, the regulation of cell separation is not universally conserved. In the Alphaproteobacterium *Hypomonas neptunium*, absence of AmiC results in a block of septal PG and cell chaining indicating that this process may be conserved in some members of the class Alphaproteobacteria (27). In contrast, absence of the only amidase, AmiC, in the Alphaproteobacterium *Caulobacter crescentus*, causes no significant alterations of cell morphology or growth rates (28, 29). In addition, deletion of the putative activator of PG hydrolysis, DipM, causes a significant increase of cell length instead of cell chaining (12, 13, 30). Thus, it is clear that the regulation of PG hydrolase activity is varied among Alphaproteobacteria.

Here, we sought to expand our knowledge of the cell separation processes in Alphaproteobacteria by exploring this process in the bacterial plant pathogen, *Agrobacterium tumefaciens*. We show that the catalytically-inactive endopeptidases, DipM and EnvC, are required for efficient cell division, proper cell shape, and cell separation. Removal of lytic transglycosylases in a strain lacking DipM delays the block in cell division suggest that DipM regulate a complex network of PG hydrolases.

RESULTS AND DISCUSSION

4.1 PG hydrolases in the genome of *A. tumefaciens*.

In an effort to understand the cell separation process, we performed a comprehensive bioinformatic analysis of cell wall hydrolases found in the genome of *A. tumefaciens*. Our analysis revealed the presence of genes encoding for canonical PG hydrolases such as endopeptidases, amidases, lytic transglycosylases, carboxypeptidases and putative inactive LytM-factors, which previously have been found to serve as regulators of cell separation in bacteria (10, 12, 13, 30, 31) (Table 4-1).

4.1.1 Peptidoglycan amidases of *A. tumefaciens*

Based on the general amino acid sequence of the amidase catalytic domain, bacterial amidases can be further classified into two types: amidase type 2 and type 3 (22). The *A. tumefaciens* genome encodes a single type 2 amidase, AmiD_{AT}, that contributes to cytoplasmic peptidoglycan recycling pathways and is required for regulation of β -lactamase expression (see Chapter 3). Type 3 amidases hydrolase the amide bond that link the peptide stem to the glycan strand (Figure 4-1, pink). In *E. coli*, type 3 amidases are involved in the cell separation process (14). Indeed, type 3 amidases in *E. coli* have been shown to localize at mid-cell or to the periplasmic region during final stages of the cell division process and require cell division proteins, such as FtsN and FtsEX, for recruitment to mid-cell (24, 32). Moreover, deletion of amidases results in the formation of chains of cells connected through their outer membrane in several

AMIDASES	Gene ID	Signal P 4	ESS#	Similarity to CCNA	Family Type or Class	Comparison to CCNA
<u>AmiC</u>	<u>Atu1340</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>61.50%</u>	<u>Type 3</u>	<u>CC 1876</u>
<u>AmiD</u>	<u>Atu2113</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>35.50%</u>	<u>Type 2</u>	<u>CC 2567</u>
MLTs¹						
<u>MitA</u>	<u>Atu0009</u> <u>MitA</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>53.00%</u>	<u>Family 2</u>	<u>CC 3740</u>
<u>MitB1</u>	<u>Atu0092</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>58.10%</u>	<u>Family 3</u>	<u>CC 3322</u>
<u>MitB2</u>	<u>Atu2122</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>57.30%</u>	<u>Family 3</u>	<u>CC 3322</u>
<u>MitB3</u>	<u>Atu2489</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>47.10%</u>	<u>Family 3</u>	<u>CC 3322</u>
<u>MitB4</u>	<u>Atu3779</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>58.70%</u>	<u>Family 3</u>	<u>CC 3322</u>
SLTs²						
<u>Slt</u>	<u>Atu0572</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>15.10%</u>	<u>Family 1</u>	<u>CC 1332</u>
	<u>Atu1022</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>18.60%</u>	<u>Family 1</u>	<u>CC 1332</u>
	<u>Atu2112</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>9%</u>	<u>Family 1</u>	<u>SdpA</u>
	<u>Atu2117</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>11.20%</u>	<u>Family 1</u>	<u>SdpA</u>
	<u>Atu2451</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>5.10%</u>	<u>Family 1</u>	<u>SdpA</u>
	<u>Atu3093</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>7.70%</u>	<u>Family 1</u>	<u>SdpA</u>
	<u>*Atu5162</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>-</u>	<u>Family 1</u>	<u>N/A*</u>
	<u>*Atu6167</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>-</u>	<u>Family 1</u>	<u>N/A*</u>
DD-EPases³						
	<u>Atu4178</u>	<u>Periplasmic</u>	<u>Essential</u>	<u>35.50%</u>	<u>M23</u>	<u>LdpA</u>
	<u>Atu1832</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>40%</u>	<u>M23</u>	<u>LdpA</u>
dLytM⁴						
	<u>Atu1700</u> <u>DipM</u>	<u>Periplasmic</u>	<u>Essential</u>	<u>41.20%</u>	<u>M23</u>	<u>DipM</u>
	<u>Atu2775</u> <u>EnvC</u>	<u>Periplasmic</u>	<u>Essential*</u>	<u>43.30%</u>	<u>M23</u>	<u>LdpF</u>
CPases⁵						
<u>PBP5</u>	<u>Atu1499</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>32.60%</u>	<u>S11</u>	<u>CC3628</u>
<u>PBP4B</u>	<u>Atu3077</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>37.80%</u>	<u>Class C</u>	<u>CC0575</u>
	<u>Atu0929</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>27.60%</u>	<u>Class C</u>	<u>CC0575</u>
	<u>Atu1917</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>34.50%</u>	<u>Class C</u>	<u>CC0575</u>
	<u>Atu2513</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>33.40%</u>	<u>Class C</u>	<u>CC0575</u>
	<u>Atu1505</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>35%</u>	<u>S11</u>	<u>CC3628</u>
	<u>Atu2321</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>35.20%</u>	<u>S11</u>	<u>CC3628</u>
	<u>Atu3634</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>30%</u>	<u>S11</u>	<u>CC3628</u>
	<u>Atu0933</u>	<u>Periplasmic</u>	<u>Not essential</u>	<u>28.10%</u>	<u>Class D</u>	<u>CC3628</u>
	<u>Atu3376</u>	<u>Cytoplasmic</u>	<u>Not essential</u>	<u>25.10%</u>	<u>Class A</u>	<u>CC3628</u>

Table 4-1. Putative PG hydrolases found in the genome of *A. tumefaciens*.

Examination of the genome of *A. tumefaciens* using the BLAST server and the CAZyme server led to the finding of amidases, lytic transglycosylases¹⁻² (Membrane-bound lytic transglycosylases¹ and soluble lytic transglycosylases²) DD-endopeptidases³ (DD-EPases) and carboxypeptidases⁴ (CPases) in the genome of *A. tumefaciens*. Columns indicate (1) the canonical name of the gene, (2) the locus tag of the gene in the genome of *A. tumefaciens* strain C58, (3) putative localization of the protein based on Signal P 5.0, (4) essentiality of the gene based on TnSeq data (33), (5) percent similarity to *C. crescentus* CCNA genes using a ClustalW protein alignment, Geneious software, % similarity (Blosum 45 with threshold 0), (6) protein family name, and (7) *C. crescentus* (CCNA strain) homologs were gathered using BLAST (best-hits) and the EggNOG orthology prediction pipeline. *Indicate no homologs in *Caulobacter crescentus* or genes involved in *A. tumefaciens* plant gall formation.

bacterial organisms (11, 15, 23) suggesting that the hydrolytic function of amidases is required at mid-cell during late stages of the cell division for the efficient cleavage of septal PG synthesis. In *C. crescentus*, however, no alterations in morphology were observed upon deletion of *amiC* (28, 29). Overexpression of AmiC in *Caulobacter* causes cell chaining and cell separation defects suggesting that too much AmiC activity is detrimental cell division (34). To better understand the cell separation process in *A. tumefaciens*, we characterized the contribution of AmiC to cell division. This amidase contains a signal sequence for periplasmic transport, an AMIN domain shown to be a PG-binding domain involved in the localization of proteins to the septum during cell division (26) and the amidase_3 catalytic domain involved in PG hydrolysis (Figure 4-2A). In comparison to the *C. crescentus* homolog for AmiC, amino acid alignment show 61.5% similarity with *A. tumefaciens* AmiC.

AmiC-sfGFP localizes at mid-cell during cell division in WT cells (Figure 4-2B). Deletion of AmiC results in a marked cell viability defect that can be complemented with a plasmid containing AmiC suggesting the viability defect observed in $\Delta amiC$ is AmiC-dependent (Figure 4-2C). In addition, we found that $\Delta amiC$ cells are highly susceptible to hypertonic environments and detergents such as SDS suggesting an increased outer membrane permeability in the absence of AmiC (Figure 4-2C,D).

To understand other contributions of AmiC, we assessed the morphology and cell length of $\Delta amiC$ cells. Our results show that $\Delta amiC$ significantly alters cell length (Figure 4-2E). Time-lapse DIC microscopy (Figure 4-2E) and cell curvature analysis (Figure 4-2F) show that $\Delta amiC$ leads to a significant increase in cell curvature. Indeed, the cells exhibit C- and S-shaped morphologies, and other cell division defects such as tip splitting, hyper constrictions and asymmetric cell divisions. Together, these results suggest that the periplasmic cell wall amidase AmiC is required for outer membrane permeability, proper cell growth and shape, cell division and separation.

4.1.2 Lytic transglycosylases of *A. tumefaciens*

Bacterial lytic transglycosylases (LTs) can be subdivided into three families. Family 1, or soluble lytic transglycosylases (SLTs), have been shown to be involved in bacterial cell separation (14, 20), PG recycling, β -lactam resistance and outer membrane permeability (18, 19, 35) (Chapter 3). Family 2 corresponds to the membrane-bound lytic transglycosylases A (MltA). In *E. coli*, deletion of MltA leads to cell separation defects, and overexpression causes severe cell lysis and spheroplasting (36, 37). Besides crosslinked PG, MltA is the only family of LTs that can accept soluble naked glycan chains as substrates. Deletion of MltA does not affect growth or morphology in *E. coli* (36, 38). Lastly, family 3 or the membrane-bound lytic transglycosylases B, MltB has been shown to be involved in exolytic and endolytic PG cleavage reactions (22, 38, 39). In addition, deletion of the MltB family results in β -lactam resistance or hypersensitivity and

Figure 4-2. AmiC is the major cell wall amidase in *A. tumefaciens*.

(A) Schematic representation of AmiC containing domains, which are highlighted in colors. The corresponding AmiC ORF number can be found in Table 4-1. (B) Subcellular localization of AmiC in WT *A. tumefaciens* cells. Cells expressing AmiC-sfGFP were grown to exponential phase and imaged by phase and

fluorescence microscopy. Arrows indicate the polar to mid cell localization of AmiC-sfGFP. (C) Viability of amidase-deficient cells spotted on LB solid media, or (D) LB + 2% salt. Exponentially growing cultures were serially diluted, spotted on solid LB agar and incubated at 28°C for 36 h before imaging. (E) Outer-membrane permeability using an SDS-based assay shows $\Delta amiC$ leads to increased permeability and lower detergent tolerance. (F) Cell length distribution of WT, amidase-deficient and complementing mutants grown in liquid media. The cell length distribution is shown in violin plots. The median values (M_{Length}) and representative values are also shown (top). The violin plot middle dash line represents the median cell length, top (Q1) and lower (Q2) quartiles represent the 75% and 25% of the population, respectively. **** = p-value <0.0001. Data was analyzed by a one-way ANOVA followed by a Dunnett's multiple comparison test. Comparisons are to WT. (G) Scatter plot indicates the relationship between the WT cell length and curvature with that of *amiC*-deficient cells. (H) Time-lapse microscopy of WT and *amiC*-deficient cells over the course of 8 h.

increased outer membrane permeability (35, 38). Bioinformatic analysis using the CAZyme (Carbohydrate Active Enzyme) database (CAZy, <http://www.cazy.org>) and the BLAST server (40), we identified thirteen lytic transglycosylases belonging to families 1-3. More specifically, we found eight LTs containing the Slt_1 domain characteristic of LT family 1, a single member belonging to family 2 (MltA) and four members of family 3 (MltB), which contain Slt_2 domains (Table 4.1). Most of the LTs found in *A. tumefaciens* genome contain signal sequence domains for periplasmic transport, which highlights their putative involvement in a wide array of periplasmic activities in *A. tumefaciens*. A transposon mutagenesis screening performed in *A. tumefaciens* revealed the LTs found in the genome are dispensable for viability suggesting a high level of redundancy (33). Indeed, single deletion of LTs causes no morphological phenotypes or alterations in cell viability (Chapter 3).

4.1.3 *LytMs* of *A. tumefaciens*

LytM-containing factors are zinc-metallo-endopeptidases that cleave crosslinks in the PG cell wall (Figure 4-1, light blue) (9). Some LytM-containing proteins lack chelating residues involved in cofactor-binding and crosslink cleavage (41) and these catalytically inactive or degenerate LytMs (dLytMs) proteins have evolved regulatory functions (Figure 4-1, dark blue). The genome of *A. tumefaciens* encodes for four LyM-containing proteins: DipM (Atu1700), EnvC (Atu2775), Atu4178 and Atu1832 (Table 4-1). In saturating a transposon mutagenesis screening, DipM, EnvC (chapter 5) and Atu4178 were found to be essential for

viability in rich medium, whereas Atu1832 (data not shown) was dispensable for viability (33). A primary structure analysis of the *A. tumefaciens* LytM-containing factors suggests that only EnvC is soluble in the periplasm, whereas DipM is a lipoprotein anchored to the outer membrane. Atu4178 and Atu1832 contain a predicted transmembrane (TM) domains at the N-terminus and are hypothesized to be anchored to the bacterial inner membrane, while the C-terminal catalytic domain lays in periplasmic space. DipM contains two LysM domains for PG-binding, predicted to be critical for its localization. Both EnvC and Atu1832 contain predicted coil-coiled regulatory domains. A comparison between the C-terminal LytM domains of the four LytM-containing factors revealed that DipM and EnvC lack several chelating residues required for endopeptidase activity (Figure 4-3A). In contrast, Atu4178 and Atu1832, were predicted to be active DD-endopeptidases involved in the cleavage of crosslinks that link peptide stems in the PG cell wall (Figure 4-1, light blue). Active DD-endopeptidases have been shown to be involved in the growth process of several bacteria (9, 42, 43). Depletion of Atu4178 leads to a block in polar elongation and complete rounding in *A. tumefaciens* by 24 h (Figure 4-3B). Analysis of cell length indicates a substantial decrease in cell length during depletion (Figure 4-3C). Together, these results suggest that Atu4178 is an active space-making DD-endopeptidase involved in the growth of *A. tumefaciens* and not in cell separation.

25% of the population, respectively. Error bar caps indicate the highest (top) and smallest (bottom) cell length values. Area outside of the error bar caps are outliers. Median cell length are displayed above each box.

4.2 DipM localizes to mid-cell during cell division

The high redundancy found in the different PG hydrolase families led us to the characterization of the function of the two putative regulators, DipM and EnvC. DipM-sfGFP displays mid-cell localization in pre-divisional cells and is retained at the growth poles following cell division (Figure 4-4B). In most bacteria, initiation of cell division is initiated and driven by the bacterial tubulin, FtsZ (44–47). Mid-cell localization and anchoring of the scaffolding protein FtsZ, leads to the hierarchical recruitment of dozens of essential and accessory cell division proteins, which together form the divisome. The primary function of the divisome is the synthesis of septal PG and formation of the incipient bacterial cell poles (48, 49). In bacteria, most core divisome proteins are essential for cell survival (44). To investigate whether DipM is recruited to mid-cell by cell division proteins, DipM-sfGFP was assessed in different strains lacking essential cell division proteins. In the presence of FtsN, DipM-sfGFP localizes to the growth pole during elongation and to mid-cell during cell division. In contrast, depletion of FtsN (-FtsN) causes DipM-sfGFP to become trapped at the growth poles suggesting that DipM requires FtsN for recruitment to mid-cell (Figure 4-4C). These results suggest that DipM is a cell division accessory proteins recruited to mid-cell by the core cell division machinery.

4.3 DipM is required for efficient cell division and separation in *A. tumefaciens*

In a saturating transposon mutagenesis screen, DipM was shown to be essential

Figure 4-4. DipM localizes to mid-cell in predivisive cells.

(A) Schematic representation of DipM. *represents LytM-domain lacking of several chelating residues involved in PG hydrolysis. (B) Dynamic subcellular localization of DipM in WT *A. tumefaciens* and in FtsN depletion ($\downarrow$) strains was observed. Cells expressing DipM-sfGFP were grown to exponential phase and imaged by phase and fluorescence microscopy. (C, left) Severe cell viability

defect in the absence of DipM (-DipM). (C, right) Increased SDS sensitivity is indicative of increased outer membrane permeability in the absence of DipM and EnvC. (D) Increased cell area and max cell width for -DipM at 16 h of depletion. The cell area distribution is shown in violin plots. The violin-plot mid-line represents the median cell length, top (Q1) and lower (Q2) quartiles represent the 75% and 25% of the population, respectively. Phase-micrographs of representative cells and their median cell area are also displayed (top). (E) Time-lapse DIC microscopy of DipM-deficient cells over the course of 16 h reveals cell division defects.

for survival (33). Consistent with this data, we were unable to delete *dipM* from the genome of *A. tumefaciens*. Thus, we constructed a *dipM* depletion strain (55, see Chapter 2), where the only copy of *dipM* is located at a neutral site on the chromosome under the inducible control of IPTG promoter. When DipM is produced in the presence of IPTG (+DipM) the cells are viable, whereas removal of IPTG (-DipM) leads to a severe cell viability defect (Figure 4-4D). DipM depletion causes cells to become sensitive to SDS (Figure 4-4E) suggesting cell surface integrity is not properly maintained. DipM depletion causes cell area to increase and ultimately leads to a complete block in cell division, consistent with the presence of mid-cell bulges and aberrant cell morphology (Figure 4-4F,G). Together, these results suggest that DipM is required for efficient cell division, separation and proper outer membrane integrity.

4.4 Decreased viability caused by DipM overexpression is AmiC dependent

Overexpression of amidases, lytic transglycosylases and regulators of PG hydrolases leads to lysis in several bacterial organisms (7, 36, 38). We reasoned that if the activity of amidases and/or LTs is regulated by DipM, then overexpression of DipM in amidase- and LT-deficient backgrounds should prevent toxicity caused by DipM overexpression. To overexpress DipM, a plasmid containing *dipM* under the control of a pTAC promoter, resulting in production of about ~30X DipM (50), was introduced into WT cells.

Overexpression of DipM led to a severe cell viability defect (Figure 4-6A) and the formation of visible membrane vesicles (Figure 4-6B). In contrast, overexpression

of DipM in the $\Delta amiC$ and $\Delta amiC \Delta amiD$ mutants does not decrease viability or cause membrane vesicle formation (Figure 4-6A-B). Together, these results suggest that DipM functions as an activator of AmiC activity.

4.5 DipM regulate lytic transglycosylase activity

Although DipM is an activator of AmiC, the phenotypic differences between the DipM depletion strain (Figure 4-5) and $\Delta amiC$ (Figure 4-2) suggest that DipM has additional functions. Indeed, the cell wall composition of cells after 16 h of DipM depletion reveals an increased abundance of M5 monomers and anhydromuropeptides (Figure 4-7). The increase in M5 monomers indicates decreased PG carboxypeptidase activity (Figure 4-1, yellow) (Figure 4-7) suggesting that DipM may be necessary for the activation of carboxypeptidases. The increased level of anhydromuropeptides, which are the product of lytic transglycosylases, suggests that DipM may contribute to regulation of LT activity. *A. tumefaciens* has many PG hydrolytic enzymes, including 13 LTs and 11 carboxypeptidases (Table 4-1) suggesting that PG hydrolase activities may be regulated. The striking increase in anhydromuropeptides abundance (Figure 4-7), which in WT cells are below detection levels, suggests that DipM may contribute to the tight LT regulation in *A. tumefaciens*. We hypothesized that DipM may regulate the activity of one or more major LTs. To test this hypothesis,

Figure 4-5. AmiC is required decreased cell viability induced by DipM overexpression.

(A) Decreased viability of WT and $\Delta amiD$ strains after overexpression of DipM.

Overexpression of DipM requires AmiC to reduce cell viability. (B) Morphology of WT, $\Delta amiC$, $\Delta amiD$ and $\Delta amiC\Delta amiD$ after overexpression of DipM for 4 h reveals the formation of membrane vesicles (white arrow) when AmiC is present.

Figure 4-6. Cell wall composition analysis of DipM depleted cells.

Depletion of DipM leads to an increase of M5s (the products of carboxypeptidases, Figure 4-1, yellow), decreased levels of LD-3,4s and increase abundance of anhydromuropeptides (the products of lytic transglycosylases, Figure 4-1, green).

we deleted single LTs in the DipM depletion strain (Figure 4-7 A-B). Single deletions of LTs in WT do not cause major changes to cell morphology or viability (Chapter 3, Figure 3-4B,C). Remarkably, single deletions of LTs in the DipM depletion strain delay the onset of the phenotypes associated with the loss of DipM (Figure 4-7 A-C). Viability assays of mutants with single LTs deletions in the DipM depletion strain reveal that these strains are viable only if DipM is present when the cells are spotted (Figure 4-7A); however, if DipM is depleted for 24 h prior to spotting, the deletion of single LTs is not sufficient to restore viability (Figure 4-7B). These findings suggest that the absence of any LT delays the onset of the DipM phenotype but does not bypass DipM. Indeed, early during DipM depletion, the absence of an LT appears to protect the cells (Figure 4-7C) but later during depletion the characteristic phenotype of DipM depletion emerges in these strains (Figure 4-7D). Together, these results suggest that DipM is directly or indirectly involved in the regulation of LTs.

CONCLUSION

Here, we found that the catalytically-inactive LytM factor DipM of *A. tumefaciens* is involved in the cell division and separation of *A. tumefaciens*. DipM is responsible either directly or indirectly for the regulation of LTs and the amidase, AmiC. Although it is tempting to argue that DipM may be an inhibitor of the activities of LTs, current data obtained in other bacterial models suggests that specific modifications on the carbohydrates found in the cell wall can protect the PG cell wall against attacks by hydrolytic enzymes (51). In addition, it has been

Figure 4-7. Deletion of LTs delay the phenotypes associated with DipM depletion.

Cell viability assay of single LT deletion mutants in -DipM after being spotted on solid medium (A) no predepletion show a restoration in the phenotype associated by DipM.

found that LTs, and other hydrolytic enzymes, can recognize specific modifications on PG and alter their activities (52). Thus, perhaps in *A. tumefaciens*, the absence of DipM leads to alterations of the functions of a wide variety of enzymes such as PG modifying enzymes or carboxypeptidases, which could alter the availability of substrates for PG synthases. Thus, more research needs to be done in order to determine whether DipM is an inhibitor of the LT activity, or if depletion of DipM leads to the inactivation of “protective” cell wall-modifying enzymes leading to an unmodified cell wall susceptible to attacks by LTs and other hydrolytic enzymes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Agrobacterium tumefaciens* C58 and derived strains were grown in LB medium at 28°C with shaking. When appropriate, antibiotics were used at the following working concentrations: kanamycin 300 µg/ml and gentamycin 200 µg/ml. When indicated, IPTG was used as an inducer at a concentration of 1 mM. *E. coli* DH5α and S17-1 were routinely cultivated in LB medium at 37°C with shaking. When needed antibiotics were used at the following concentrations: kanamycin 50 µg/ml and gentamycin 20 µg/ml.

Construction of expression plasmids and strains. All strains and plasmids used are listed in Table 4-2. Primers used in this study are listed in Table 4-3. To construct expression vectors containing *dipM-sfgfp*, *envC-sfgfp*, *amiC-sfgfp*, and

amiD-sfgfp corresponding coding sequence was amplified from purified *Agrobacterium tumefaciens* C58 genomic DNA. The amplicons and plasmids pSRKKM-Plac-*sfgfp* or pSRKKM-T7-*sfgfp* were digested overnight and ligated overnight. at 4°C using NEB T4 DNA ligase.

Construction of expression plasmids and strains. Gene deletions were achieved by allelic exchange and vectors were constructed as previously described (53). Briefly, 500 bp fragments upstream and downstream of the gene of interest were amplified using primer pairs P1/P2 and P3/P4. Overlapping PCR was used to the amplicons generated by P1/P2 and P3/P4, 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 Kan resistant, sucrose sensitive primary exconjugants. Primary exconjugants 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. To construct the DipM depletion strain, *dipM* was amplified, digested and ligated into pUC18-mini-Tn7T-GM-Ptac. The mini-Tn7 vector, along with the pTNS3 helper plasmid, were introduced into C58 Δ *tetRA::a-attTn7* as described previously (50). Transformants were selected for gentamicin 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.

Bioinformatic analyses. Proteins of interest were found using BLAST-TP and Cazy (<http://www.cazy.org/b42.html>), and analyzed using TMHMM (54) and SignalP 4.1 (55). Phylogenetic trees were constructed using a ClustalW protein alignment and creating a PhyML tree, version 3.0, as a Geneious plugin using the Geneious Tree Builder with the following settings: Le Gascuel substitution model with 100 bootstrap models.

Fluorescence and time-lapse microscopy. A small volume (~0.8 μ l) of cells in exponential phase was applied to a 1% agarose pad as described previously (56). For time-lapse imaging, cells were imaged every 10 minutes for the duration of the experiment. Differential interference microscopy and epifluorescence microscopy was performed with an inverted Nikon Eclipse TiE with a QImaging Rolera em-c² 1K EMCCD camera and Nikon Elements Imaging Software.

Cell viability assays. The cell viability assay was performed as described (56). For cell viability spot assays, cultures were grown overnight and diluted to an OD₆₀₀ = 0.05 and serially diluted in LB. 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 48 h before imaging. When appropriate LB plates contained KM 300 μ g/ml, IPTG 1mM as indicated in figure legends. For the DipM

or DipM-derived depletion strains, cells were grown in LB media in the presence of 1 mM IPTG to an $OD_{600} = 0.6$. The cells were washed 3 times by centrifugation in LB media to remove the inducer and resuspended to an $OD_{600} = 0.05$ in LB media. Serial dilutions were performed, and 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 48 h before imaging. Each condition was tested in duplicate during three independent experiments.

SDS hypersensitivity/outer membrane integrity assay. For the SDS hypersensitivity assay, exponentially growing cells were diluted to an $OD_{600} = 0.5$. 0.25ml of each culture was aliquoted into 1.5 ml tubes and centrifuged at 7000 x rpms for 5 minutes. Cells were resuspended in 0.25 ml of various concentrations of SDS* solubilized in HEPES buffer at a pH 7.4, and incubated for 5 min. Untreated controls were resuspended in 0.25 ml of HEPES** buffer only. 4 μ l of each dilution was spotted onto LB and incubated at 28 °C for 24 h before imaging. When treating depletion strains with SDS, cells were depleted for a specific amount time, treated with SDS for 5 min and plated in medium containing IPTG and no SDS.

Peptidoglycan analysis. The cell viability assay was performed as described (53). Briefly, for cell wall analysis, cells were grown in 10 ml of LB with and without IPTG (if applicable) exponential phase. The 10 ml cell cultures were added to 40 ml of fresh media. The 50 ml cultures were grown to exponential phase and pelleted by centrifugation at 4000 x g for 10 minutes. Cell pellets were

washed three times with LB by centrifugation and resuspension to remove IPTG (if applicable). After the final wash 3 cell pellets were resuspended in 50 ml LB and the remaining 3 pellets were resuspended in 50 ml ATGN with 1 mM IPTG. Each culture was grown for 14 h. The optical densities of the cells were monitored to ensure the optical density of the cultures never went above OD₆₀₀ = 0.7 to avoid changes to peptidoglycan content due to stationary phase. If necessary, fresh medium was added to dilute the cultures to maintain exponential growth. After 14 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 1 ml of LB and 2 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 min at 60000 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 15000 rpm in a table top microcentrifuge. The muropeptides were mixed with 15 µl 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). Muropeptides were analyzed on a Waters UPLC system equipped with an ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μ m, 2.1 mm $\times$ 150 mm (Waters) and a dual wavelength absorbance detector. Elution of muropeptides was detected at 204 nm. Muropeptides were separated at 45°C using a linear gradient from buffer A [formic acid 0.1% (v/v)] to buffer B [formic acid 0.1% (v/v), acetonitrile 20% (v/v)] in a 12 min run with a 0.250 ml/min flow. Peptidoglycan compositional analysis on triplicate samples was completed on two separate occasions.

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TABLES

Table 4-2

Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference/ Source
<i>Source Plasmids</i>		
pSRKKm-Plac	Kmr ^r ; broad host range vector containing lacI ^q and lac promoter	(57)
pSRKKm-Plac-sfgfp	pSRKKm vector containing lacI ^q and lac promoter with sfGFP	(50)
pSRKKm-Pcym-sfGfp	pSRKKm vector containing lac promoter flanked by <i>cuO</i> operator site along with the <i>cymR</i> repressor gene	This study
pSRKKm-T7-sfgfp	pSRKKm vector containing T7 and lac promoters with sfGFP	Ikeda et. al.
pNTPS139	Km ^r ; Suicide vector containing <i>oriT</i> and <i>sacB</i>	D. Alley
pTNS3	Ap ^r ; helper plasmid encoding the site-specific TnsABCD Tn7 transposition pathway	(58)
pUC18-mini-Tn7T-GM-Ptac-HA	Ap ^r Gm ^r ; mini-Tn7 vector containing lacI ^q and tac promoter	(43)
<i>Replicating Plasmids</i>		
pSRKKm-T7ST- <i>Atu1700-sfgfp</i>	pSRKKm vector containing <i>Atu1700-sfgfp</i> lacking of stop codon and under the constitutive control of the T7 strong promoter	This study
pSRKKm-Ptac- <i>Atu1700-sfgfp</i>	pSRKKm vector containing <i>Atu1700-sfgfp</i> and under the control of the Ptac promoter	This study
pSRKKm-T7MD- <i>Atu1340-sfgfp</i>	pSRKKm vector containing <i>Atu1340-sfgfp</i> lacking of stop codon and under the constitutive control of the T7 medium promoter	This study
<i>Deletion plasmids</i>		
pNTPS139Δ <i>Atu0009</i>	Km ^r Suc ^S ; deletion plasmid for <i>mltA</i> (<i>Atu0009</i>)	Cava Lab
pNTPS139Δ <i>Atu0092</i>	Km ^r Suc ^S ; deletion plasmid for <i>Atu0092</i>	Cava Lab
pNTPS139Δ <i>Atu1022</i>	Km ^r Suc ^S ; deletion plasmid for <i>Atu1022</i>	Cava Lab
pNTPS139Δ <i>Atu2112</i>	Km ^r Suc ^S ; deletion plasmid for <i>Atu2112</i>	Cava Lab

pNTPS139Δ <i>Atu2117</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu2117</i>	Cava Lab
pNTPS139Δ <i>Atu2122</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu2122</i>	Cava Lab
pNTPS139Δ <i>Atu3093</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu3093</i>	This study
pNTPS139Δ <i>Atu3779</i>	Km ^r Suc ^s ; deletion plasmid for <i>Atu3779</i>	Cava Lab
pNTPS139Δ <i>Atu1340</i>	Km ^r Suc ^s ; deletion plasmid for <i>amiC</i>	This study
pNTPS139Δ <i>Atu2113</i>	Km ^r Suc ^s ; deletion plasmid for <i>amiD</i>	This study
pNTPS139Δ <i>Atu1700</i>	Km ^r Suc ^s ; deletion plasmid for <i>dipM</i>	This study
<i>Depletion plasmids</i>		
pUC18-mini-Tn7TGM-TAC- <i>dipM</i>	Apr Gmr; mini-Tn7 vector containing <i>dipM</i> under control of the tac promoter	This study
pUC18-mini-Tn7TGM-LAC- <i>Atu4178</i>	Apr Gmr; mini-Tn7 vector containing <i>Atu4178</i> under control of the lac promoter	This study
pUC18-mini-Tn7TGM-LAC- <i>Atu1710</i>	Apr Gmr; mini-Tn7 vector containing FtsN under control of the lac promoter	This study
<i>E. coli strains</i>		
DH5α	Cloning strain	Life Technologies
S17-1	Sm ^r ; RP4-2, Tc::Mu,Km-Tn7; for plasmid mobilization	(59)
<i>A. tumefaciens strains</i>		
C58	Nopaline type strain; pTiC58; pAtC58	(43)
C58Δ <i>tetRA</i> ::a- <i>attTn7</i>	Replacement of the Δ <i>tetRA</i> locus with an artificial <i>attTn7</i> site	(43)
C58Δ <i>tetRA</i> ::a- <i>attTn7</i> pSRKKm-Ptac- <i>sfgfp</i>	C58 with empty pSRKKm plasmid	(43)
C58Δ <i>tetRA</i> ::a- <i>attTn7</i> pSRKKm-Ptac- <i>Atu1700-sfgfp</i>	C58Δ <i>tetRA</i> ::a- <i>attTn7</i> with inducible expression of <i>Atu1700-sfGFP</i>	This study

C58 $\Delta tetRA::mini-Tn7T$ -GM-Ptac- <i>sfgfp</i> pSRKKm-Ptac- <i>sfgfp</i>	Empty pSRK-Ptac- <i>sfgfp</i> in C58 $\Delta tetRA::mini-Tn7T$ -GM-Ptac- <i>sfgfp</i>	(43)
C58 $\Delta tetRA::a-attTn7 \Delta amiC$	Deletion strain for $\Delta amiC$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiC$ pSRKKm-Ptac- <i>sfgfp</i>	Empty pSRKKm-Ptac- <i>sfgfp</i> in $\Delta amiC$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiC$ pSRKKm-Ptac- <i>Atu1700-sfgfp</i>	$\Delta amiC$ deletion strain with inducible expression of <i>Atu1700-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiD$	Deletion strain for $\Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiD$ pSRKKm-Ptac- <i>sfgfp</i>	Empty pSRKKm-Ptac- <i>sfgfp</i> in $\Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiD$ pSRKKm-Ptac- <i>Atu1700-sfgfp</i>	$\Delta amiD$ deletion strain with IPTG-inducible expression of <i>Atu1700-sfGFP</i>	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiC, \Delta amiD$	$\Delta amiC \Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiC, \Delta amiD$ pSRKKm-Ptac- <i>sfgfp</i>	Empty pSRKKm-Ptac- <i>sfgfp</i> in $\Delta amiC \Delta amiD$	This study
C58 $\Delta tetRA::a-attTn7 \Delta amiC, \Delta amiD$ pSRKKm-Ptac- <i>Atu1700-sfgfp</i>	$\Delta amiC \Delta amiD$ deletion strain with inducible expression of <i>Atu1700-sfGFP</i>	This study
C58 $\Delta 0009$	Deletion strain for $\Delta mltA$	This study
C58 $\Delta 0092$	Deletion strain for $\Delta mltB1$ (<i>Atu0092</i>)	This study
C58 $\Delta 1022$	Deletion strain for $\Delta slt1$ (<i>Atu1022</i>)	This study
C58 $\Delta 2112$	Deletion strain for $\Delta slt2$ (<i>Atu2112</i>)	This study
C58 $\Delta 2117$	Deletion strain for $\Delta slt3$ (<i>Atu2117</i>)	This study

C58 $\Delta 2122$	Deletion strain for $\Delta mltB2$ (<i>Atu2122</i>)	This study
C58 $\Delta tetRA::a-attTn7 \Delta 3093$	Deletion strain for $\Delta slt4$ (<i>Atu3093</i>)	This study
C58 $\Delta 3779$	Deletion strain for $\Delta mltB3$ (<i>Atu3779</i>)	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700$	Mini-Tn7T-GM-Ptac- <i>dipM</i> inserted into <i>a-attTn7</i> site to express DipM under the <i>lac</i> promoter	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700$ pSRKKm-Ptac- <i>sfgfp</i>	Chromosome-based complementation of $\Delta Atu1700$ with C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-Atu1700$ allowing depletion of <i>Atu1700</i> under control of the <i>tac</i> promoter	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700, \Delta 1700$	Empty pSRKKm-Ptac- <i>sfgfp</i> in the <i>Atu1700</i> depletion strain	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700, \Delta 1700 \Delta 0009$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu0009$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 0092$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu0092$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 1022$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu1022$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 2112$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu2112$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 2117$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu2117$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 2122$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu2122$	This study
C58 $\Delta tetRA::mini-Tn7T-GM-Ptac-1700 \Delta 1700 \Delta 3093$	<i>Atu1700</i> (DipM) depletion background; $\Delta Atu3093$	This study
C58 $\Delta tetRA::mini-$	<i>Atu1700</i> (DipM) depletion background;	This

Tn7T-GM-Ptac-1700 Δ 1700 Δ 3779	Δ Atu3093	study
C58 Δ tetRA::mini-Tn7T-GM-Plac-4178	Mini-Tn7T-GM-Plac-4178 inserted into a-attTn7 site to express DipM under the <i>lac</i> promoter	This study
C58 Δ tetRA::mini-Tn7T-GM-Plac-4178 Δ 4178	Chromosome-based complementation of Δ Atu4178 with C58 Δ tetRA::mini-Tn7-GM-Ptac-Atu4178 allowing depletion of <i>Atu4178</i> under control of the <i>tac</i> promoter	This study
C58 Δ tetRA::mini-Tn7T-GM-Plac-1710	Mini-Tn7T-GM-Plac-1710 inserted into a-attTn7 site to express FtsN under the <i>lac</i> promoter	This study
C58 Δ tetRA::mini-Tn7T-GM-Plac-1710 Δ 1710	Chromosome-based complementation of Δ Atu1710 with C58 Δ tetRA::mini-Tn7-GM-Ptac-Atu1710 allowing depletion of <i>Atu1710</i> under control of the <i>tac</i> promoter	This study

Table 4-3. Synthesized DNA primers used in this study.

Synthesized DNA	Sequence
Primers	
Atu1340 NdeI F	5'-GTCGTCCATATGGATTTCACGCGCAGC-3'
Atu1340 BamHI (-STOP) R	5'-GTCGTCCGATCCGCCGCCATTTCGC-3'
Atu2775 NdeI F	5'-GTCGTCCATATGGATACTGAGCCT-3'
Atu2775 BamHI (-STOP) R	5'-GTCGTCCGATCCCGAATCATTGTGCGC-3'
Atu1700 NdeI F	5'-GCACGCCATATGGGCCTTGCAAGAAGTG-3'
Atu1700 BamHI (-STOP) R	5'-GTCGCTGGATCCTTCAAGGAAACCTGAT-3'
Atu1340 SpeI P1	5'-GTCGTCACTAGTAGGCGCTTGGCCTGCTCCTGGTAT-3'
Atu1340 P2	5'-AAGCTTGGTACCGAATTCGCGCGTGAAATCCATGGCCCT-3'
Atu1340 P3	5'-GAATTCGGTACCAAGCTTGCCCTGGCGAATGGCGGCTGA-5'
Atu1340 P4	5'-GTCGTCCGATCCATTCTGCCATCAGAGCA CCGTTGC-3'
Atu1340 P5	5'-GTCGTCCGATCCGGTTCCCACGGA-3'
Atu1340 P6	5'-TGGAAGCGCCGACCGGGC-3'
Atu2775 SpeI P1	5'-GTCGTCACTAGTGTGCTTCCAATGCCGAGACCCGCA-3'
Atu2775 P2	5'-AAGCTTGGTACCGAATTCTTGAGGCTCAGTATCCATTTGAAC-3'
Atu2775 P3	5'-GAATTCGGTACCAAGCTTGGAAGGGCGCACATTGATTGTA-3'
Atu2775 BamHI P4	5'-GTCGTCCGATCCCACCCTTGCGGATCACC GTCAGCT-3'
Atu2775 P5	5'-CCGCAGGCTATGCTGAATAGAGCG-3'
Atu2775 P6	5'-CAGGTCGAGGACATAGCCCTTCAG-3'
Atu1700 SpeI P1	5'-

	GCTAGACTAGTTCCACCGCCGCCTTTAC-3'
Atu1700 P2	5'-AAGCTTGGTACCGAATTCAGACGAATGT-3'
Atu1700 P3	5'-GAATTCGGTACCAAGCTTGGTTTCCTTGA-3'
Atu1700 BamHI P4	5'-GCATAGGGATCCAAGCACGCCCATCATG-3'
Atu1700 P5	5'-TTGAGATCGGCACCGGCAGCGGTTTTA-3'
Atu1700 P6	5'-ATCGTCACCGAATTGACGGCGCGCGGCT-3'
Atu0009 P4	5'-ATTCCGGTTTGGCGAACCACATTGG-3'
Atu0009 P5	5'-GGCCTGTGCTGACGATAACAA-3'
Atu0009 P6	5'-CGGCCTCCGCAAAAGGCGTCATGATT-3'
Atu0092 P4	5'-CGATCCGCTCAATCGTTTCGTGGA-3'
Atu0092 P5	5'-CGGCATTGCATCTCATCAAGAAG-3'
Atu0092 P6	5'-AGACGCTCACCTTCGGCGGGCATCG-3'
Atu0572 SpeI P1	5'-GTCGTCACTAGTAGTCGTGCATACACTGAAGCTGCA-3'
Atu0572 P2	5'-AAGCTTGGTACCGAATTCAATTCCTTCCCGGCACGCCATA-3'
Atu0572 P3	5'-GAATTCGGTACCAAGCTTGCGAAAACTTTGCGACGGATAG-3'
Atu0572 BamHI P4	5'-GTCGTGCGATCCGCCGTGTCGTGCGATCCCTGATC-3'
Atu0572 P5	5'-TTTCTCCTCCTCCCGCTCGGGCAC-3'
Atu0572 P6	5'-ATTCAAGGATGCGACGCTCGCGGGC-3'
Atu1022 P4	5'-GCTGCTGAATACGCGATCAAGGCAAC-3'
Atu1022 P5	5'-GCAACCTCGATGCACAGCTCAAC-3'
Atu1022 P6	5'-CGCTGTCCCAGGAGCCTGATAACAAT-3'
Atu2112 P4	5'-GAAAGCGCCCGCGATACATAGTCTC-3'
Atu2112 P5	5'-GCCGATCCGCAAGGTCGATCCG-3'
Atu2112 P6	5'-CGCGTCATGACCTCGACGCTACG-3'
Atu2117 P4	5'-AGGCCGCTTTTGGAGAGTGCCT-3'
Atu2117 P5	5'-TGGCGTCGTACGGCCATGACGAT-3'

Atu2117 P6	5'-CGATGTGGTGCGGGTTGAGGT-3'
Atu2122 P4	5'-CGTGTCATCTACGGTTCTTAAGT-3'
Atu2122 P5	5'-GGCGAATATTCTGCATGCGCCTTA-3'
Atu2122 P6	5'-GCGTTCCTCAGATTCTGGATGAGCGT-3'
Atu2451 SpeI P1	5'- GTCGTCACTAGTTTTTGAATATGCGCGAG CACC-3'
Atu2451 P2	5'- AAGCTTGGTACCGAATTCAAATACGCCAC GCATAACGGA-3'
Atu2451 P3	5'- GAATTCGGTACCAAGCTTCAGCAGTGCGG ATATCGCTGA-3'
Atu2451 BamHI P4	5'- GTCGTCCGATCCGGCGCGGTTCTTCCGG CG-3'
Atu2451 P5	5'-CCCCGTTTGCGATCCGCCGGCTTG-3'
Atu2451 P6	5'-TCCCGGTGCCGCGACCTTCTCTCC-3'
Atu2489 SpeI P1	5'- GTCGTCACTAGTCGAAAACGAGATCACTG CCGATCA-3'
Atu2489 P2	5'- AAGCTTGGTACCGAATTCCTTCATTGCCC GCATCAAAAAT-3'
Atu2489 P3	5'- GAATTCGGTACCAAGCTTAAGGCGATCGA CGGCCAATAA-3'
Atu2489 BamHI P4	5'- GTCGTCCGATCCCGGGAGAACTTTTCGTT GTTTCGCA-3'
Atu2489 P5	5'-TTCCGGCGCCGGCAAGGAAGGCAT-3'
Atu2489 P6	5'-TTCAACGCGCCGAGTTCCTCTCTT-3'
Atu3093 SpeI P1	5'- GCTGCAACTAGTGCGCCACAGCCCATCTC G-3'
Atu3093 P2	5'- AAGCATGGTACCGAATTCGCGGCTTGTTG ATATTCCT-3'
Atu3093 P3	5'- GAATTCGGTACCATGCTTTAGCAGCGGCG GGCGCCGGA-3'
Atu3093 HindIII P4	5'- GCACGTAAGCTTGCGGTGATCTTGATGAT- 3'
Atu3093 P5	5'-GAGCTTCTCGGGCAATTCCG-3'
Atu3093 P6	5'-GCCTCACGAAGCCGCACGATC-3'

Atu3779 P4	5'-CAGGAAATCACCGATGACCAGAAC-3'
Atu3779 P5	5'-AGGCTCATCGCCGACGATATCAC-3'
Atu3779 P6	5'-TATTCCTTGAACCAGACTTCCGTGC-3'
Atu4178 SpeI P1	5'- GTCGTCAGTAAATCAAACCCCAAAA GCTAAA-3'
Atu4178 P2	5'- AAGCTTGGTACCGAATTCGATCCGCAGTC ATACTAGC-3'
Atu4178 P3	5'- GAATTCGGTACCAAGCCTATCAGGTGGCG AGCCGCTGA-3'
Atu4178 BamHI P4	5'- GTCGTCGGATCCACAACGACGATGTGGAT TCGGTTCGCG-3'
Atu4178 P5	5'- TGCTCGCGAAATTGGTGTGTCTCATTAA- 3'
Atu4178 P6	5'-CCTATCAGGTCGTGTTGTTTCGAT-3'
Atu1710 SpeI P1	5'- GCTGCAACTAGTTGACCGAACAATCGAAG GATAA-3'
Atu1710 P2	5'- AAGCATGGTACCGAATTCTACGTGTTGCA CCATCTA-3'
Atu1710 P3	5'- GAATTCGGTACCATGCTTTGATAACGGCC AATACGGT-3'
Atu1710 HindIII P4	5'- GCACGTAAGCTTGATAGGCTTGCAGGATC GGCG -3'
Atu1710 P5	5'- CGTCGCATTCATGATGCTCTATCGGAAG- 3'
Atu1710 P6	5'- GCCAGGCAGCGCGCAGACCGGCACTCCG GT-3'

*R = Reverse, F=Forward primer

**AtuXXXX- *Agrobacterium tumefaciens* gene loci

***P1-P6 primers amplify the 500 upstream and downstream regions of the gene loci

****-STOP = Reverse (R) primer does not contain a stop codon

CHAPTER 5

PG Hydrolases and their Regulators are Involved in Cell Division, Separation and
Beta-lactam Resistance in *A. tumefaciens*

INTRODUCTION

This dissertation provides mechanistic insights into the function of peptidoglycan (PG) hydrolases in *A. tumefaciens*. We developed a new tool that has permitted the depletion and characterization of essential genes in *A. tumefaciens* (Chapter 2), enabling advances in the mechanism underlying essential processes including cell growth, division and separation of *A. tumefaciens* (1). Indeed, using this system, we have shown that DipM is an essential, degenerate LytM-containing factor involved in the regulation of PG hydrolysis in *A. tumefaciens* (Chapter 4). In addition, we characterized the function of two families of PG hydrolases in *A. tumefaciens*, namely amidases and lytic transglycosylases (Chapters 3 and 4). We found that amidases and LTs are involved in a wide variety of processes such as growth, fitness, cell division, separation, outer membrane permeability, PG recycling and beta-lactam resistance of *A. tumefaciens* (Chapters 3 and 4). Although existing knowledge describing some of these mechanisms is available in bacterial model organisms, such as *E. coli* and *P. aeruginosa*, we have shown that the regulation of, and the key players involved in, some of these conserved processes is quite different in non-model organisms such as bacteria belonging to the order *Rhizobiales* (2–9). Overall, these findings promote the view that studies of diverse bacteria are necessary to fully understand complex bacterial processes.

5.1 DipM is required for the regulation of AmiC and LTs

We showed that the essential degenerate LytM-containing factor, DipM, is necessary for the regulation of cell wall hydrolysis in *A. tumefaciens* (Chapter 4). DipM activates AmiC (Chapter 4); however, AmiC is dispensable for viability (Chapter 4) and deletion of *amiC* in the depletion of DipM background worsens the phenotype associated with DipM (data not shown). This suggests that DipM regulates the activities of other proteins. To determine if loss of DipM changes the PG structure due to misregulation of hydrolases, we conducted a cell wall compositional analysis. The absence of DipM leads to an increase in anhydromuropeptides, the products made by lytic transglycosylases (Chapter 4). Single deletions of LTs in the DipM depletion background causes a delay of the characteristic phenotype associated with loss of DipM. It remains to be determined if the PG hydrolase regulatory function of DipM is direct or indirect.

In canonical cell division models, such as *E. coli*, degenerate LytM-factors mediate the cell separation process by controlling the function of amidases (4). Indeed, inactivation of NlpD and EnvC leads to cells connected through their outer membrane. Importantly, absence of the cognate amidases mimics absence of their activators, NlpD and EnvC (4). Thus, in *E. coli* the function of degenerate LytMs may be limited to activation of amidases. In *A. tumefaciens*, the essential DipM activates AmiC; however, DipM is an essential protein while AmiC is dispensable for viability. Moreover, the phenotypes observed in the absence of DipM or AmiC are strikingly different, and deletion of AmiC in the depletion of DipM worsens the phenotype associated by the absence of DipM suggesting that

DipM regulates the activity of other proteins in the cell. Thus, more studies need to be conducted to address the regulatory mechanism of DipM in *A. tumefaciens*.

Currently, two hypotheses may explain the regulatory function of DipM in *A. tumefaciens*. One hypothesis is that DipM could be inhibiting the activity of cell wall-modifying enzymes. It has been shown that specific modifications to the cell wall, such as *O*-acetylation, make the PG cell wall more resistant to PG hydrolase degradation by lytic transglycosylases (10). These modifications are thought to protect the cell wall from attacks by PG hydrolases. Thus, one possibility is that the absence of DipM may be impacting the regulation of a specific type of cell wall-modifying enzymes namely deacetylases. In this scenario, the absence of DipM could cause an increase in deacetylation on the cell wall, making it more susceptible to hydrolysis by PG hydrolases.

Alternatively, another hypothesis is that DipM could be an adaptor protein in *A. tumefaciens*. We showed that the absence of DipM leads to an increase of the products made by LTs and carboxypeptidases. Thus, DipM may serve as a PG hydrolase adaptor protein, whose main activity is to recruit and regulate a wide variety of proteins in the periplasm. In this scenario, DipM could potentially recruit PG hydrolases—specifically—to locations of active growth during both growth and cell division. We have shown in Chapter 4 that DipM localizes at the growing cell pole during growth and to mid-cell during cell division in an FtsN-dependent manner. Thus, the putative scaffolding activity of DipM could be facilitating the regulation of local bursts of PG hydrolysis near active PG synthesis areas.

5.2 EnvC contributes to efficient cell division and separation in *A.*

tumefaciens

DipM is not the only degenerate LytM-factor predicted to play an important role in PG metabolism of *A. tumefaciens*. Preliminary characterization of EnvC (Atu2775) suggests that this protein also functions to regulate LT activity. EnvC-sfGFP localizes strictly to mid-cell during cell division (Figure 5-1A). Although deletion mutants of *envC* are recovered, $\Delta envC$ leads to a marked cell viability defect (Figure 5-1B). This defect can be complemented with a plasmid-containing *envC* under the native promoter control, suggesting that the defect is EnvC-dependent (Figure 5-1B). $\Delta envC$ results in changes in outer membrane permeability (Figure 5-1C), hypersensitivity to increased salt concentrations (Figure 5-1D), and a significant increase of cell area and curvature compared to WT (Figure 5-1E). Time-lapse microscopy reveals that $\Delta envC$ cells exhibit several cell division defects, including hypercurvature, asymmetric growth, tip splitting, hyperconstriction and cell lysis (Figure 5-1F). In some instances, failed cell separation events lead to ectopic pole formation. Finally, depletion of DipM in $\Delta envC$ worsens the phenotype associated with the absence of either DipM or EnvC as indicated by a more rapid increase in cell area (Figure 5-1E,F). These observations suggest EnvC and DipM may have distinct contributions to the regulation of cell division. To corroborate the impact of EnvC on lytic transglycosylases, we constructed single LT deletions in $\Delta envC$ (Figure 5-2A). Surprisingly, we found that single deletion of LTs, in most cases, restores the

Figure 5-1. Deletion of *envC* causes cell division and separation defects.

(A) Dynamic subcellular localization of EnvC in WT *A. tumefaciens* strain was observed. Cells expressing EnvC-sfGFP were grown to exponential phase and

imaged by phase and fluorescence microscopy. (B) Severe cell viability defect in the absence of *envC* and $\Delta envC$ -DipM. (C) Increased SDS sensitivity is indicative of increased outer membrane permeability in the absence of EnvC. (D) $\Delta envC$ is hypersensitive to increased concentrations of salt. (E) Increased cell area and max cell width for $\Delta envC$, $\Delta envC$ -DipM at 6 h of depletion. The cell area distribution is shown in violin plots. The violin-plot mid-line represents the median cell length, top (Q1) and lower (Q2) quartiles represent the 75% and 25% of the population, respectively. Phase-micrographs of representative cells are displayed (top). (F) Time-lapse DIC microscopy of $\Delta envC$, DipM-deficient, and $\Delta envC$ -DipM cells over the course of 16 h reveals the unique and additive cell division defects.

Figure 5-2. Deletion of $\Delta ldt6$ in $\Delta envC$ restores the viability of cells.

(A) Cell viability of $\Delta envC$ is improved upon single deletion of LTs. (B) Cell viability of $\Delta envC$ is restored after deletion of *Atu0009*, *Atu0092*, *Atu1022*, *Atu2112*, *Atu2117* and *Atu2122* (strain 19 or $\Delta ldt6$).

phenotype of $\Delta envC$. Interestingly, deletion of the major LT Atu3779 is synthetically lethal in the $\Delta envC$ background. Using the single LT $\Delta envC$ data, we next constructed a strain that lacks six of the LTs that contributed to the restoration of the $\Delta envC$ phenotype, namely Atu0009, Atu0092, Atu1022, Atu2112, Atu2117 and Atu2122 ($\Delta lt6$). Remarkably, we found that deletion of $\Delta envC$ in $\Delta lt6$ completely restores the viability of EnvC (Figure 5-2). This suggests that EnvC may regulate these LTs.

CONCLUSION

PG hydrolases mediate a wide variety of processes in the bacterial cells. Indeed, PG hydrolases permit the insertion of secretion systems, are involved in beta-lactam resistance and allow the cells to expand, divide and separate. Currently, the mechanisms by which these fascinating enzymes achieve the aforementioned activities are still under study.

The goal of the work presented in the previous chapters is to provide mechanistic insights into the activities of the PG hydrolases found in plant pathogen *A. tumefaciens*. We focused our efforts into the understanding of lytic transglycosylases (LT) and amidases as well as their regulators. We found that LTs and amidases are versatile enzymes with roles in beta-lactam resistance and PG recycling (Chapter 3) but also cell division and separation (Chapter 4) (Figure 5-3). Indeed, in the case of amidases, we showed that inactivation of the cytoplasmic amidase, AmiD, leads to increased beta-lactam resistance (Chapter

3). Our current hypothesis is that a failure in the recycling of LT degradation products in the cytoplasm by AmiD, leads to an increase of these products and the activation of inducible beta-lactamases. We also found that deletion the periplasmic amidase, AmiC, leads to cell division defects suggesting that this amidase may be involved in septal PG hydrolysis (Chapter 4) (Figure 5-3). We also studied the function of lytic transglycosylases (LTs) (Chapter 3). Some LTs can be found dispersed in the cytoplasm (Atu0009, Atu0572, Atu3093), while others may form “punta” throughout the cell (Atu0092 and Atu3779). Interestingly, we found that majority of the LTs of *A. tumefaciens* display a polar to mid-cell localization (Atu2122, Atu2489, Atu1022, Atu2117, Atu2112 and Atu2451), a characteristic feature of cell division proteins. More research needs to be done to address the specific roles of these LTs in cell division. Overall, our finding verify that PG hydrolases involved in cell division require complex regulatory mechanisms to ensure the proper activity of these enzymes at the right place and the right time.

LTs are not just involved in cell division and cell separation, but some LTs contribute to beta-lactam resistance (11, 12). Here, we found that the most significantly expressed LT, Atu3779, is involved in beta-lactam resistance (Figure 5-3). Indeed, deletion of Atu3779 causes hypersensitivity to ampicillin (Chapter 3). Currently, two hypotheses may explain the role of Atu3779 in beta-lactam resistance. The first hypothesis is that deletion of Atu3779 may be causing outer membrane permeability defects, thus allowing the free passage of small

Figure 5-3. Regulation and activity of PG hydrolases in *A. tumefaciens*.

Our model shows the diverse networks by which *A. tumefaciens* PG hydrolases may function in the cell. Degenerate LytM-containing factors (blue) may regulate the activities of lytic transglycosylases (green) and the amidase (pink) AmiC to promote cell division. The cytoplasmic amidase, AmiD, and Atu3779 are involved in beta-lactam resistance. Arrows indicate activation, capped lines indicate inhibition, question marks indicate associations that remain to be explored in more depth.

molecules, such as antibiotics and detergents, into the periplasm leading to cell death. It remains to be tested whether increased outer membrane permeability defects are the cause of the hypersensitivity to beta-lactams. Future experiments will assess the status of the outer membrane of this strain.

Alternatively, deletion of *Atu3779* may cause an overall reduction in production of cell wall degradation products or anhydromuropeptides. Treatment with beta-lactam antibiotics leads to an increase in the production and recycling of anhydromuropeptides due to a block of in PG synthesis (5). The increase of cell wall degradation products in the cytoplasm serve as signal for the induction of beta-lactamases, which inactivate beta-lactam antibiotics. Thus, inactivation of *Atu3779* may significantly reduce anhydromuropeptide production and subsequent induction of beta-lactamases. Future experiments will assess beta-lactamase induction in $\Delta 3779$ cells following treatment with beta-lactams. We expect that additional knowledge about proteins that promote or inhibit beta-lactam resistance may aid in the development more efficient tools to improve the efficiency of *Agrobacterium*-mediated plant genetic transformations. For example, if infective, a mutant to *A. tumefaciens* which is sensitive to antibiotics may be more easily removed from newly transformed plant tissue, potentially maximizing crop yield and food production.

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VITA

Wanda M. Figueroa-Cuilan was born and raised in Arroyo, Puerto Rico. She graduated from Natividad Rodríguez-González High School in 2007. She next went to the University of Puerto Rico-Cayey, where she worked in the laboratory of Dr. Jannette Gavillán from 2009-2012. Wanda graduated in 2012, earning her Bachelor Degree in Biological Sciences, with an emphasis in Natural Sciences. For the next two years, Wanda was part of the NIH-PREP Program, where she worked in the laboratory of Dr. David Pintel at the University of Missouri-Columbia.

Wanda started graduate school at the University of Missouri in Fall 2014, and she joined the laboratory of Dr. Pamela Brown in the Spring of 2015. She received an NIH Initiative for Maximizing Student Diversity (IMSD) Fellowship and the Gus T. Ridgel Fellowship in 2015. During this time, Wanda participated in national and on-campus organizations such as the Society for the Advancement of Chicanos/Latinos and Native Americans in Science (SACNAS), Minorities in STEM, the Graduate Collaborative Group (GCG), the Biology Graduate Association (BGSA) and was also a Mizzou Graduate Student of Excellence of the Office of Diversity, Inclusion and Equity. Wanda also participated as a panelist (8 different panels) for different organizations and university offices with the goal of providing undergraduate students with the tools necessary to attend graduate school. During her graduate career, Wanda mentored a total of 6 undergraduate students. Wanda gave a total of 8

presentations and also participated as a science fair or conference judge twice. During graduate school, Wanda also was part of the Divisional Council, where she was the graduate student representative for a year. Wanda was also part of the Mizzou national recruitment team, where she attended to ABRCMS and SACNAS conferences (a total of 3 times) with the goal of recruiting URM students to Mizzou. Lastly, Wanda has accepted a postdoctoral position at Johns Hopkins University, School of Medicine, in the laboratory of Dr. Erin Goley.